

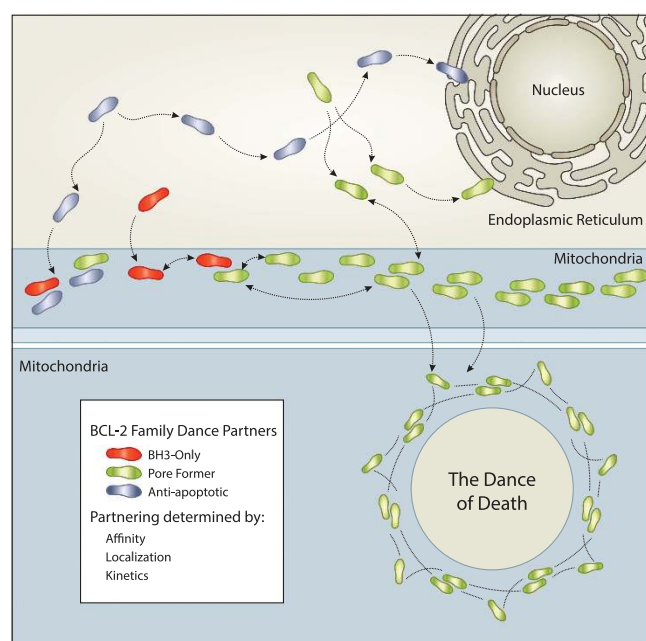
Review

BCL-2 family proteins: changing partners in the dance towards death

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The BCL-2 family of proteins controls cell death primarily by direct binding interactions that regulate mitochondrial outer membrane permeabilization (MOMP) leading to the irreversible release of intermembrane space proteins, subsequent caspase activation and apoptosis. The affinities and relative abundance of the BCL-2 family proteins dictate the predominate interactions between anti-apoptotic and pro-apoptotic BCL-2 family proteins that regulate MOMP. We highlight the core mechanisms of BCL-2 family regulation of MOMP with an emphasis on how the interactions between the BCL-2 family proteins govern cell fate. We address the critical importance of both the concentration and affinities of BCL-2 family proteins and show how differences in either can greatly change the outcome. Further, we explain the importance of using full-length BCL-2 family proteins (versus truncated versions or peptides) to parse out the core mechanisms of MOMP regulation by the BCL-2 family. Finally, we discuss how post-translational modifications and differing intracellular localizations alter the mechanisms of apoptosis regulation by BCL-2 family proteins. Successful therapeutic intervention of MOMP regulation in human disease requires an understanding of the factors that mediate the major binding interactions between BCL-2 family proteins in cells.

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Graphical Abstract

Facts:

- Direct physical interactions between the BCL-2 family proteins dictated by BCL-2 homology 3 (BH3) regions regulate mitochondrial outer membrane permeabilization (MOMP).

- The membrane plays an active role in most BCL-2 family interactions by changing the affinities and local relative abundance of these proteins.
- The majority of studies examining the interactions between BCL-2 family proteins use truncated proteins or peptides of the BH3 region at physiologically irrelevant concentrations or in the absence of membranes leading to confusion in defining the core mechanisms of the BCL-2 family proteins.
- Differential expression in various tissues, targeting to different subcellular localizations and post-translational modifications all contribute to regulation of BCL-2 family-binding interactions.
- Targeting the BH3 domain-binding groove of anti-apoptotic BCL-2 family proteins with BH3 mimetics has proven useful in generating anti-cancer therapeutics but future improvements depend on accounting for more of the factors that govern BCL-2 family interactions.

Questions:

- Other than the BH3 region, which factors determine the dominant interactions between the BCL-2 family proteins?
- How do the affinities between, and physiologically relevant concentrations of, BCL-2 family proteins in cells dictate the regulation of apoptosis?
- How do BAX and BAK transition from inactive monomers to membrane-embedded oligomers that permeabilize membranes?
- How do BCL-2 family proteins control cell death at intracellular locations other than the mitochondria?

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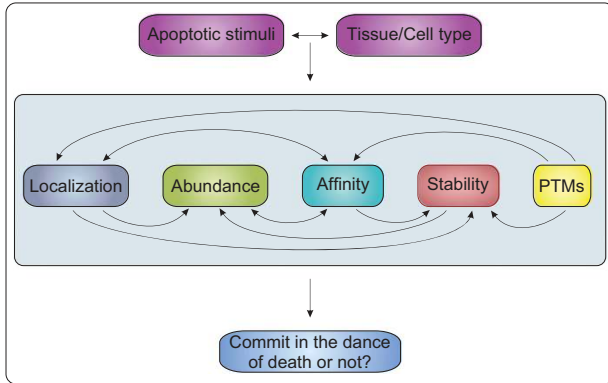


Figure 1 Cellular factors regulating commitment to the apoptotic dance of death by the BCL-2 family proteins. BCL-2 family proteins function by direct binding interactions that lead to mutual sequestration and/or membrane permeabilization. These direct protein–protein binding interactions are functionally regulated in cells by a number of interrelated processes. Differences in apoptotic stimuli and cell types lead to different responses due to the integration of the effects of localization, abundance, affinity, stability and post-translational modifications (PTMs). Related interactions are connected by arrows. For example, PTMs affect protein stability, localization and binding affinities. Affinities determine which interactions dominate but also affect localization and stability (heterodimerization with BH3-only proteins stabilizes MCL-1). The affinities of BCL-2 family proteins for intracellular membranes and other binding partners at these membranes dictate BCL-2 family localization. Co-localization increases local concentrations (abundance). Protein stability also impacts abundance of the BCL-2 family proteins. The relative abundance and affinity ultimately determine which binding interactions dominate and whether or not the cell undergoes MOMP committing it to apoptosis

Introducing the dance of death

Dancing is a joy of life; however, cells have a dance between the BCL-2 family proteins that can lead to death. Exchange of dance partners within the BCL-2 family proteins regulates apoptosis. The outcome of this dance – the fate of the cell – is decided by the net interactions between the BCL-2 family proteins. These interactions are governed by the abundance of the proteins and the ‘attraction’ (affinity) between the partners. The affinities between the dance partners are the result of protein conformation changes, most of which occur on the dance floor – the intracellular membranes where the interactions between the BCL-2 family proteins take place. Further, the apoptotic stimuli, cell type and post-translational modifications differentially regulate the interactions that predominate to control MOMP. This concept of context-dependent regulation of apoptosis is outlined in Figure 1.

In one view, the ultimate goal of the dance between the BCL-2 family proteins is to trigger apoptosis by forming pores within the mitochondrial outer membrane. MOMP results in the release of pro-apoptogenic factors (e.g., cytochrome *c*) from the mitochondrial intermembrane space (IMS) into the cytosol ultimately causing the activation of a caspase cascade that functions to dismantle and destroy the cell.¹ Caspases cleave hundreds of proteins vital for proper cellular function and homeostasis culminating in the typical biochemical and morphological features of apoptosis.²

Commitment to the apoptotic dance of death is due in part to MOMP being rapid and complete – cytochrome *c* is released from most mitochondria within 5 mins and complete caspase activation occurs within 15 minutes inevitably resulting in cell

death.^{3–5} As BCL-2 family proteins regulate this critical cell fate decision an abundance of research has focused on understanding the interactions between the BCL-2 family proteins, the regulation of these interactions within a cell, and how these interactions lead to MOMP.

Dancing on and within membranes – molecular mechanisms of BCL-2 family regulation

The BCL-2 family is divided into three groups based on their primary function (1) anti-apoptotic proteins (BCL-2, BCL-X_L, BCL-W, MCL-1, BFL-1/A1), (2) pro-apoptotic pore-formers (BAX, BAK, BOK) and (3) pro-apoptotic BH3-only proteins (BAD, BID, BIK, BIM, BMF, HRK, NOXA, PUMA, etc.). All BCL-2 family proteins contain a BH3 domain; one of four BH domains involved in interactions between these proteins.⁶ The anti-apoptotic and pore-forming proteins contain all four BH domains (multi-BH domain proteins) and adopt a highly conserved tertiary structure forming a hydrophobic BH3 domain-binding groove that acts as a receptor for BH3 domains of other family members.⁷ The BH3-only proteins are subdivided into activator and sensitizer proteins, which contain only the BH3 domain and with the exception of BID are unstructured in solution, reviewed in ref. 8. With the stage set and the dancers named, who ‘dances’ with whom dictates whether or not MOMP occurs.

Various competing models (reviewed in ref. 9) describe how BCL-2 family proteins interact with each other to control MOMP. Figure 2 highlights the ‘embedded together’ model that puts an emphasis on the role of the membrane as the ‘locus of action’ for most BCL-2 family proteins.¹⁰ In all models, the BH3 domain is necessary for the primary apoptotic function of BCL-2 family members and the interactions between them at intracellular membranes. The BH3 domain of activator BH3-only proteins binds to the BH3 domain-binding groove in BAX/BAK.^{11,12} This activates BAX and BAK, eliciting a series of conformation changes that result in BAX/BAK homooligomerization and pore formation within the MOM (Figure 2b). The BH3 domain-binding groove in anti-apoptotic proteins binds the BH3 domains of the pore-formers and the activator BH3-only proteins, inhibiting their function by sequestering them (Figure 2c).^{13,14} The BH3 domain of sensitizer BH3-only proteins binds to the BH3 domain-binding groove of anti-apoptotic proteins, inactivating them.¹⁵ This interaction is competitive with binding of activator BH3-only proteins and pore-formers leading to their displacement from anti-apoptotic proteins (Figures 2d and e). Many, if not most, of these interactions occur at, on and within the MOM.¹⁶ The lipid bilayer has an active role in facilitating structural changes of the BCL-2 family proteins that alter their affinities and consequently the interactions between them thus governing whether or not MOMP occurs.¹⁷

The embedded together model is more complicated than the original rheostat model that proposed apoptotic fate is determined by the ratio of pro-apoptotic to pro-survival proteins in a cell.¹⁸ The rheostat model was conceived when the BCL-2 family consisted of BCL-2 and BAX and posited that the more abundant protein decided life versus death, respectively. In the context of the more complicated interactions between the expanded families of BCL-2 proteins the

basic posit of the rheostat model remains relevant. Ultimately, the fate of the cell is decided by the interactions between the BCL-2 family dancers. The interactions are determined by the relative abundance and affinities of the partners. Both are impacted by the affinity for membranes, which regulates the localization, conformation and therefore, function of BCL-2 family members. However, similar to the rheostat model, if the sum of interactions results in BAX/BAK oligomers and MOMP— then the cell is committed to death and will dance no more.

Attraction among the BCL-2 family proteins dictates dance partners

Understanding the primary interactions that occur between the BCL-2 family members requires knowledge of the affinity and concentration of each member. These factors dictate the predominate interactions and thus if MOMP occurs. The consequences of differing affinities between the BCL-2 family proteins can be illustrated with a simple case of binding competition between cBID, BCL-X_L and BAX. We have previously demonstrated that BCL-X_L functions as a dominant negative BAX.¹⁹ When cBID, BCL-X_L and BAX were incubated together, BAX-mediated membrane permeabilization was completely inhibited. If a mutant of cBID that interacts with BAX but not BCL-X_L was used, BAX-mediated membrane permeabilization was not inhibited to the same extent. This suggests that cBID preferentially interacts with BCL-X_L over BAX. Using purified full-length recombinant BAX, BCL-X_L and cBID we have found that the affinity (dissociation constant, K_D) between BCL-X_L and cBID is 3 nM (unpublished data), whereas the affinity between cBID and BAX is high micromolar in the absence and 25 nM in the presence of membranes.²⁰ The interaction between cBID and BAX in the absence and presence of BCL-X_L can be modeled using these K_D values (Figure 3a).²¹ The higher affinity of cBID for BCL-X_L versus that of BAX prevents BAX activation and only a supraphysiological concentration of BAX can out-compete BCL-X_L for cBID binding. This simple example does not account for interactions between BAX and BCL-X_L, the differing affinities of the proteins for membranes, changing conformations within the bilayer or the addition of sensitizer BH3-only proteins like BAD. The situation becomes more complex in cells where BCL-2 family proteins interact with known and unknown binding partners at different subcellular locations. Transcriptional and post-translational control of the BCL-2 family proteins further changes the abundance and affinities of these proteins modifying their interactions and localizations within the cell. These factors result in a complex interaction network, the sum of which determines cellular fate.

The first step in understanding the BCL-2 family interaction network is to establish the affinities between binding partners. The reported affinities of known BCL-2 family protein interactions are summarized in (Tables 1A and 1B). Most of the affinity data were generated with BH3 peptides and c-terminally truncated multi-BH domain proteins. Organizing this affinity data by BH3 peptide length in Tables 1A and 1B, revealed a distressing trend. The length of BH3 peptides used in the literature varies from 16 amino acids to >30. As observed for BAK, and BAD, the BH3 peptide length can have

a large effect on the measured affinity.^{22,23} Adding to the confusion, different peptide lengths are often used for BH3-only proteins within the same paper.^{24,25} Generally, BH3 peptide length correlates with increased affinity for the binding partner suggesting that residues outside of the BH3 domain facilitate proper interactions among BCL-2 family proteins.²⁶ This summary found BIM to be the most studied BH3 peptide but also revealed a scarcity in studies that measure affinities for BAX or BAK binding. In fact, little data have been reported for many of the BH3 proteins including BAP31, BCL-B, BCL-G, BCL-RAMBO, BCL-W, BECLIN-1, BFK, BFL-1/A1, BOK and SPIKE binding to multi-BH domain proteins.

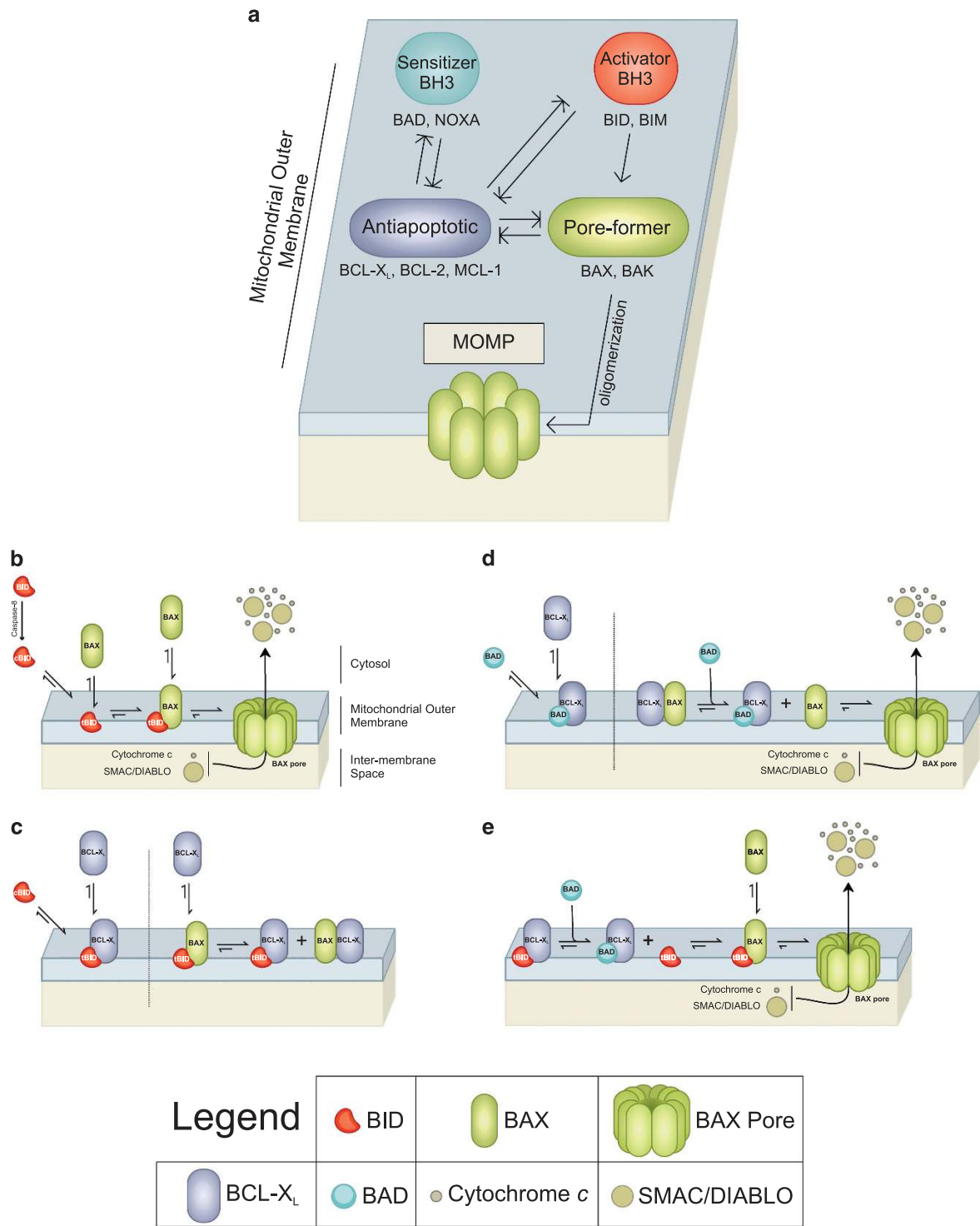
To understand the reported affinities in a cellular context, the physiologically relevant concentration ranges for BCL-2 family proteins must be known. It is frequently stated that cellular concentrations of BCL-2 family proteins are in the low nanomolar range. However, with the exception of BAX, with endogenous levels reported between 3 nM and 170 nM, it appears that the low nanomolar statements are not well supported by data.^{27,28} Direct measurement of concentrations in cells is highly desirable as the affinities between full-length BCL-2 family proteins are in the low nanomolar range. Thus, the micromolar dissociation constants measured using peptides could be irrelevant if other BCL-2 family protein concentrations are in the low nanomolar range like BAX.

Studies with full-length proteins can reveal physiologically relevant interactions previously overlooked owing to the use of peptides. For example, many studies state that NOXA binds MCL-1 and BFL-1/A1 but does not bind BCL-2, BCL-X_L or BCL-W, whereas BAD binds BCL-2, BCL-X_L and BCL-W but not MCL-1 or BFL-1/A1.^{29,30} This matches well with the reported affinities for NOXA and BAD peptides binding truncated anti-apoptotic proteins (Tables 1A and 1B). NOXA has nanomolar affinity for MCL-1 and BFL-1/A1 and micromolar affinity (which may not be relevant physiologically) with BCL-2, BCL-X_L or BCL-W, whereas BAD is the reverse. However, these affinities and binding specificities change when full-length proteins are used. Full-length NOXA bound with dissociation constants of 3.4 nM for MCL-1, 70 nM for BCL-X_L and 250 nM for BCL-2, demonstrating full-length NOXA binding selectivity but not specificity.³¹ Consistent with these affinities, NOXA and BCL-2 interactions contributed to drug resistance in lymphoid cells suggesting that BCL-2 can prevent NOXA-mediated apoptosis.³¹

Most methods used to measure protein–protein interactions between BCL-2 family proteins neglect the influence of membranes. For example, full-length cBID and BAX interact in the presence of lipid membranes but not in solution because cBID requires a conformation change at membranes to interact with and activate BAX.^{17,20} A complete map of these interactions with affinities of full-length proteins in solution and in membranes will be essential to the delineation of the interactions between BCL-2 family proteins that regulate cell death.

Defining roles in the dance – what factors make a BH3-only protein an activator?

BH3-only proteins are classified as activator or sensitizer proteins based on whether or not they bind to and activate BAX and BAK. Activator BH3-only proteins promote MOMP



directly by triggering oligomerization of BAX and BAK. In contrast, sensitizer BH3-only proteins promote MOMP indirectly via binding to and inhibiting the anti-apoptotic proteins. However, activator BH3-only proteins bind both BAX and BAK and anti-apoptotic proteins. Furthermore, binding of an activator BH3-only protein to an anti-apoptotic protein also inhibits the anti-apoptotic protein. Therefore, who

is inhibiting whom, comes down to the relative abundance and affinities between activator BH3-only proteins and anti-apoptotic proteins. This redefines the interaction between activators and anti-apoptotic proteins as 'mutual sequestration', whereby BH3-only proteins bind to and inhibit anti-apoptotic proteins and vice versa.⁹ Furthermore, activator BH3-only proteins promote the membrane-embedded

Figure 2 The dance of the BCL-2 family within the bilayer regulates mitochondrial outer membrane permeabilization (MOMP) and apoptosis (a) Schematic of the embedded together model. All binding interactions are reversible and equilibria are governed by local affinities. Interactions with the lipid bilayer change the affinities of the interactions and therefore have an active role in the functions of the proteins. Binding of the activator BH3-only proteins (e.g., BID, BIM) to membranes increases their affinity for the pore-formers (e.g. BAX, BAK), which are activated (arrows) to permeabilize the mitochondrial outer membrane. The anti-apoptotic proteins (e.g., BCL-X_L, BCL-2, MCL-1) inhibit both the activator BH3-only proteins and the pore-forming proteins by mutual sequestration (T'd arrows). The sensitizer BH3-only proteins (e.g., BAD, NOXA) bind to and inhibit the anti-apoptotic proteins also by mutual sequestration. Recruitment of the complexes to the membrane by constitutive interactions (e.g., BAK) and dynamic interactions (e.g., BAX, BID, BIM) increases the affinities and local concentrations and reduces the diffusion of the BCL-2 family proteins. Localization at different intracellular membranes also dictates the binding equilibria between each family member. The efficiency of inhibition by mutual sequestration of anti-apoptotic proteins depends on both affinities and off-rates of the interactions. Interaction of the BH4 region of the anti-apoptotic proteins with BAX shifts the BAX-membrane binding equilibrium to favor the unbound state (retrotranslocation, not shown). (b–d). Interactions of the BCL-2 family that promote or inhibit MOMP illustrated for cBID, BAX, BCL-X_L and BAD as examples of different functional categories. (b) BID is activated by caspase-8 mediated cleavage to cBID (cleaved BID) a protein comprised of two fragments BID-P7 and BID-P15 held together by hydrophobic interactions. Rapid high-affinity binding to membranes dissociates the p7 fragment to solution and favors insertion of the p15 fragment (tBID; truncated BID) into the membrane. Membrane-bound tBID recruits inactive BAX from the cytosol. Binding to tBID activates BAX to insert in the bilayer, oligomerize and permeabilize the mitochondrial outer membrane releasing intermembrane space proteins including cytochrome c and SMAC. (c) Active tBID and BAX can recruit BCL-X_L to the membrane resulting in inhibition of both pro and anti-apoptotic proteins by mutual sequestration. BCL-X_L prevents tBID from activating BAX and prevents BAX from oligomerizing resulting in the inhibition of MOMP. BAX bound to BCL-X_L is in the active (oligomerization competent) conformation. (d, e) BAD inhibits unbound BCL-X_L by mutual sequestration. The affinity of BCL-X_L is higher for tBID than for active BAX (Tables 1A and 1B) therefore, in the absence of other regulatory interactions or PTMs if BCL-X_L is bound to tBID and BAX then high concentrations of BAD will displace active BAX (d) and then tBID (e) from BCL-X_L resulting in MOMP

conformation of anti-apoptotic proteins and thus also 'activate' these proteins.¹⁹ As a result a BH3-only protein with lower affinity for the membrane-bound form of an anti-apoptotic protein may function primarily to activate it. At high relative concentrations activator BH3-only proteins are expected to inhibit anti-apoptotic proteins while also activating BAX/BAK. For example, mutants of both BIM and BID that cannot bind BAX but can bind anti-apoptotic proteins promote apoptosis by functioning as sensitizer BH3-only proteins.^{32–34} Similarly, specific mutations to the BH3 domains of sensitizer BH3-only proteins BAD and NOXA turn these proteins into direct BAX activators without abrogating interactions with BCL-X_L.¹² Therefore, an activator BH3-only protein is a sensitizer-protein that has gained an additional dance partner aiding in the dance of death through activation of BAX or BAK.

Does the capacity to bind and activate pore-formers constitute the 'activator' classification of a BH3-only protein? Many BH3 peptides bind both anti-apoptotic proteins and pore-formers owing to the similarity between the BH3 domain-binding grooves. For example, the BH3 sensitizer NOXA primarily targets MCL-1 with an affinity of 3.4 nM.³¹ However, both NOXA and the *bona fide* activator BH3, BID can activate BAX to permeabilize membranes *in vitro*, causing NOXA to be classified as an activator BH3-only protein in some reports.^{35,36} This confusion is cleared up by examining affinities; the affinity of BID to BAX is 25 nM, whereas the affinity of NOXA to BAX is 1000 times higher (25 μM) (Figure 3b). BAX can be activated by NOXA, however, physiologically irrelevant micromolar concentrations are required. Therefore, the capacity to bind and activate pore-formers at physiologically relevant concentrations defines a BH3-only protein as an activator.

The question now is: what confers activator function and an increased affinity to BAX/BAK for full-length BH3-only proteins compared with peptides? The BH3 domain has a significant role in determining binding affinity, and mutations to the BH3 domains of BID and BIM fully abrogate their activator function.¹² However, the BH3 domain is not the only portion of the protein promoting BAX/BAK activation. Low nanomolar (<10 nM) concentrations of recombinant full-length BID or BIM activate BAX *in vitro*, whereas micromolar concentrations of BH3 peptides are needed to achieve the same level of BAX activation.³⁷ The creation of SAHBs (Stabilized Alpha-Helices

of BCL-2 domains) highlighted the importance of BH3 domain secondary structure.³⁸ SAHB peptides are forced into a tight alpha helical structure through a 'staple' – a covalent bond between two regions of the peptide. SAHBs of BID and BIM are more efficacious in BAX activation compared with their 'unstapled' BH3 peptides but still require 200–600 nM concentrations to fully activate BAX.^{39,40} Thus, an alpha helical BH3 domain is not enough for full efficacy.

High nanomolar concentrations of helical BH3 domain peptides may be required because the peptides do not bind to membranes as do cBID and BIM.^{41–43} Tethering a BID BH3 peptide, or a BID SAHB to the membrane resulted in efficient BAX activation at low nanomolar concentrations albeit with slower membrane permeabilization kinetics compared with full-length tBID.^{40,44} Membrane targeting of the BID BH3 peptide increases the alpha-helicity of the BH3 domain such that the peptide activates BAX as efficiently as a membrane-targeted SAHB.^{40,44} This indicates two things (1) binding to membranes increases the alpha-helicity of the BH3 domain increasing affinity for BAX and (2) membrane binding likely increases the activity of these peptides by increasing the local concentration of the peptide resulting in more efficient BAX activation. However, membrane-targeted peptides are still 5–10 times less efficacious at activating BAX compared with full-length proteins, suggesting other regions outside the BH3 domain increase the affinity between activator BH3-only proteins and BAX/BAK.⁴⁰

The dance steps of the pore-formers – mechanisms of BAX/BAK-mediated MOMP

The BAX and BAK activation mechanism is a multi-step dance within the bilayer. We and others have delineated steps in the BAX/BAK activation pathway that are generally agreed upon.^{20,45–47} Activator BH3-only proteins contribute to recruitment of BAX to membranes, activating BAX/BAK monomers, which then undergo a substantial conformation change and embed within the bilayer. Activated BAX/BAK monomers form symmetric homodimers that oligomerize to form pores within the bilayer resulting in MOMP. Understanding these steps is imperative because each represents an opportunity for therapeutic intervention in human disease. Several studies have focused on determining the exact conformations of inactive and

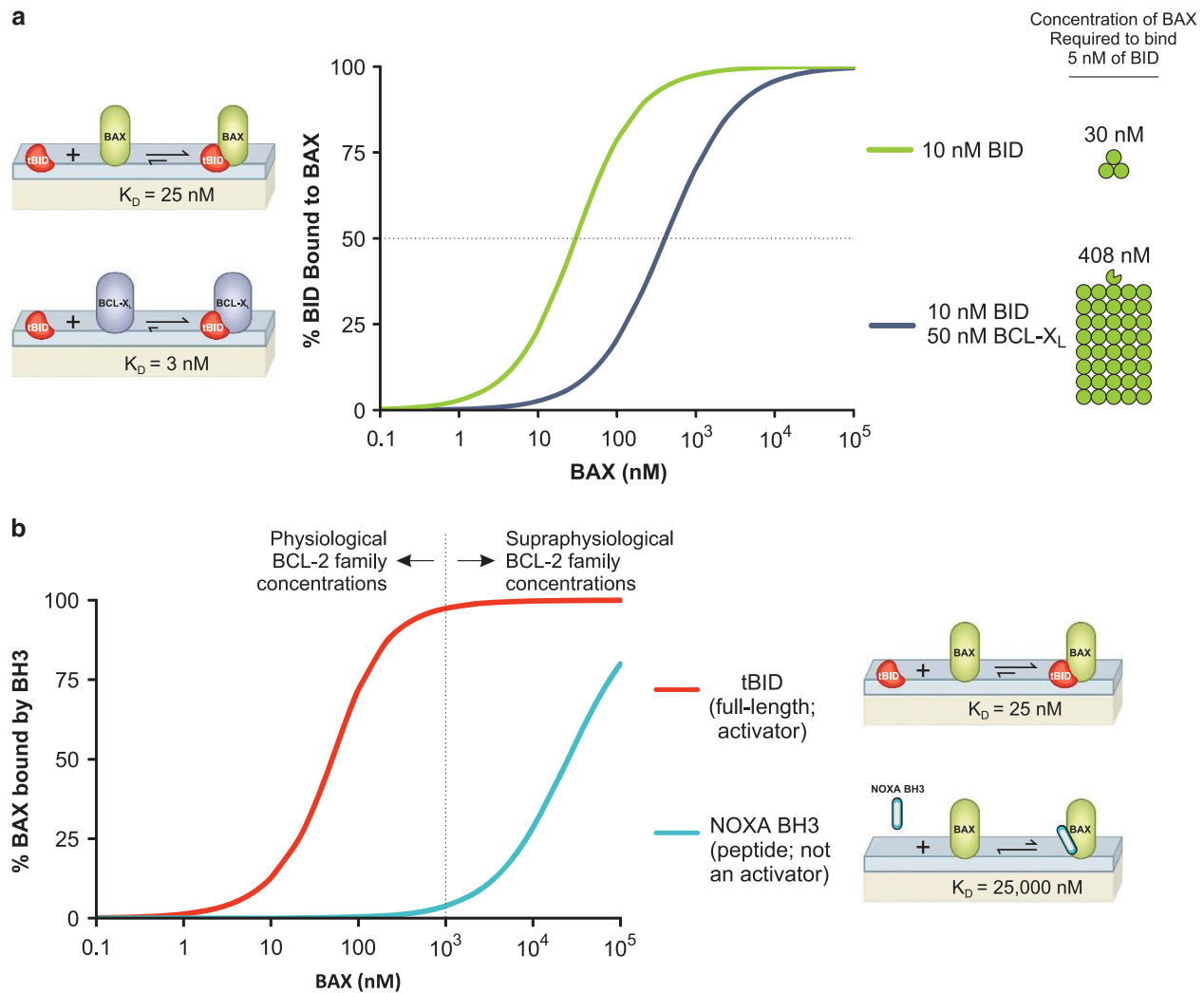


Figure 3 Affinities and concentrations dictate the predominate interactions of the BCL-2 family members (a) At membranes cBID binds BAX and BCL-X_L with an affinity of 25 nM and 3 nM, respectively. Equations describing competitive binding of two different 'ligands' (BAX, BCL-X_L) to one protein molecule (cBID)²¹ were used to model the interaction of 10 nM cBID with increasing concentrations of BAX in the absence (green) and presence (purple) of 50 nM BCL-X_L. In the absence of BCL-X_L, 50% of 10 nM cBID is bound to BAX at a BAX concentration of 30 nM. Upon the addition of BCL-X_L, ~ 13 times more BAX (408 nM) is required to bind 50% of the cBID. In this case, BCL-X_L is binding the majority of the cBID, preventing BAX activation. The functional consequence of the differing affinities results in BCL-X_L out-competing BAX for binding to cBID, effectively inhibiting apoptosis at physiologically relevant concentrations of BAX. (b) The affinities between BH3-only proteins and BAX determine whether a BH3-only protein functions as an activator or sensitizer. Equations describing the binding of two proteins²¹ were used to model the interaction of 50 nM BAX with increasing concentrations of the indicated BH3-only protein/peptide; full-length tBID (red) and NOXA BH3 peptide (cyan). The affinities of full-length tBID or NOXA BH3 peptide for BAX are 25 nM²⁰ and 25 000 nM (estimated from ref. 35) respectively. Typical *in vitro* BAX activation assays use peptide concentrations in the micromolar range, well above the nanomolar concentrations predicted for BCL-2 family proteins in cells. At supraphysiological concentrations (> 1000 nM) of peptides (e.g., NOXA, cyan) some BH3 sequences that typically function as sensitizers can bind to and activate BAX

active BAX/BAK, how their affinities for BCL-2 family proteins change and the transitions between conformations that occur for BAX/BAK to elicit MOMP. Despite years of examination, the complex mechanism is not fully understood partly owing to the difficulties in studying membrane-embedded proteins.

The specifics of BAX/BAK conformations during their activation and how they form pores have been reviewed recently.^{45,47} Rather than re-examine the precise conformations of BAX/BAK we will highlight some assumptions and unknowns regarding these models and how steps are regulated by changes in binding partners, affinities and conformations within the bilayer (Figure 4).

Activator BH3-only proteins start the dance of death by interacting with BAX/BAK at the bilayer. Activator BH3-only proteins interact with BAX/BAK at the bilayer owing to the high affinity of activator BH3-only proteins for membranes (Figure 4; step 1). For BAK, it is obvious that this interaction occurs at the bilayer because BAK is constitutively localized to the MOM. However, many models assume that BAX interacts with activator BH3-only proteins in solution resulting in a soluble activated BAX that then inserts into the MOM.^{47–49} The data supporting interactions between activator BH3-only proteins and BAX in solution are from experiments using truncated BAX, BH3 peptides or

Table 1A BCL-2 family interactions; dissociation constants reported for BH3 peptides, truncated and full-length proteins

Receptors

	Activity	length BH3	Reference																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																
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The affinity of a protein interaction is generally reported as a dissociation constant (K_D). Unless otherwise specified, K_D values obtained from the literature are for interactions between truncated multi-BH region protein 'receptors' (rows) and the corresponding BH3 'ligand' (columns). For each BH3 ligand, the first column indicates the K_D represented on a color scale (legend bottom right of chart). The second column indicates the length of the BH3 peptide used. 'ΔC' represents a BH3-only protein lacking its C-terminal tail anchor and 'FL' indicates full-length BH3-only protein. Use of a full-length multi-BH region protein 'receptor' is indicated by black shading in the second column. The letter in the third column indicates the PMID and method, listed in B. For each interaction, K_D values were sorted by peptide length (increasing length downward across rows). A reference with an asterisk (*) indicates the original report indicated that detergent was present when the K_D was measured

Table 1B PubMed ID number and method used to measure the K_D values listed in A

Label	PMID	Method
A	11206074	Fluorescence polarization (FP)
B	16697956	
C	17446862	
D	12242151	
E	19748896	
F	11248023	
G	11904405	
H	19351886	Surface plasmon resonance (SPR)
I	21727192	
J	21454712	
K	12660157	
L	15694340	
M	24265320	
N	22156224	SPR and time-resolved (TR)-FRET
O	21395401	
P	19062087	Forster resonance energy transfer (FRET)
Q	20392693	
R	23996493	Isothermal titration calorimetry (ITC)
S	21060336	
T	17337444	Yeast surface display
U	18589438	
V	17389404	
W	28411240	
X	18641390	
Y	20363230	
Z	23363053	
Za	21713285	Bacterial surface display
Zb	19766123	
Zc	9020082	Tryptophan fluorescence quenching
Zd	9372935	
Ze	23192964	NMR spectroscopy
Zf	24434006	
Zg	23301700	Stopped flow technique
Zh	25052212	Fluorescence anisotropy
Zi	27108441	FRET

detergents that promote the conformational changes of BAX that normally occur at a lipid bilayer. Indeed, activator BH3 peptides remain in solution when binding to BAX/BAK, however, micromolar amounts of activator are required to fully activate BAX/BAK with slow membrane permeabilization kinetics compared with activation with full-length activator BH3-only proteins.^{37,40} In comparison, full-length activator BH3-only proteins have a high affinity for membranes and efficiently activate BAX/BAK at nanomolar concentrations resulting in rapid membrane permeabilization kinetics.^{20,35,37,40} Activator BH3-only proteins bind membranes (half-time <50 s) before interacting with BAX (half-time ≈ 500 s).⁵⁰ Furthermore, activator BH3-only proteins require membrane binding for efficient BAX/BAK-mediated membrane permeabilization *in vitro* and in cells.^{20,41,51} Thus, binding between activator BH3-only proteins and BAX/BAK in solution may reflect what can happen with these proteins but in cells the relevant interactions occur at and within intracellular membranes.

Inviting BAX and BAK to dance without activator BH3-only proteins. BAX or BAK can be activated by antibodies,⁵²

small molecules,^{53,54} heat,⁵⁵ pH,⁵⁶ detergents^{57–59} and metabolites,⁶⁰ suggesting that all of the information for oligomerization and pore formation is contained within BAX and BAK. Thus, spontaneous activation of BAX/BAK could occur due to any perturbation of their compact globular structure that results in exposure of their hydrophobic cores to membranes. In line with these data, HCT116 cells with a genetic loss of 8 known BH3-only proteins (BAD^{-/-}, BID^{-/-}, BIK^{-/-}, BIM^{-/-}, BMF^{-/-}, HRK^{-/-}, NOXA^{-/-} and PUMA^{-/-}) underwent apoptosis in a BAX/BAK-dependent manner once anti-apoptotic BCL-2 proteins were genetically or pharmacologically inactivated.⁶¹ Further, exogenous expression of either BAX or BAK in cells lacking the majority of BCL-2 family proteins (the above HCT116 cells lacking 8 BH3-only proteins with additional genetic loss of BCL-2, BCL-XL, MCL-1, BCL-W, BFL/A1, BNIP3, NIX, BAX and BAK) resulted in spontaneous targeting of BAX and BAK to mitochondria and subsequent apoptosis, suggesting that BAX and BAK do not necessarily require BH3-only proteins for activation *in vitro*.⁶¹ However, it must be kept in mind that cultured cells are abnormal, under stress owing to high oxygen levels, often transformed and are usually growing on plastic potentially altering the cytoplasmic environment, cell metabolism or lipid composition of the mitochondria resulting in conditions that spontaneously activate BAX and BAK.

Dance moves of the pore-formers – activated BAX/BAK undergo multiple conformation changes within the bilayer. BAX and BAK are comprised of nine alpha-helices and in their inactive forms adopt a globular structure.^{11,62} Inactive monomers of BAX and BAK are slightly different; the carboxyl-terminal transmembrane helix, α_9 , is constitutively inserted into the MOM for BAK, whereas for BAX α_9 is sequestered to the protein core within the hydrophobic BH3 domain-binding groove resulting in a cytoplasmic localization.^{63,64} Activator BH3-only proteins bind to the BH3 domain-binding groove, displacing α_9 , which then inserts into the bilayer resulting in BAX adopting a similar conformation to that of inactive BAK.^{64–66} BAX/BAK disengages the amino (α_1) and carboxyl-terminal (α_6 – α_8) regions from the hydrophobic core of the protein that is formed by the BH3 domain-binding groove (α_2 – α_5).^{12,67–69} Then BAX/BAK embed into the bilayer with α_5 and α_6 helices partially inserted and α_9 , adopting a transmembrane configuration (Figure 4; step 2).^{49,67,70–72} Determining whether there is a precise order of these changes and the equilibria between each step is important since a physiologically relevant intermediate conformation of BAX or BAK could be targeted pharmaceutically to modulate the regulation of MOMP. But so far, a defined temporal intermediate between inactive monomeric and membrane inserted dimeric BAX/BAK has not been found in physiologically relevant experimental conditions.

Dancing with a twin – activated monomers of BAX/BAK form symmetric homodimers. Of the BCL-2 family proteins BAX/BAK are unique in that they first form heterodimers with activator BH3-only proteins and then transition by an unclear mechanism to homodimers with other active BAX/BAK monomers (Figure 4; step 3).^{73,74} The conundrum arises

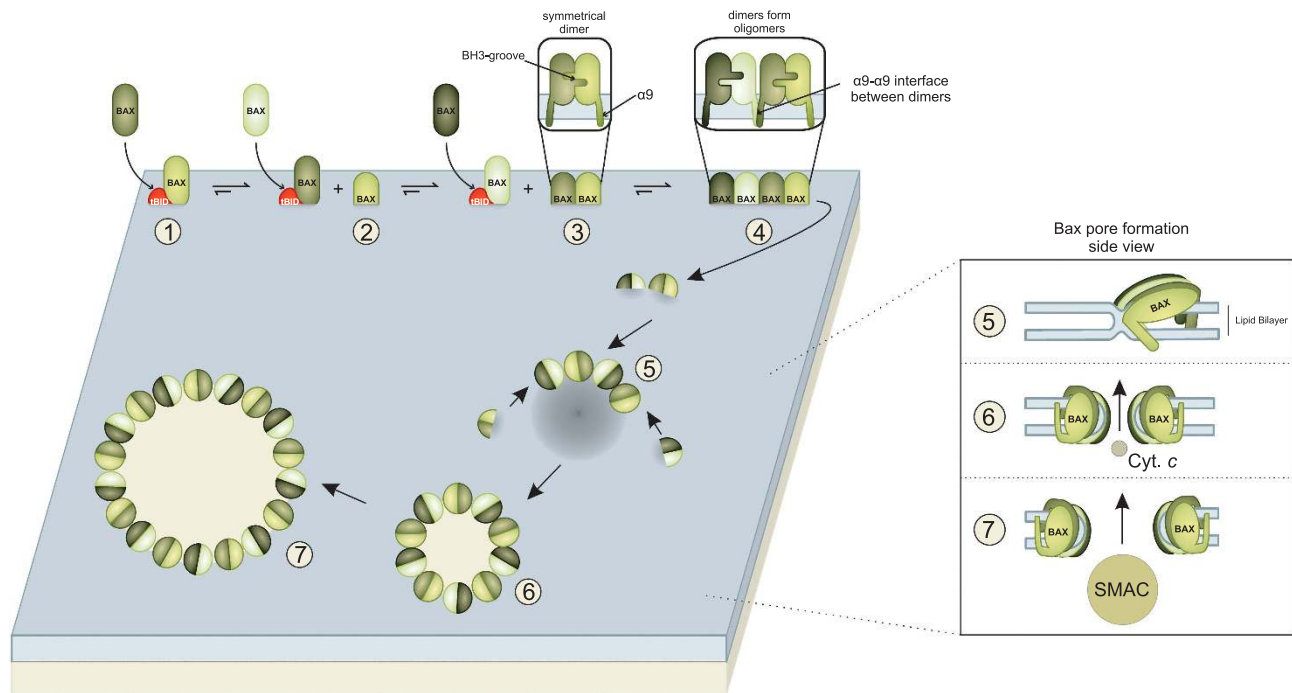


Figure 4 BAX activation is a multi-step process characterized by hetero- and homotypic interactions that result in MOMP. Full-length activator BH3-only proteins, like tBID, have a high affinity for membranes and bind them rapidly before interacting with the pore-formers, BAX and BAK. The following steps are shown for BAX activation; however, the BAK activation mechanism appears to be very similar. **(Step 1)** BAX interacts with tBID at the membrane. **(Step 2)** BAX then undergoes multiple conformational changes, inserting into the bilayer with $\alpha 9$ spanning the membrane. Insertion into the bilayer is the rate-limiting step in the BAX activation mechanism. **(Step 3)** The transition from the BAX and BH3-only protein heterodimer to BAX homodimers is not well understood but would occur spontaneously if the homotypic interaction is of higher affinity than that of the heterotypic interaction between BAX and activator BH3-only proteins. The end result is that the membrane-embedded active BAX monomers dimerize via reciprocal interactions between their BH3-grooves. **(Step 4)** BAX dimers interact with each other via multiple lower affinity interactions of which parallel $\alpha 9$ - $\alpha 9$ interactions between dimers appear particularly important for the stabilization of large pores. **(Step 5)** BAX oligomers composed of symmetrical dimer subunits start to destabilize and thin the bilayer. Weak affinity interactions between dimers allow additional dimer subunits to add to the oligomer at any point. **(Step 6)** BAX oligomers form small pores in the bilayer that can initially release smaller intermembrane space (IMS) proteins like cytochrome *c* (12 kDa). **(Step 7)** Continuing activation of BAX results in a higher concentration of dimers in the bilayer that add to the oligomer, resulting in pore expansion and the release of larger IMS proteins like SMAC (54 kDa dimer).

because the BH3 domain of the activator BH3-only protein binds inactive BAX/BAK in the BH3 domain-binding groove and BAX/BAK dimerization involves symmetric binding between these BH3 domain-binding grooves, which also contain the BH3 domain, of each activated monomer.^{12,67,71,72,75} With similar interaction sites, activator BH3-only proteins would be expected to inhibit rather than activate BAX/BAK dimerization, which has not been observed for BAX/BAK even in the presence of excess activator.^{36,76} In support of this, BID SAHBs, previously shown to activate BAK, inhibited BAK oligomerization when disulfide cross-linked to the BAK BH3 domain-binding groove.¹¹ This suggests that activator BH3-only proteins leave the BH3 domain-binding groove in order for BAX/BAK oligomerization to proceed. However, at steady state BH3 activators can stay bound to BAX even after pore formation.^{20,50,77} These studies measured the entire population of BAX and BID via FRET (Förster resonance energy transfer) thus, it remains possible that there are sub-populations, one of cBID and BAX heterodimers and one of BAX oligomers. It is also possible that binding partners exchange rapidly at equilibrium despite their high affinities.

An attractive hypothesis for what could be occurring is that at the bilayer, activator BH3-only proteins convert BAX/BAK from inactive monomers into active membrane-embedded

monomers.⁷³ This structural rearrangement could reduce the affinity between activator BH3-only proteins and active BAX/BAK monomers such that BAX/BAK homodimerization displaces the activator BH3-only protein (Figure 4; step 2). Displacement is favored by inactive monomers being higher in concentration and having a higher affinity for the activator BH3-only proteins compared with the active monomers. This would explain why BH3-only proteins function as catalysts for BAX activation in certain experimental conditions.⁷⁸ The displaced monomer is limited to two-dimensional diffusion within the bilayer, resulting in a high local concentration of active monomers. This results in the recruitment of multiple activated BAX/BAK monomers in close proximity and thereby assists dimer formation via high-affinity BH3 domain-binding groove interactions (Figure 4; step 3). The high-affinity BH3 domain-binding groove interactions in a dimer along with insertion into the bilayer drive BAX/BAK activation irreversibly towards dimers. Release of the BH3 protein would repeat this process resulting in a high local concentration of BAX/BAK dimers in the bilayer that further oligomerize thereby permeabilizing the membrane (Figure 4; step 4).

Dancing in a circle – low-affinity interactions allow for a dynamic BAX/BAK pore. Fluorescence-based kinetic studies revealed BAX insertion into the bilayers was rate-limiting

and followed rapidly by BAX oligomerization.²⁰ The last step to be characterized is determining exactly how BAX/BAK dimers dance as oligomers that permeabilize the MOM. Recently, single-molecule imaging of BAX showed BAX monomers form dimers in the bilayer that self-assemble into higher-order oligomers.⁷³ This is consistent with biochemical data where BAX/BAK symmetrical homodimers can be disulfide cross-linked into higher-order oligomers^{71,74} and with structural data of active BAX/BAK at membranes.^{67,72} In addition, the BAX/BAK inhibitors MSN-125, MSN-50 and DAN004 prevent BAX oligomerization but not symmetrical dimer formation.⁷⁹ These inhibitors prevented some but not all of the interfaces within symmetrical dimers suggesting that proper dimer formation is prerequisite for oligomerization and pore formation.

Chemical cross-linking studies report inter-dimer cross-links at a number of positions between one BAX molecule in a dimer (α^1) and another in a separate dimer (α^2) such as $\alpha 6^1$: $\alpha 6^2$, $\alpha 1^1$: $\alpha 1^2$, $\alpha 3^1$: $\alpha 5^2$ and $\alpha 9^1$: $\alpha 9^2$.^{71,72,80} The interface formed between $\alpha 9$ helices within the bilayer is particularly important for stabilizing large pores. Deletion of $\alpha 9$ or mutations to $\alpha 9$ that disrupt the inter-dimer interface results in the release of smaller IMS proteins like cytochrome *c* (12 kDa) but not larger proteins like SMAC (54 kDa dimer in cells).^{71,81} Consistent with observations that BAX/BAK form dynamic pores that enlarge over time with variable sizes dependent on BAX/BAK concentration,^{82,83} direct visualization of BAX in cells revealed that homo-oligomers form lines, arcs and rings of various sizes on mitochondria.⁸⁴ In this study 97% of BAX rings formed pores in isolated lipid bilayers versus 12% of BAX arcs. This supports observations that BAX lines the edges of both small

and large pores with similar protein density.⁸⁵ Together, these data suggest a model whereby oligomers are formed by dimers linked together by many weak affinity interactions (Figure 4; step 4). This model is attractive because weak affinity interactions between dimers explain how pores enlarge over time: newly formed dimers join together end-to-end as lines and arcs that eventually close to form pores that are expanded by additional dimer subunits (Figure 4; step 5–7).

Changing Dance Partners Within the Cell

PTMs alter the abundance and affinities of BCL-2 family proteins in cells. Just as different songs affect dancing styles, post-translational modifications (PTMs) of BCL-2 family proteins have a role in regulating their interactions. PTMs can affect stability, localization and function of BCL-2 family proteins and can promote or inhibit apoptosis. PTMs regulating BCL-2 family proteins were reviewed comprehensively.⁸⁶ However, the majority of PTMs are single reports that have not been independently confirmed. In other cases, two or more reports conclude opposite functional effects. For example, phosphorylation of BCL-2 at S70 was reported to increase^{87–89} or inhibit apoptosis.^{90–92}

To illustrate the range of activities ascribed to post-translational modifications we generated an updated list of PTMs reported for just BAX (Table 2). Even for the well-studied protein BAX there are only two independently confirmed PTMs, polyubiquitination and phosphorylation of S184 of BAX. Polyubiquitination targets BAX for proteasomal degradation. Proteasomal degradation of monomeric BAX would

Table 2 Post-translational modifications of BAX

Modification and residue	Modified by	Functional consequence	Year	PMID
Phosphorylation S184 (H9)	AKT	Inactivates BAX, prevents translocation from cytosol to mitochondria	2004	14766748
			2005	15642728
			2009	19376889
			2016	26763134
			2016	28357332
Phosphorylation S163 (H8-H9 loop)	PKC-zeta	Inactivates and destabilizes BAX	2007	17525161
	GSK-3P	Activates BAX by increased targeting to mitochondria	2004	15525785
Phosphorylation T167 (H8-H9 loop)	JNK and p38	Phosphorylation inactivates BAX Inhibits apoptosis	2006	16709574
Phosphorylation T135 or T140 (H6)	Unknown	Unknown. Observed in a pre-malignant (AT1), but not a malignant cell line (CA1a)	2009	19194518
Phosphorylation T22(H1)	Unknown	HTS, needs validation	2011	21712546
Phosphorylation T85 (H3-H4 loop)	Unknown		2015	25814448
Dephosphorylation T172, T174, T186	WIP1	Downregulates BAX. Inhibits apoptosis	2013	23907458
Dephosphorylation	PP2A	Promotes apoptosis	2006	16679323
Cleavage G28/G29 (H1)	Calpain	Releases C-terminal BAX lacking amino acids 129 of the N-terminus, increases its apoptotic activity by unknown mechanism Promotes apoptosis	2003	12490315
PolyUbiquitination non-specific site (9 lysines in BAX)	unknown	Inhibits apoptosis targets BAX for proteasomal degradation	1998	9438391
			2000	10725400
			2012	22460798
			2010	20300062

Reported post-translational modifications (PTMs) of BAX. Helix in BAX where modification takes place is indicated (H#). Only phosphorylation of S184 and ubiquitination of BAX have been independently confirmed by multiple labs (shaded). Where the same functional consequence is reported in multiple studies rows were merged for clarity. The functional consequence of S184 phosphorylation is controversial as it has been reported that it either activates or inhibits BAX. The corresponding publication year and Pubmed ID (PMID) are listed for each study

decrease BAX concentration and shift activator BH3 binding to anti-apoptotic proteins (Figure 3a).

Phosphorylation could alter the core mechanism of 'dance' steps detailed above by introducing a large negatively charged phosphate to regions critical for BAX function. Phosphorylation of residue S184 by AKT is known to inhibit BAX.^{93,94} Currently, the precise mechanism of how S184 phosphorylation affects BAX activation remains to be elucidated. However, to understand BCL-2 family protein regulation *in vivo* it will be

imperative to understand how PTMs change BCL-2 family-binding affinities for proteins and membranes, and their subcellular localizations both of which can alter the fate of the cell.

Alternate dance venues in cells – changing localizations of the BCL-2 family. The BCL-2 family proteins have diverse localizations within cells, likely dictated by their different affinities for various intracellular membranes and

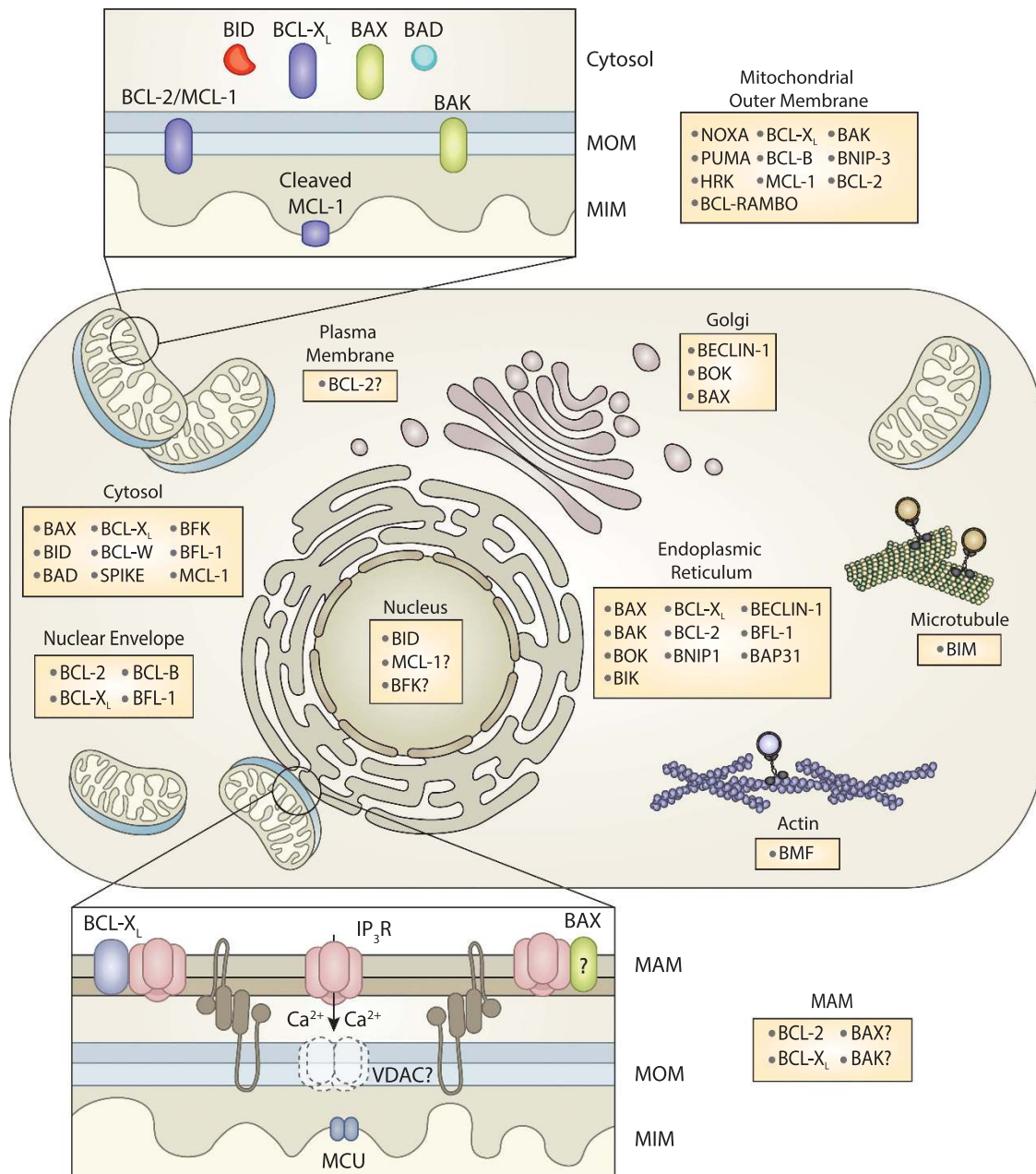


Figure 5 BCL-2 family protein subcellular localization in non-apoptotic cells BCL-2 family proteins reported at each location are listed in the corresponding box. Uncertainty or data for which there are conflicting reports are indicated by a '?'. Localization that changes during apoptosis is summarized in Tables 3A and 3B. Interactions at the mitochondria are shown in the enlargement top left. Interactions at the mitochondrial associated membrane (MAM), a subdomain of the endoplasmic reticulum in close contact with mitochondria are enlarged below left. Among other proteins, mitofusin proteins (shown as dimer linking the two membranes) bring a specialized subdomain of ER membranes in contact with the mitochondrial outer membrane forming the MAM

Table 3A Localization of BCL-2 Family proteins

Protein	Localization									
	ER	NE	TGN	Cytosol	MOM	MM	MIM	Cyto-skeleton	Plasma membrane	Nucleus
BCL-2	XX				XX					
BFL-1										
BCL-X _L					XX					
BCL-W										
MCL-1					XX					
					XX					
BCL-G					XX					
BAX				XX						
					XX					
BAK					XX					
BOK	XX									
BMF										
BIM										
BID				XX						
PUMA					XX					
NOXA					XX					
					XX					
HRK										
BAD				XX						
BIK	XX									
BECLIN-1	XX									
BNIP										
SPIKE				XX						
BAP31	XX									
BCL-G										
BFK				XX						
BCL-Rambo					XX					

Abbreviations

ER	Endoplasmic Reticulum		normal conditions
NE	Nuclear Envelope		
MOM	Mitochondrial Outer Membrane		
MIM	Mitochondrial Inner Membrane		apoptotic conditions
MM	Mitochondrial Matrix		
TGN	trans Golgi network	XX	predominant localization

Heat map of reported localizations for BCL-2 family proteins. Proteins reported in multiple locations or that change location in growth and apoptotic conditions are shaded light and dark gray, respectively. XX indicates predominant localization for proteins reported at multiple locations.

Table 3B Detailed localization information and references

	Senior Author	Year	Localization		Modification(s) affect localization
			Resting	Apoptosis	
BCL-2 (B-cell lymphoma 2)					
8402648	JC Reed	1993			
8043515	A Strasser	1994	ER, NE, & MOM	no change	
8144576	DW Andrews	1994			
BCL-X_L (B-cell lymphoma-extra large)					
9108035	RJ Youle	1997	Cytosol and MOM	MOM, ER & NE	
12763855	X Wang	2003			
2254066	JT Opferman	2012			
BCL-W / BCL2-L2 (BCL-2-like protein 2)					
12952938	DCS Huang	2003	cytosol & loose association at MOM	C-terminal inserted MOM	
BFL-1(human) / A1(murine) (BCL-2-related protein A1)					
11992871	J Borst	2002			
23499873	C Ferran	2013	ER, NE, & MOM	no change	
16094403	C Gélinas	2005			
MCL-1 (Induced myeloid leukemia cell differentiation protein)					
24525728	H Harada	2014		MOM	
7896880	RW Craig	1995			
15798198	HFY Yen	2005	MOM, Cytosol and ER		
22544066	JT Opferman	2012		MOM, MM, MIM	N-terminal proteolytic cleavage
20627101	HFY Yen	2010	MIM		proteolytic cleavage
15554878	V Duroio	2005	Nucleus		
BCL-B / BCL2-L10 (BCL-2-like protein 10)					
11278245	JC Reed	2000			no change
23563182	J Borst	2013	MOM		target to cytosol
22233804	H Varmus	2011			Mono-Ubiquitination
BAX (BCL-2 Associated X)					
9382873	RJ Youle	1997			
9670005	SJ Korsmeyer	1998	Cytosol & Golgi	MOM, & ER	
12847083	CB Thompson	2003			
22560721	M Deshmukh	2012			S184E
10228148	RJ Youle	1999			Phosphorylation (S184)
19376889	AB Waxman	2009	translocation blocked		
BAK (Bcl-2 homologous antagonist/killer)					
12847083	CB Thompson	2003			
10085290	JA Hickman	1999	MOM & ER	no change	
BOC (BCL-2 related ovarian killer)					
23429263	T Kaufmann	2013	ER & Golgi		
27053113	JH Wojcikiewicz	2016	MOM	no change	
26949185	DR Green	2016			
BMF (BCL-2-modifying factor)					
14561217	MG Hinds	2004	Associated with (DLC)-2 (Actin Cytoskeleton)	MOM	Phosphorylation, release from actin cytoskeleton
12591950	K Lei & RJ Davis	2003			
BIM-L and EL (BCL-2-interacting mediator of cell death), BIM-S= constitutively mitochondria					
17517961	G Hacker	2015			
12142566	Y Fukuchi	2002	Associated with (DLC)-1 (microtubules)	MOM	Phosphorylation, release from microtubules
12591950	RJ Davis	2003			
BID (BH3 interacting-domain death agonist)					
10807576	N Tanaka	2000	Cytosol	MOM	Cleavage required for translocation
14500711	TH Kim	2003			
PUMA(BBC3) (p53 upregulated modulator of apoptosis)					
11463392	KH Vousden	2001			
17968660	KH Vousden	2008	MOM	no change	
12574499	L Zhang	2002			
26431330	A Vasquez	2015			
26212789	H You	2015	cytosol	MOM	Phosphorylation (S10) promotes translocation to MOM
NOXA					
10807576	N Tanaka	2000			Phosphorylation (S13) targets to cytosol
14500711	TH Kim	2003	MOM	MOM	
21145489	A Kelekara	2010	cytosol		
HRK (Harakin)					
15031724	C Kitahara	2004	not transcribed	MOM	
17434443	J Villalón	2007			
BAD (BCL-2-associated death promoter)					
19641507	SJ Korsmeyer	2003			
16603546	UR Rapp	2006	Cytosol (bound to 14-3-3)	MOM	Dephosphorylation (S136), release Bad from 14-3-3, translocates to MOM
10230394	SJ Korsmeyer	1999			
10195903	JC Reed	1999	MOM		
BIK (BCL-2 Interacting Killer)					
18299962	S Ge	2008			
11884414	G Shore	2002	ER	no change	
BECLIN-1 (Coiled-coil myosin-like BCL2-interacting protein)					
11306553	T Yoshimori	2000	ER, TGN & MOM	no change	
16179260	B Levine	2005			
BNIP (BCL2/adrenovirus E1B 19 kd-interacting protein)					
15272311	M Tagawa	2004			
7954800	G Chinnadurai	1994	BNIP1, 2; ER; BNIP3; MOM	no change	poly-ubiquitination relocates BNip1 to MOM
23896122	J. Tang	2013			
SPIKE					
20361936	M Mitrovic	2010	cytosol	no change	
BAP31 (B-cell receptor-associated protein 31)					
9334338	GC Shore	1997			
12668660	GC Shore	2003	ER	no change	
BCL-G (BCL-2-like protein 14)					
11054413	JC Reed	2001	BCL-G ₁ : diffuse BCL-G ₂ : MOM	no change	
BRK (BCL-2 family kin)					
12700646	A Strasser	2003	Cytosol	no change	
17412810	M Poutanen	2007	Nucleus	no change	
BCL-RAMBO					
22921587	JH Park	2012	MOM	no change	

Legend

	Human	NE	Nuclear Envelope
	Murine	MOM	Mitochondrial Outer Membrane
	Human and Murine	MIM	Mitochondrial Inner Membrane
	Dynein Light Chain-1	TGN	trans Golgi network
	Dynein Light Chain-2	<u>underlined</u>	denotes predominant localization
	Endoplasmic Reticulum		

binding partners at each location (Figure 5, Tables 3A and 3B). The localization of BCL-2 family proteins results from complex binding equilibria with membranes and other proteins within the cell. Localization dictates the available binding partners and therefore BCL-2 family response to different cell death stimuli. The subcellular localization of BCL-2 family proteins may also be important for monitoring various cellular processes and/or may be requisite to alternate functions performed by the proteins in non-apoptotic cells.

For example, detachment from the matrix and some drug treatments result in perturbations of the cytoskeleton that in many cell types causes a specialized form of apoptosis called anoikis. In response to anoikis BIM and BMF have been reported to translocate from the cytoskeleton, their location in non-apoptotic cells, to the MOM (Tables 3A and 3B, Figure 5). Moreover, loss of either protein results in protection from anoikis and resistance to cytoskeleton altering drugs like paclitaxel.^{95,96} It seems likely therefore, that localization of BIM and BMF at the cytoskeleton permits monitoring cellular fitness and initiation of an apoptotic signal in response to cytoskeletal perturbation.

The most widely recognized non-mitochondrial localization for BCL-2 family proteins is the endoplasmic reticulum (ER) where they regulate calcium homeostasis, ER stress, autophagy and apoptosis.⁹⁷ The mechanism by which BCL-2 proteins regulate ER stress and calcium homeostasis and how this relates to apoptosis is not clear.⁹⁸ BCL-X_L, BCL-2, MCL-1, BAX and BAK have all been reported to regulate calcium release from the ER by interacting with inositol triphosphate receptors (IP3R), a ligand-gated calcium channel.⁹⁸ There is accumulating evidence that this interaction is mediated by the BH4 domain of the multi-domain anti-apoptotic proteins.⁹⁹ Moreover, BCL-2 and BCL-X_L can localize at the mitochondria-associated ER membranes, with Type III IP3Rs favouring transmission of calcium to the mitochondria, reducing calcium stores (Figure 5, panel B inset).^{100,101} Although many details remain unclear, it has been hypothesized that anti-apoptotic proteins promote the slow leak of calcium from the ER, decreasing the potential for strong signaling to mitochondria and thereby inhibiting apoptosis.¹⁰¹ BAX/BAK and BH3-only proteins may oppose calcium leakage by binding BCL-2/BCL-X_L or IP3R, increasing calcium stores and promoting apoptosis.^{102,103} However, there are many areas of controversy and additional models for the role of BCL-2 family proteins in calcium signaling and ER stress.⁹⁸ Nevertheless, the preponderance of evidence suggests that BCL-2 family proteins function differently at the ER and mitochondria.

Using Small Molecules with High Affinities to BCL-2 Family Proteins to Control the Dance of Death

Developing small molecule modulators of BCL-2 family proteins has been a high priority for major pharmaceutical companies for more than a decade. Many cancer cells are 'addicted' to the expression of anti-apoptotic BCL-2 family proteins because in their absence the cells die faster than they grow. Such cells are described as 'primed for death' requiring only a 'push' in the right direction to trigger MOMP and

subsequent apoptosis.¹⁰⁴ Inactivating the anti-apoptotic BCL-2 family proteins with small molecule BH3 mimetic drugs is one potential 'push' as displacing the active but sequestered pro-apoptotic proteins results in MOMP.^{9,105,106} Small molecule BH3 mimetics, like ABT-263 (Navitoclax) and ABT-199 (Venetoclax), mimic the binding of BH3 peptides to the hydrophobic BH3 domain-binding groove of anti-apoptotic proteins and thus displace BH3-only proteins and active BAX/BAK from anti-apoptotic proteins.^{107,108} By binding to the BH3 domain-binding grooves of anti-apoptotic proteins, ABT-263 inhibits BCL-2, BCL-XL and BCL-W, whereas ABT-199 only inhibits BCL-2. ABT-199 is approved for use in chronic lymphocytic leukemia and both drugs are being used in dozens of clinical trials as single agents and in combination with other therapies.¹⁰⁹

Some cancers depend primarily on MCL-1 for survival^{110–112} and others acquire resistance to drugs that target BCL-2/BCL-XL/BCL-W by upregulating MCL-1.¹¹³ The small molecule MCL-1 inhibitor, S63845 shows promise as a therapeutic.¹¹⁴ S63845 was efficacious in killing multiple cancer-derived cell lines *in vitro* and had potent anti-tumor activity in pre-clinical mouse models of hematological malignancies *in vivo* while sparing normal tissues. Another cancer treatment strategy would be small molecule activation of BAX and/or BAK. However, it has yet to be determined whether such small molecules have any therapeutic index in cancer.⁵³

ABT-263 and ABT-199 were optimized to displace BAD BH3 peptides from C-terminally truncated anti-apoptotic proteins in solution. However, the significant differences in BCL-2 family interactions between full-length proteins at membranes compared with truncated proteins in solution described above extend to interactions with drugs. For example, even though ABT-263 and the functionally similar BH3 mimetic ABT-737 displace BID and BAD, unlike what was seen *in vitro* using peptides and truncated proteins, in live cells the compounds do not displace BIM from BCL-2 or BCL-XL.^{115,116} Recently, this result was independently confirmed for BIM with the BCL-2 inhibitor ABT-199 and BCL-XL inhibitor WEHI-539 and extended to include resistance in live cells of PUMA interactions with full-length BCL-2 and BCL-XL, but not C-terminally truncated cytoplasmic anti-apoptotic proteins.¹¹⁷ These studies are consistent with the tenets of the embedded together model in which membrane binding alters the functional interactions of BCL-2 family proteins via conformation changes that alter binding affinities. It has not been determined whether membrane binding, additional interactions outside of the BH3 domains or both contribute to the apparent enhanced affinity between these BCL-2 family proteins at membranes. However, these studies highlight the need to examine BCL-2 family interactions, and their pharmacological manipulation, in live cells.

Modeling the dance of death

Pharmacological manipulation of the BCL-2 family activities will be limited until there is a more quantitative understanding of how BCL-2 family proteins regulate apoptosis in cells. Quantitative modeling in combination with experimental data can be used to explain, predict and understand the behavior of complex biological processes while validating and

discriminating competing models. Quantitative modeling was successfully used to explain the substantial cell-to-cell variability in cell death kinetics upon induction of extrinsic apoptosis.⁵ A similar approach using Bayesian statistics discriminated between the competing models of BCL-2 family interactions by incorporating previously published binding affinities between BCL-2 family proteins.¹¹⁸ This study concluded that only the 'embedded together' model reproduces MOMP dynamics observed in single-cells. These quantitative models provided important insights into the complex regulation of the BCL-2 family proteins that likely would have been missed otherwise. The large number of BCL-2 family proteins and differences in their binding affinities and mechanisms of action make predicting the apoptotic response of cells very difficult. We expect additional fine tuning of quantitative models will require inclusion of all (or most) of the determinants discussed in this review such as the range of concentrations of BCL-2 family proteins and their various affinities and binding partners in cells. As dysregulation of BCL-2 family proteins has wide-ranging implications in human disease, as difficult as it will be, quantitatively modeling the 'dance of death' by the Bcl-2 family proteins will be required to understand how apoptosis can be modulated to most effectively treat human disease.

Conflict of Interest

The authors declare no conflict of interest.

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