

## Interplay of Bcl-2 Proteins Decides the Life or Death Fate

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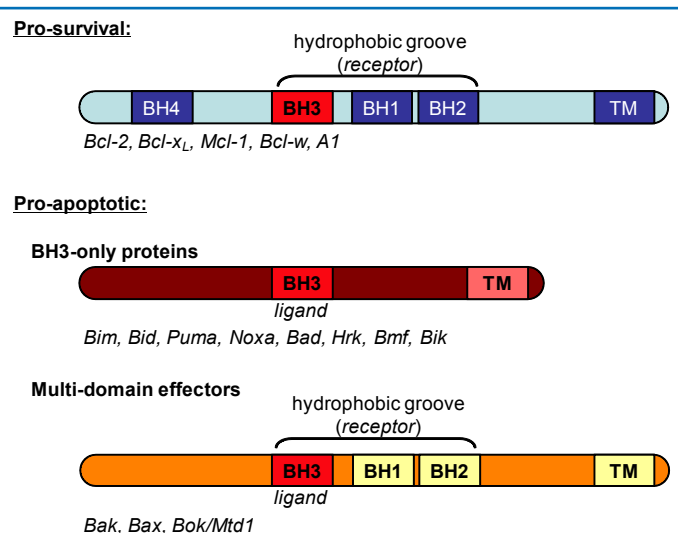
Ever since the discovery that the founding member of the Bcl-2 family of proteins contributes to tumour development by inhibiting cell death rather than by encouraging proliferation (1), interest (and controversy) in these functionally diverse homologues has raged. Characterised by conservation within Bcl-2 homology (BH) domains, the Bcl-2 proteins fall into three subgroups (Fig. 1): 1) the multi-domain pro-survival proteins (Bcl-2, Bcl-x<sub>L</sub>, Mcl-1, A1, Bcl-w); 2) the BH3-only proteins (e.g., Bim, Bid, Puma, Noxa, Bad), which are regulated transcriptionally or post-translationally to initiate the apoptotic response, and thereby act as the sensors of cellular damage; and 3) the multi-domain pro-apoptotic effectors (Bak, Bax, Bok/Mtd1).

In response to disparate death stimuli, including growth factor deprivation (discussed by Paul Ekert in this Showcase on Research) and DNA damage, the interplay of Bcl-2 proteins mediates the integrity of the mitochondrial outer membrane (MOM). Consequently, an apoptotic cell is on the receiving end of a two-pronged attack. Firstly, a breached mitochondrial membrane ensures loss of oxidative phosphorylation and therefore energy production. Secondly, apoptogenic factors such as cytochrome *c* and Smac/DIABLO are released from the intermembrane space to activate proteolytic caspases that orchestrate destruction of the cell. Disruption of the cell's power plant appears to be of paramount importance to the final outcome, because even if caspase activity is inhibited, the majority of cells with permeabilised MOM are destined to die. Bak and Bax therefore deliver the *coup de grace* by irreparably damaging the MOM. Despite intense investigation and numerous proposed mechanisms, how Bak and Bax deliver the death blow is unknown. Although it seems clear that the balance of pro-survival and pro-apoptotic proteins governs the life-death decision, how the complex interplay of Bcl-2 proteins allows for Bak and Bax activity to be tempered in a healthy cell and unleashed in response to cytotoxic insult is controversial. Determining how and when (or indeed if) these proteins interact is pivotal for deciphering the control of the apoptotic pathway and for potential therapeutic intervention.

### Bak and Bax - the Harbingers of Doom

Bak and Bax functionally overlap and, to an extent, can substitute for each other in their ability to cause mitochondrial perturbation and cell death. Thus the single knockout mice phenotypes are rather mild; *bak*<sup>-/-</sup> mice have no overt phenotype except modest thrombocytosis, whilst *bax*<sup>-/-</sup> mice exhibit lymphoid hyperplasia and defects in spermatogenesis (2-4), and cells from these mice are susceptible to most forms of cell death. In contrast, mice lacking both Bak and Bax die either soon after birth on a mixed background (3) or before birth on an inbred C57BL/6 background (David Huang, unpublished data), and cells from these mice are completely resistant to most activators of the intrinsic cell death pathway. The close relative Bok/Mtd1 is less well understood. Although it cannot substitute for Bak or Bax in fibroblasts (Grant Dewson, unpublished data), it remains to be seen whether Bok/Mtd1 plays a more cell type-specific Bak/Bax-like role during cell death.

Bak and Bax adopt a similar fold as their inactive conformer (Fig. 2A) (5,6), but despite both conserved structure and function, they reside in distinct subcellular compartments in a healthy cell. Whereas Bak is constitutively integrated into the MOM, Bax is normally cytosolic and translocates to mitochondria only after apoptotic challenge. In their native



**Fig. 1. Regulators of apoptosis: the Bcl-2 family.**

The Bcl-2 proteins form a complex network of interactions that govern susceptibility to death stimuli. Bcl-2 homologues are characterised by regions of sequence homology, the Bcl-2 homology (BH) domains 1-4. The majority of Bcl-2 homologues also have a hydrophobic C-terminal transmembrane (TM) domain that anchors them to intracellular membranes. Pro-survival proteins, such as Bcl-2, have BH domains 1-4. Pro-survival proteins have a hydrophobic groove derived from BH domains 1-3 that acts as a receptor surface for BH3 domain ligands from specific BH3-only proteins. The pro-apoptotic BH3-only proteins share little sequence homology except in the BH3 domain. These proteins sense cellular stress and are activated either by transcriptional upregulation (e.g., Noxa, Puma) or post-translational modification, such as phosphorylation (e.g., Bad) or proteolytic cleavage (Bid is converted to its truncated form, tBid). The pro-apoptotic effector proteins Bak, Bax and Bok/Mtd1 have BH1-3 domains and are often described as multi-domain pro-apoptotic proteins. Bak and Bax seemingly have both the receptor and ligand attributes, allowing their critical self-association during cell death.

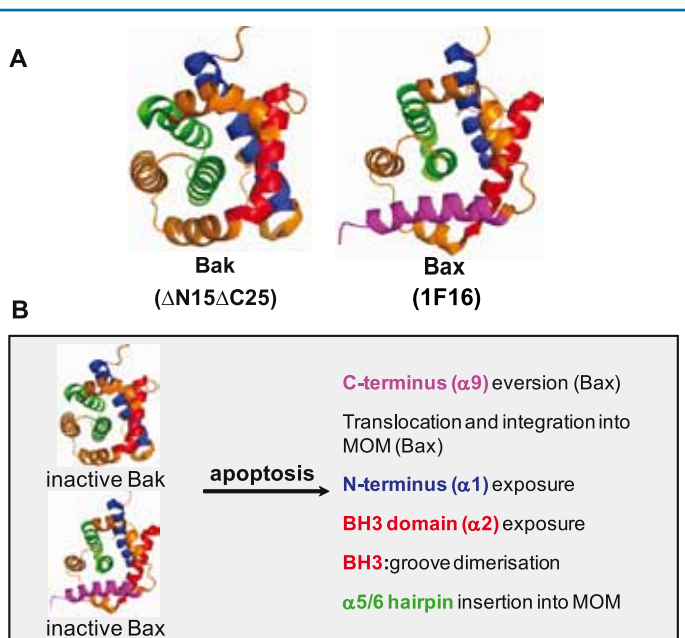
state, Bak and Bax are metastable with both undergoing structural changes to attain their activated states during cell death. Biochemical analyses have revealed that activation results in significant alterations to the N-terminus, exposure of the normally buried BH3 domain and reorientation of the core  $\alpha 5/6$  helices (Fig. 2B) (7-10). Unfortunately, due largely to the difficulties in obtaining structures of membrane-associated proteins, there is currently no high resolution structure for this activated form.

What provides the initial driving force for the transition to the activated conformation is a prevailing controversy in the field. Interaction with 'activator' BH3-only proteins Bim, tBid and Puma, or with non-Bcl-2 proteins such as p53, is a potential activating mechanism (11,12). Alternatively, post-translational modification or changes in cellular environment such as hyperthermia or an increase in cellular pH may provide the impetus (13-15). Once activated, Bak and Bax may interact with the inactive pool, resulting in auto-activation that amplifies the apoptotic response.

### Restraining Bak and Bax Pro-apoptotic Function

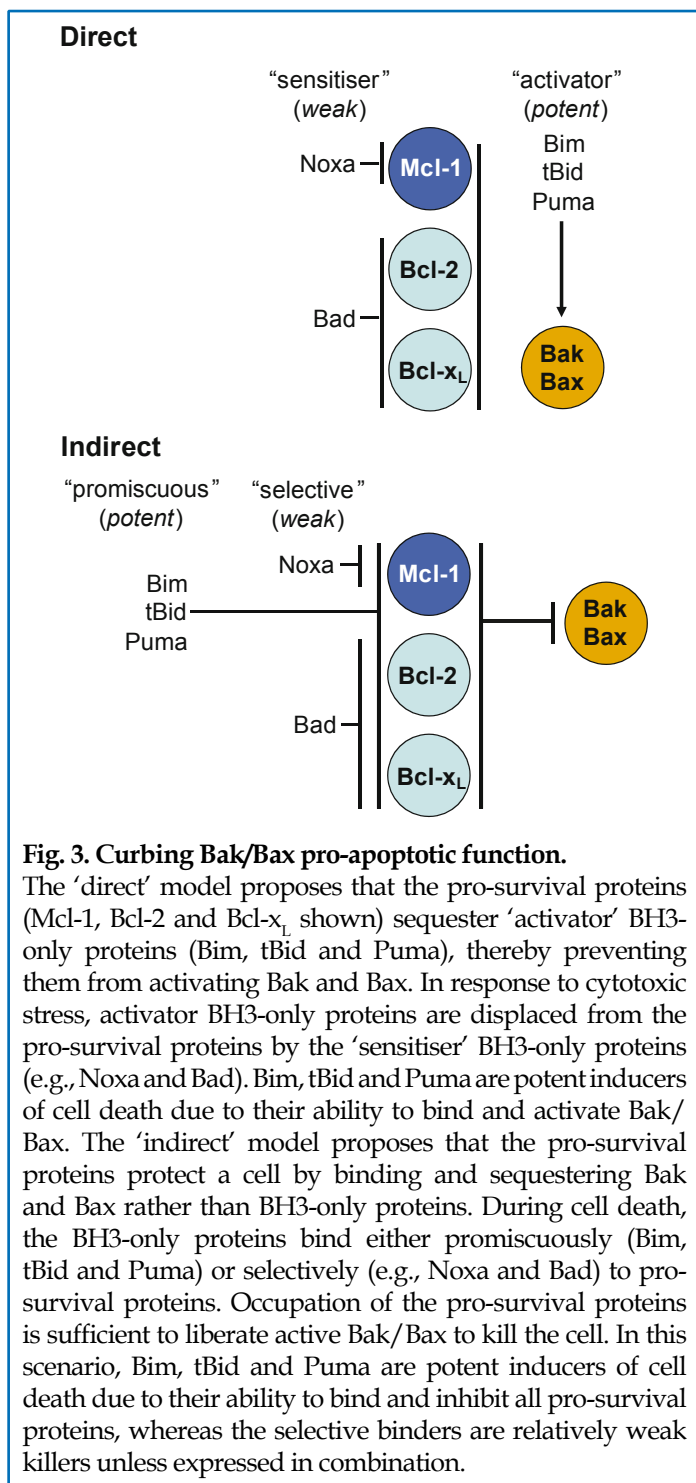
As the activity of Bak and Bax is the point of no return in an apoptotic response, its proper control is clearly crucial. Ineffectual restraint results in excessive cell death and can contribute to degenerative disorders, whilst excessive restraint inappropriately inhibits cell death and can contribute to tumourigenesis. At present, there are two models on curbing Bak/Bax pro-apoptotic function (Fig. 3), although neither model is entirely consistent with all available data. The 'direct' model proposes that BH3-only proteins belong to one of two distinct classes, the aforementioned 'activators' (Bim, tBid, Puma) and the 'sensitisers' (Bad, Noxa). The pro-survival proteins temper Bak/Bax activation by sequestering the activator BH3-only proteins. During an apoptotic response, when displaced by the sensitiser, the activators bind to Bak and Bax, causing their activation. However, the transient 'hit and run' nature of this interaction has made it difficult to confirm in cells. This model is consistent with the greater propensity of the activator BH3-only proteins to induce cell death upon overexpression compared to the sensitiser BH3-only proteins. Inconsistent with this model is the finding that the putative activator BH3-only proteins are seemingly not required for Bax and Bak activation (16).

Alternatively, the 'indirect' model proposes that the pro-survival proteins protect cells by binding Bak/Bax. The capacity of BH3-only proteins to induce apoptosis is due solely to their selective binding to pro-survival proteins. Upon upregulation, the BH3-only proteins liberate Bak/Bax from pro-survival interactions, allowing them to self-associate and auto-activate. Because Bim, tBid and Puma are promiscuous and bind with high affinity to all pro-survival proteins, while Bad and Noxa are selective and bind only to a subset (17), the greater killing capacity of Bim, tBid and Puma can be explained by their ability to bind and inhibit all pro-survival proteins rather than directly activating Bak or Bax. This model is supported by the observation that Bak/Bax-dependent apoptosis can be initiated by overexpression of Noxa and Bad in combination in the absence of the activator BH3-only proteins (16). However, in healthy cells, Bax is generally regarded as monomeric, and Bak is not predominantly sequestered by pro-survival proteins. Furthermore, this model requires that liberated Bak or Bax already be in its active conformation or at least have undergone some conformation change. Structural analysis suggests Bak inserts its BH3 domain into the hydrophobic groove of Bcl-x<sub>L</sub> (18) (although this has not been confirmed in a complex of full-length proteins). Thus this Bak/Bax conformation at least involves exposure of the normally buried BH3 domain. So what serves as the initial activating stimulus in this case? The inherent instability of Bak and Bax may allow them to spontaneously activate, with erroneously activated Bak/Bax being restrained from self-association by binding to pro-survival proteins until inhibition is relieved upon cytotoxic stress. A propensity to spontaneously activate may explain why Bak and Bax can become activated to kill a cell in the absence of the putative activator BH3-only proteins simply by occupying their pro-survival guardians (16). Therefore, interaction with direct activators or, alternatively, post-translational modification may expedite, but may not be necessary for, Bak/Bax activation.



**Fig. 2. Bak and Bax change conformation significantly during apoptosis.**

- A.** Inactive Bak and Bax adopt a similar tertiary structure. Structures of Bak ( $\Delta N15/\Delta C25$ , 2IMS) and Bax (1F16) as determined by crystallography and nuclear magnetic resonance, respectively (5,6). Regions that undergo significant rearrangement during activation are indicated:  $\alpha 1$  (blue),  $\alpha 2$  (BH3, red),  $\alpha 5/6$  (green) and  $\alpha 9$  (TM, magenta). The Bak structure lacks both its N- and C-termini. Images were generated with MacPyMOL.
- B.** Bak/Bax conformation change during apoptosis. Bak and Bax undergo a series of conformation changes that facilitate subcellular redistribution, MOM integration and oligomerisation. Bax is normally cytosolic due to sequestration of its hydrophobic C-terminal TM domain in its groove, so that its activation involves exposure of its TM domain and translocation to and integration into the MOM. In contrast, Bak is constitutively mitochondrial via integration of its C-terminus and therefore bypasses this redistribution phase. The precise order of these events is uncertain.



**Fig. 3. Curbing Bak/Bax pro-apoptotic function.**

The ‘direct’ model proposes that the pro-survival proteins (Mcl-1, Bcl-2 and Bcl-x<sub>L</sub> shown) sequester ‘activator’ BH3-only proteins (Bim, tBid and Puma), thereby preventing them from activating Bak and Bax. In response to cytotoxic stress, activator BH3-only proteins are displaced from the pro-survival proteins by the ‘sensitiser’ BH3-only proteins (e.g., Noxa and Bad). Bim, tBid and Puma are potent inducers of cell death due to their ability to bind and activate Bak/Bax. The ‘indirect’ model proposes that the pro-survival proteins protect a cell by binding and sequestering Bak and Bax rather than BH3-only proteins. During cell death, the BH3-only proteins bind either promiscuously (Bim, tBid and Puma) or selectively (e.g., Noxa and Bad) to pro-survival proteins. Occupation of the pro-survival proteins is sufficient to liberate active Bak/Bax to kill the cell. In this scenario, Bim, tBid and Puma are potent inducers of cell death due to their ability to bind and inhibit all pro-survival proteins, whereas the selective binders are relatively weak killers unless expressed in combination.

### Mitochondrial Perturbation by Bak/Bax Oligomers

Irrespective of the activating mechanism, the resultant conformation change in Bak and Bax facilitates their self-association. Bak/Bax homo-oligomerisation is a consistent feature during apoptosis and these complexes are believed to be responsible for the damage to the MOM. Deciphering how Bak and Bax coalesce to form the killing complex is critical in determining how they permeate the MOM. We have recently shown that during apoptosis, Bak (and Bax) expose their BH3 domains to insert into the hydrophobic groove of a partner molecule (8). We propose that a symmetric BH3:groove homodimer forms the basic oligomeric subunit. How this subunit nucleates to form the larger complexes necessary to permeabilise the MOM is

unclear, but obviously must involve interfaces distinct from both the BH3 domain and groove surface (8).

Bak and Bax can potentially cooperate to kill a cell, as hetero-oligomers of activated Bak and Bax can be detected under certain conditions. Although a wildtype apoptotic response in fibroblasts lacking either Bak or Bax indicates such an association is not necessary for their pro-apoptotic function (19), it is possible that cooperativity accelerates MOM permeabilisation or sensitises certain cells (i.e., those which express limiting Bak and Bax) to apoptotic stimuli.

The exact nature of the Bak/Bax ‘pore’ remains enigmatic, with high molecular weight complexes comprised of potentially hundreds of molecules detected in dying cells (20). Whether the oligomeric complex exists as a finite proteinaceous pore or whether their aggregation non-specifically destabilises the lipid bilayer is disputed, although the concomitant release of a plethora of intermembrane space proteins of varying sizes during cell death may be more consistent with non-specific membrane disruption. At least in artificial membranes, activated Bak or Bax can permeabilise membranes without assistance from other proteins. In cells, however, a variety of mitochondrial proteins have been proposed to assist Bak/Bax in MOM permeabilisation. For example, hijacking of the mitochondrial fission/ fusion machinery via association of Bak/Bax with Drp1 and mitofusins has been proposed (21).

Structural homology of Bcl-2 proteins to the pore-forming domains of bacterial colicins and diphtheria toxin hints at a potential mechanism for membrane perturbation (22). The pore-forming domain theory posits that mitochondrial perturbation is due to the hydrophobic  $\alpha 5/6$  helices of Bax/Bak inserting into the MOM. Indeed, Andrews and colleagues have elegantly shown membrane insertion of  $\alpha 5/6$  of Bax, and intriguingly also Bcl-2, in response to an apoptotic stimulus (7,23). The pore-forming toxins are thought to form lipidic ‘toroidal’ pores, whereby lipid bilayer curvature results in a solvent-exposed pore surface comprised of both  $\alpha$ -helical peptides and phospholipid headgroups. Bcl-2 homologues are reported to induce similar membrane curvature, with a Bax  $\alpha 5$  peptide recently shown to induce a lipid pore structure (24). As both pro-apoptotic Bax and pro-survival Bcl-2 similarly insert their  $\alpha 5/6$  into MOM, what distinguishes a pro-apoptotic protein from a pro-survival protein is unclear, but presumably relates to their ability to oligomerise and nucleate a ‘pore’. As  $\alpha 5$  forms the backbone of the hydrophobic groove of Bak and Bax, its insertion into the MOM may disrupt this critical binding surface. Therefore, what role BH3:groove-mediated oligomerisation plays in  $\alpha 5/6$  insertion is currently unclear.

### It’s a Groove Thing

While it is clear that the intricate interplay of the Bcl-2 family of proteins exerts critical control over whether a cell lives or dies, controversy still reigns regarding what the crucial interactions are and when they occur. There is substantial evidence that the interplay of Bcl-2 proteins occurs via BH3:groove associations, including structural and binding studies that show pro-survival proteins are able to bind BH3-only proteins, as well as Bak and Bax, in this way. More recently, we have shown that Bak and Bax self-association also involves a BH3:groove interaction. As

the hydrophobic groove of Bak/Bax can act as a receptor surface for BH3 domains, insertion of the BH3 domain from activator BH3-only proteins into the groove of Bak/Bax is therefore a plausible mechanism for direct activation. As Bak/Bax activation involves exposure of the BH3 domain, this is likely to disrupt the binding groove and thereby provide a molecular mechanism for displacing the BH3-only protein, as proposed by the 'hit and run' model. Somewhat surprisingly, a recent study showed that a BH3 peptide from Bim binds to Bax  $\alpha$ -helices 1 and 6, rather than its groove (25). Whether such an interface is retained in full-length proteins and is conserved for direct activation of Bak, and how this binding signal is subsequently translated into the Bak/Bax structural alterations outlined in **Fig. 1**, is of intense interest.

Insight into the molecular interactions of the Bcl-2 homologues is critical in understanding how the apoptotic machinery is regulated and therefore how it can be manipulated for therapeutic benefit. The recent development of BH3 mimetic compounds that interfere with the interactions between the Bcl-2 proteins heralds a new era for targeted cancer therapy. Conservation of the BH3 domain:groove interaction highlights the need for a clear understanding of the molecular interplay of these critical proteins. These myriad associations ultimately regulate Bak/Bax activity and thus commitment to death. Elucidating the structure of the activated, oligomerised conformer of Bak/Bax, importantly in the context of a membrane, will finally reveal how Bak and Bax permeabilise the MOM and assert their deadly influence.

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