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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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?
BCL-2 family proteins and the dance towards death

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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-apoptotic 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-XL, 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 predominating 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-XL and BAX. We have previously demonstrated that BCL-XL functions as a dominant negative BAX. When cBID, BCL-XL and BAX were incubated together, BAX-mediated membrane permeabilization was completely inhibited. If a mutant of cBID that interacts with BAX but not BCL-XL was used, BAX-mediated membrane permeabilization was not inhibited to the same extent. This suggests that cBID preferentially interacts with BCL-XL over BAX. Using purified full-length recombinant BAX, BCL-XL and cBID we have found that the affinity (dissociation constant, \(K_d\)) between BCL-XL 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. The interaction between cBID and BAX in the absence and presence of BCL-XL can be modeled using these \(K_d\) values (Figure 3a). The higher affinity of cBID for BCL-XL versus BAX prevents BAX activation and only a supraphysiological concentration of BAX can out-compete BCL-XL for cBID binding. This simple example does not account for interactions between BAX and BCL-XL, the differing affinities of the proteins for membranes, changing conformations within the bilayer or the addition of sensitiser 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. Adding to the confusion, different peptide lengths are often used for BH3-only proteins within the same paper. 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. 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, when endogenous levels reported between 3 nM and 170 nM, it appears that the low nanomolar statements are not well supported by data. 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-XL or BCL-W, whereas BAD binds BCL-2, BCL-XL and BCL-W but not MCL-1 or BFL-1/A1. 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-XL 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-XL and 250 nM for BCL-2, demonstrating full-length NOXA binding selectivity but not specificity. 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.

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. 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 sensitiser 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. Furthermore, activator BH3-only proteins promote the membrane-embedded...
conformation of anti-apoptotic proteins and thus also ‘activate’ these proteins. 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. 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. 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. However, both NOXA and the bona fide activator BID, BID can activate BAX to permeabilize membranes in vitro, causing NOXA to be classified as an activator BH3-only protein in some reports. 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. 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. The creation of SAHBs (Stabilized Alpha-Helices of BH3 domains) highlighted the importance of BH3 domain secondary structure. 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. 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. Tethering a 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. 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. 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.

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. 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
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

| Ligands | Receptors |
|---------|-----------|
| BAX     | BAK       |
| BIM     | BID       |
| PUMA    | NOXA      |
| BMF     | BAD       |
| HRK     | BIK       |
| BECLIN-1| BNIP      |

| Affinity | length BH3 | Reference |
|----------|-------------|-----------|
| 10 to 50 | <10 K (nM)  |           |
| 50 to 100| >1000       |           |
| 100 to 300|            |           |
| 300 to 1000|          |           |
| >10000 |                 |           |

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.
BAX or BAK can be activated by antibodies, only proteins. Inviting BAX and BAK to dance without activator BH3-only proteins has a high affinity for membranes and 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. Activator BH3-only proteins have a high affinity for membranes and efficiently activate BAX/BAK at nanomolar concentrations resulting in rapid membrane permeabilization kinetics compared with activation with full-length activator BH3-only proteins. Activator BH3-only proteins bind to the BH3 domain-binding groove resulting in a cytoplasmic localization. Activator BH3-only proteins bind to the BH3 domain-binding groove, displacing α9, which then inserts into the bilayer resulting in BAX adopting a transmembrane configuration in vitro. Activator BH3-only proteins bind to the BH3 domain-binding groove, displacing α9, which then inserts into the bilayer resulting in conditions that spontaneously activate BAX and BAK.

### Table 1B: PubMed ID number and method used to measure the KD 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     | 23926939   |                                |
| R     | 23996493   |                                |
| S     | 21060336   |                                |
| T     | 17337444   |                                |
| U     | 18589438   |                                |
| V     | 17389404   |                                |
| W     | 28411240   |                                |
| X     | 18641390   |                                |
| Y     | 20363230   | Yeast surface display          |
| 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                          |

**Inviting BAX and BAK to dance without activator BH3-only proteins.** BAX or BAK can be activated by antibodies, heat, pH, detergents and metabolites, 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. 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-, 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. 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.

**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). The conundrum arises...
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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{20,60,77} These studies measured the entire population of BAX and BID via FRET (Förster resonance energy transfer) thus, it remains possible that there are subpopulations, 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.\textsuperscript{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.\textsuperscript{76} 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. 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. This is consistent with biochemical data where BAX/BAK symmetrical homodimers can be disulfide cross-linked into higher-order oligomers and with structural data of active BAX/BAK at membranes. In addition, the BAX/BAK inhibitors MSN-125, MSN-50 and DANO04 prevent BAX oligomerization but not symmetrical dimer formation. 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 and another in a separate dimer such as α1:α6, α1:α12, α3:α5 and α9:α9. The interface formed between α9 helices within the bilayer is particularly important for stabilizing large pores. Deletion of α9 or mutations to α9 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). Consistent with observations that BAX/BAK form dynamic pores that enlarge over time with variable sizes dependent on BAX/BAK concentration, direct visualization of BAX in cells revealed that homo-oligomers form lines, arcs and rings of various sizes on mitochondria. 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. 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. 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 or inhibit apoptosis.

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

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**Table 2** Post-translational modifications of BAX

| Modification and residue | Modified by   | Functional consequence                              | Year       | PMID          |
|-------------------------|---------------|-----------------------------------------------------|------------|---------------|
| Phosphorylation S184    | AKT           | Inactivates BAX, prevents translocation from cytosol| 2004       | 14766748     |
|                         | PKC-zeta      | Inactivates and destabilizes BAX                    | 2007       | 17525161     |
| Phosphorylation S163    | GSK-3P        | Activates BAX by increased targeting to mitochondria| 2004       | 15525785     |
| Phosphorylation T167    | JNK and p38   | Phosphorylation inactivates BAX                     | 2006       | 16709574     |
| Phosphorylation T135 or T140 | Unknown | Unknown. Observed in a pre-malignant (AT1), but not a malignant cell line (CA1a) | 2009 | 19194518 |
| Phosphorylation T22     | Unknown       | HTS, needs validation                               | 2011       | 21712546     |
| Phosphorylation T85     | Unknown       |                                                      | 2015       | 25814448     |
| Dephosphorylation T172 | WIP1          | Downregulates BAX. Inhibits apoptosis                | 2013       | 23907458     |
| Dephosphorylation T174 | PP2A          | Promotes apoptosis                                   | 2006       | 16679323     |
| Cleavage G28/G29        | Calpain       | Releases C-terminal BAX lacking amino acids          | 2003       | 12490315     |
| PolyUbiquitination non-specific site (9 lysines in BAX) | PARKIN | Inhibits apoptosis targets BAX for proteasomal degradation | 1998 | 9438391 |
|                         | IBRDC2        |                                                      | 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   | ER   | NE | TGN | Cytosol | MOM | MM | MIM | Cytoskeleton | Plasma membrane | Nucleus |
|-----------|------|----|-----|---------|-----|----|-----|--------------|-----------------|---------|
| BCL-2     | XX   |    |     |         |     |    |     |              |                 |         |
| BFL-1     |      |    |     |         |     |    |     |              |                 |         |
| BCL-X₁    | XX   |    |     |         | XX  |    |     |              |                 |         |
| BCL-W     |      |    |     |         |     |    |     |              |                 |         |
| MCL-1     |      |    |     | XX     |     |    |     |              |                 |         |
| BCL-G     |      |    |     | XX     |     |    |     |              |                 |         |
| BAX       | XX   |    |     | XX     |     |    |     |              |                 |         |
| BAK       | XX   |    |     | XX     |     |    |     |              |                 |         |
| BOK       | XX   |    |     |         |     |    |     |              |                 |         |
| BMF       |      |    |     |         |     |    |     |              |                 |         |
| BIM       |      |    |     |         |     |    |     |              |                 |         |
| BID       | XX   |    |     |         |     |    |     |              |                 |         |
| PUMA      |      |    |     | XX     |     |    |     |              |                 |         |
| NOXA      |      |    |     | XX     |     |    |     |              |                 |         |
| HRK       |      |    |     | XX     |     |    |     |              |                 |         |
| BAD       | XX   |    |     |         |     |    |     |              |                 |         |
| BIK       |      |    |     | XX     |     |    |     |              |                 |         |
| BECLIN-1  | XX   |    |     |         |     |    |     |              |                 |         |
| BNIP      |      |    |     |         |     |    |     |              |                 |         |
| SPIKE     |      |    |     | XX     |     |    |     |              |                 |         |
| BAP31     | XX   |    |     |         |     |    |     |              |                 |         |
| BCL-G     |      |    |     |         |     |    |     |              |                 |         |
| BFK       | XX   |    |     |         |     |    |     |              |                 |         |
| BCL-Rambo |      |    |     |         |     |    |     |              |                 |         |

**Abbreviations**
- ER: Endoplasmic Reticulum
- NE: Nuclear Envelope
- MOM: Mitochondrial Outer Membrane
- MIM: Mitochondrial Inner Membrane
- MM: Mitochondrial Matrix
- TGN: trans Golgi network

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.
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). However, loss of either protein results in protection from anoikis and resistance to cytoskeleton altering drugs like paclitaxel.\textsuperscript{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.\textsuperscript{97} The mechanism by which BCL-2 proteins regulate ER stress and calcium homeostasis and how this relates to apoptosis is not clear.\textsuperscript{98} BCL-X\textsubscript{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.\textsuperscript{98} There is accumulating evidence that this interaction is mediated by the BH4 domain of the multi-domain anti-apoptotic proteins.\textsuperscript{99} Moreover, BCL-2 and BCL-X\textsubscript{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).\textsuperscript{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.\textsuperscript{101} BAX/BAK and BH3-only proteins may oppose calcium leakage by binding BCL-2/BCL-X\textsubscript{L} or IP3R, increasing calcium stores and promoting apoptosis.\textsuperscript{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.\textsuperscript{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. 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. Small molecule BH3 mimetics, like ABT-263 (Nivitoclax) 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. 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.

Some cancers depend primarily on MCL-1 for survival and others acquire resistance to drugs that target BCL-2/BCL-XL/BCL-W by upregulating MCL-1. The small molecule MCL-1 inhibitor, S63845 shows promise as a therapeutic. 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.

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. 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. 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. 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. 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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