

THE BCL2 FAMILY: REGULATORS OF THE CELLULAR LIFE-OR-DEATH SWITCH

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Tissue homeostasis is regulated by apoptosis, the cell-suicide programme that is executed by proteases called caspases. The Bcl2 family of intracellular proteins is the central regulator of caspase activation, and its opposing factions of anti- and pro-apoptotic members arbitrate the life-or-death decision. Apoptosis is often impaired in cancer and can limit conventional therapy. A better understanding of how the Bcl2 family controls caspase activation should result in new, more effective therapeutic approaches.

Selective cell suicide is crucial for sculpting the embryo, maintaining tissue homeostasis, shaping the immune repertoire, terminating immune responses and restricting the progress of infections. Moreover, disturbed regulation of this vital physiological process underlies many diseases, including cancer, autoimmunity and degenerative disorders. As cells perform their choreographed 'dance of death', they shrink and bleb violently, undergoing chromatin condensation and internucleosomal DNA cleavage, before being tidily packaged into vesicles that are rapidly engulfed by other cells¹. Although the importance of cell death during development had long been recognized, Kerr, Wyllie and Currie were the first to propose that the stereotypic nature of 'apoptosis', as they coined the process, reflected an underlying genetic programme¹.

The cancer connection

A central player in that genetic programme, and the link between apoptosis and cancer, emerged when *BCL2* (B-cell lymphoma 2), the gene that is linked to an immunoglobulin locus by chromosome translocation in follicular lymphoma, was found to inhibit cell death, rather than promote proliferation². This unexpected discovery gave birth to the concept, now widely embraced³⁻⁶, that impaired apoptosis is a crucial step in tumorigenesis. Indeed, a defective suicide programme endows nascent neoplastic cells with multiple selective advantages. The cells can persist in hostile niches (for

example, where cytokines or oxygen are limiting), escape the death that is often imposed as a fail-safe mechanism by other oncogenic changes and evolve into more-aggressive derivatives. Finally, defective apoptosis facilitates metastasis, because the cells can ignore restraining signals from neighbours and survive detachment from the extracellular matrix. So, neoplastic progression in no small measure reflects loss of normal apoptotic mechanisms.

Impaired apoptosis is also a significant impediment to cytotoxic therapy^{6,7}. The mutations that favoured tumour development dampen the response to chemotherapy and radiation, and treatment might select more refractory clones. Nevertheless, most tumour cells still remain sensitive to some apoptotic stimuli, and more rational therapy should emerge from clarifying how particular agents elicit apoptosis and which apoptotic pathways remain open in individual tumours.

So, how do *Bcl2* and related proteins monitor cellular well-being and decide whether the suicide programme should be activated? Re-evaluation of this hotly debated issue⁸⁻¹⁵ is timely, because recent findings challenge many prevailing notions. How oncogenic changes impinge on the Bcl2 family, how impaired apoptosis affects therapy and how direct targeting of these regulators could lead to more effective treatment of cancer and other diseases in which apoptosis is perturbed are also explored.

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Death circuits

The molecular circuitry for apoptosis began to emerge when an exciting convergence of studies in mammals and the nematode *Caenorhabditis elegans*¹⁶ revealed that the worm protein **CED-9** is the functional homologue of Bcl2 (REF. 17), and that CED-9 regulates activation of an aspartate-directed cysteine protease (**CED-3**)¹⁸, now called a caspase. To prevent unscheduled cell suicide, each caspase is synthesized as a pro-enzyme that typically requires processing at caspase cleavage sites to generate the active enzyme¹⁹. Once an initiator caspase is activated, it processes others that cleave a host of cellular proteins. So, a chain reaction of caspase activation is the cell's death sentence.

So far, two principal pathways for activating caspases have been discovered (FIG. 1 and BOX 1). The more ancient, which is induced by diverse intracellular stresses, including cytokine deprivation and genotoxic damage, is regulated by Bcl2 and its relatives. Progression through the pathway usually leads to the activation of **caspase-9** on a scaffold that is formed by apoptotic protease-activating factor 1 (**Apaf1**). This activation occurs after Apaf1 has interacted with cytochrome *c* that is released from damaged mitochondria²⁰. A more-recently evolved pathway is triggered when 'death receptors' on the plasma membrane, engaged by cognate ligands of the tumour-necrosis factor (**TNF**) family, recruit **caspase-8** through the adaptor protein **FAS-associated death domain (FADD)**²¹.

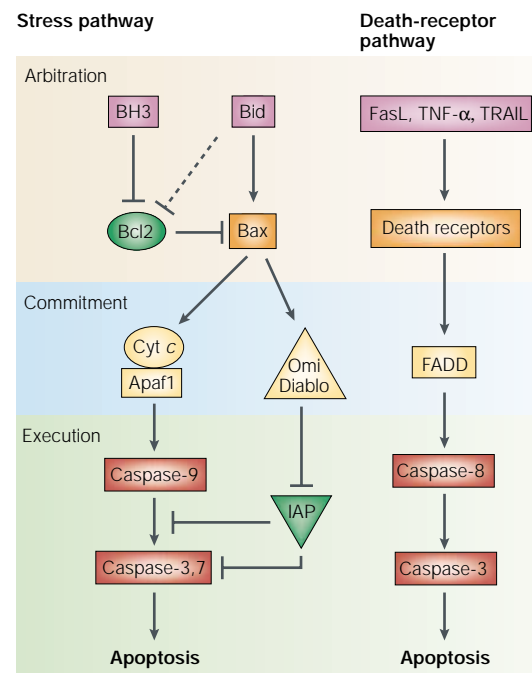


Figure 1 | **Two main pathways to apoptosis.** Intracellular stress signals are mediated through the Bcl2 family, whereas the death-receptor pathway is activated by signals from other cells. The Bcl2 family does not directly regulate the death-receptor pathway^{12,21,153}, although caspase-8 can make a link by activation of Bid in certain cell types (see text for details). Activity of caspase-9 and caspase-3 is restrained by inhibitor of apoptosis proteins (IAPs), but the IAPs can be countermanded by Diablo/Smac and Omi/HtrA2, which are released from damaged mitochondria¹⁹. Apaf1, apoptotic protease-activating factor 1; cyt *c*, cytochrome *c*; FADD, Fas-associated death-domain; FasL, Fas ligand; TNF- α , tumour-necrosis factor- α ; TRAIL, tumour-necrosis-factor-related apoptosis-inducing ligand.

Summary

- **Apoptosis, the cell-death programme that is mediated by proteases called caspases, is essential for tissue homeostasis, and its perturbed regulation underlies many diseases, including cancer. Commitment to apoptosis in response to diverse physiological cues and cytotoxic agents is governed by proteins of the Bcl2 family.**
- **Bcl2 and several pro-survival relatives associate with the mitochondrial outer membrane and the endoplasmic reticulum/nuclear membrane and maintain their integrity. Initiation of apoptosis requires not only pro-apoptotic family members such as Bax and Bak that closely resemble Bcl2, but also distant cousins that are related only by the small BH3 protein-interaction domain.**
- **The BH3-only proteins are sentinels that detect developmental death cues or intracellular damage. In healthy cells, they are restrained in diverse ways, including sequestration on the cytoskeleton. When unleashed by death signals, they switch off survival function by inserting their BH3 domain into a groove on their pro-survival relatives.**
- **Either Bax or Bak is required for apoptosis, but how they are activated or countermanded by Bcl2 remains uncertain. During apoptosis, Bax and Bak oligomerize in the mitochondrial outer membrane and probably breach its integrity, freeing pro-apoptotic proteins such as cytochrome *c*, which allows activation of caspase-9.**
- **The pro-survival Bcl2-like proteins can prevent cytochrome *c* release, and hence caspase-9 activation. They probably also regulate the activation of several other caspases, independently of mitochondrial damage.**
- **Impaired apoptosis is a central step towards neoplasia. Pro-survival Bcl2-like proteins can promote tumorigenesis, and certain pro-apoptotic relatives act as tumour suppressors. Moreover, the expression of family members is affected by other tumorigenic alterations (for example, p53 mutation).**
- **Conventional cytotoxic therapy indirectly induces apoptosis, but more effective outcomes should be achieved by direct activation of the apoptotic machinery. Promising approaches include impairing expression of pro-survival Bcl2-like proteins or identifying drugs that mimic the action of BH3-only proteins.**

The Bcl2 clan

In mammals, Bcl2 has at least 20 relatives, all of which share at least one conserved Bcl2 homology (BH) domain (FIG. 2). The clan includes four other anti-apoptotic proteins: **Bcl-x_L**, **Bcl-w**, **A1** and **Mcl1**, and two groups of proteins that promote cell death: the **Bax** and the BH3-only families. Members of the Bax death family²² have sequences that are similar to those in Bcl2, especially in the BH1, BH2 and BH3 regions, but the other pro-apoptotic proteins have only the short BH3 motif (hence their name) — an interaction domain that is both necessary and sufficient for their killing action. Both types of pro-apoptotic proteins are required to initiate apoptosis: the BH3-only proteins seem to act as damage sensors and direct antagonists of the pro-survival proteins, whereas the Bax-like proteins act further downstream, probably in mitochondrial disruption (see below).

The pro-survival family. Bcl2 and its closest homologues, Bcl-x_L and Bcl-w, potently inhibit apoptosis in response to many, but not all, cytotoxic insults (FIG. 1). Their hydrophobic carboxy-terminal domain helps

Box 1 | Caspases: the engine of cellular destruction

The dozen or so caspases in a mammal are synthesized as inactive precursors^{19,148}. The long prodomain on those that initiate apoptosis promotes self-association and binding to activating adaptor or scaffold proteins. When procaspase-8 molecules are concentrated through their recruitment to ligated death receptors by Fas-associated death domain (FADD), they undergo autocatalysis, releasing the p10 and p20 subunits that form the active (tetrameric) enzyme. Caspase-9 is instead activated — in the presence of ATP and cytochrome *c* — by an allosteric change on a heptameric scaffold of apoptotic protease-activating factor 1 (Apaf1) proteins termed the apoptosome^{118,149,150}. Effector caspases (3, 6 and 7) have short prodomains and are activated by the initiator caspases; once processed by caspase-8 or -9, caspases -3 and -7, in turn, process caspase-6. Other caspases with a long prodomain (1, 2, 4, 5 and 10 in humans and 1, 2, 11 and 12 in mice) might also serve as initiators (see text for details), as might granzyme B, a serine protease that is released from cytotoxic lymphocytes¹⁵¹.

SCAFFOLD PROTEINS
Proteins that provide a platform for the assembly of other proteins.

target them to the cytoplasmic face of three intracellular membranes: the outer mitochondrial membrane, the endoplasmic reticulum (ER) and the nuclear envelope. Bcl2 is an integral membrane protein, even in healthy cells²³, whereas Bcl-w and Bcl-x_L only become tightly associated with the membrane after a cytotoxic signal (J. Wilson-Annan and D. Huang, unpublished observations); this is indicative of an induced conformational change. The core three-dimensional structure is well conserved between Bcl-x_L²⁴, Bcl2 (REF. 25) and Bcl-w (C. Day, D. Huang and M. Hinds, personal communication) — as well as a viral Bcl2 homologue²⁶ — and comprises a globular bundle of five amphipathic α -helices that surround two central hydrophobic α -helices (FIG. 3a). Notably, a hydrophobic groove, formed by residues from BH1, BH2 and BH3, can bind the BH3 α -helix of an interacting BH3-only relative²⁷ (FIG. 3b). In Bcl-w, at least, the groove can be occupied by its carboxy-terminal tail (C. Day, D. Huang and M. Hinds, personal communication), as is the case with

Bax²⁸ (FIG. 3c), so the BH3 ligand might need to displace the tail. The less well studied A1 and Mcl1 seem to have weaker survival activity and are more divergent in sequence — perhaps indicative of additional functions.

It is becoming increasingly evident that every nucleated cell requires protection by at least one Bcl2 homologue, and that the abundance of these 'guardians' regulates tissue homeostasis. Bcl2 overexpression in haematopoietic lineages yields excess B, T and myeloid cells that are refractory to diverse cytotoxic insults^{29–33}. Conversely, inactivation of the Bcl2 homologous genes augments apoptosis in specific cell types, presumably because the concentrations of other guardians are too low to compensate. Bcl2 itself is required for the survival of kidney and melanocyte stem cells and mature lymphocytes³⁴, Bcl-x_L for neuronal and erythroid cells³⁵, Bcl-w for sperm progenitors in adult mice^{36,37}, an A1 gene for neutrophils³⁸ and Mcl1 for zygote implantation³⁹.

The BH3-only tribe. BH3-only proteins seem to be sentinels that are charged with triggering apoptosis in response to developmental cues or intracellular damage⁴⁰. All programmed death of somatic cells in *C. elegans* requires the single BH3-only protein EGL-1 (REF. 41). The eight or more mammalian BH3 proteins (FIG. 2) — most of which are widely expressed — presumably allow more-refined control over cell death. With the possible exception of Bid (see below), they are thought to act by binding to and neutralizing their pro-survival relatives. Perhaps the small allosteric change that is induced in the pro-survival proteins by the engagement of a BH3 protein affects their association with another protein (see below). The BH3-only proteins cannot kill in the absence of Bax and Bak^{42,43}, and hence must function upstream in the same pathway.

Individual BH3-only proteins are normally held in check by diverse mechanisms (FIG. 4). Bim and Bmf are sequestered by binding to dynein light chains that are associated with the microtubules (Bim) and actin cytoskeleton (Bmf)^{44,45}. Bad, after phosphorylation by kinases such as Akt and protein kinase A, is bound by 14-3-3 SCAFFOLD PROTEINS⁴⁶, whereas Bid is relatively inactive until proteolytically cleaved^{47,48}. Noxa, Puma and Hrk/DP5, however, are controlled primarily at the transcriptional level^{49–52}, as is their worm counterpart EGL-1 (REF. 41).

Initial knockout studies indicate that individual BH3-only proteins could have specialized physiological roles. Bid, although dispensable for proper development and tissue homeostasis, facilitates the death of hepatocytes that is provoked by anti-Fas antibody⁵³. Bim is a principal regulator of haematopoietic homeostasis⁵⁴: in its absence, leukocyte numbers rise and plasma-cell accumulation provokes the onset of an autoimmune disease that is equivalent to that elicited by the overexpression of Bcl2 (REF. 31); this onset probably occurs, in part, because Bim is essential for the elimination of autoreactive lymphocytes⁵⁵. Bim also participates in neuronal death⁵⁶.

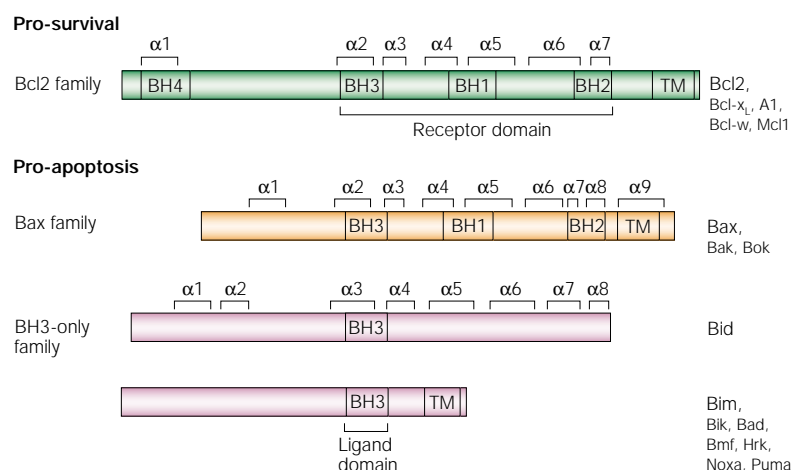


Figure 2 | **Three subfamilies of Bcl2-related proteins.** Known α -helical regions are indicated, as are the four regions (BH1–4) that are most highly conserved among family members. Most members have a carboxy-terminal hydrophobic domain that aids association with intracellular membranes, the exceptions being A1 and many of the BH3-only proteins (Bad, Bid, Noxa, Bmf and Puma). Several other multidomain homologues (for example, Boo/Diva, Bcl-Rambo, Bcl-G, Bcl-B) have been described, but their function is not yet clear. TM, transmembrane domain.

ANOIKIS
Death promoted by detachment from the extracellular matrix.

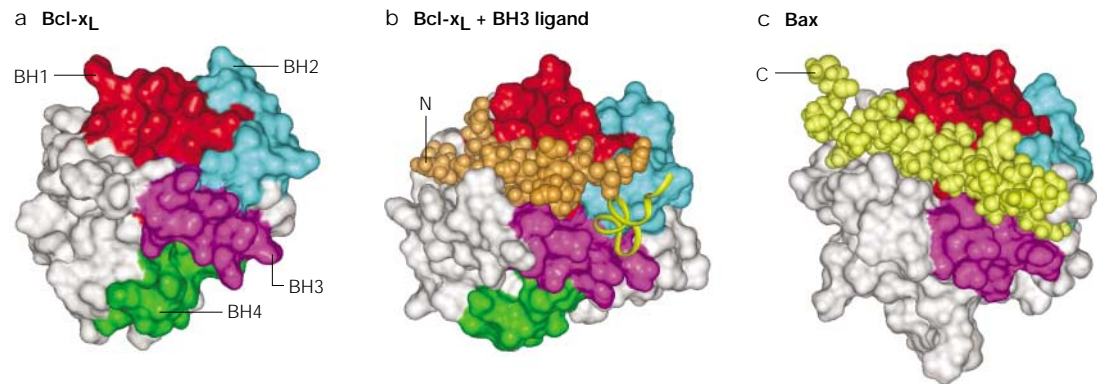


Figure 3 | Three-dimensional structures of Bcl-x_L and Bax, showing their similarity. **a** | Bcl-x_L, without its carboxy (C)-terminal tail and the unstructured loop between the BH4 and BH3 regions²⁴ (see FIG. 2). **b** | Bcl-x_L with the BH3 peptide of Bak (brown) bound to its surface groove²⁷. The yellow ribbon indicates Bcl-x_L residues that precede the hydrophobic C-terminal tail, which had been deleted to facilitate structural analysis. **c** | Bax, showing its C-terminal tail (yellow) tucked into the groove, but running in the opposite orientation to a BH3 ligand²⁸. Bak, Bcl2 antagonist/killer; Bax, Bcl2-associated X protein; N, amino terminus of the Bak BH3 peptide. Figure kindly prepared by Dr Brian Smith, Walter and Eliza Hall Institute, Melbourne, Australia.

Tissue homeostasis seems to be set by the balance between the pro-survival and BH3-only proteins. Interestingly, the apoptosis that normally decimates the neonatal kidney and immune system of *Bcl2*^{-/-} mice³⁴ was ablated by the concomitant loss of even a single allele of *Bim*⁵⁷.

Individual BH3-only proteins might transduce specific death signals⁴⁰. Loss of Bim impairs the cytotoxic response of lymphocytes to cytokine deprivation, calcium flux or paclitaxel (**Taxol**), but not, notably, the cytotoxic response to γ -irradiation⁵⁴. Similarly, Bmf might be required for ANOIKIS⁴⁵. As Noxa⁴⁹ and Puma^{50,51} are both induced by p53, they might mediate the apoptosis that is elicited by genotoxic damage or oncogene activation. Clarifying these pathways should have important implications for tumorigenesis and therapy (see below).

Bid seems to promote death by activating Bax and Bak, and it might also inactivate pro-survival relatives⁵⁸. Exposure of its buried BH3 domain^{59,60} requires cleavage within the amino-terminal region — for example, by caspases or **granzyme-B** (FIG. 4). The cleaved (p7/p15) complex is then myristoylated on p15 (REF. 61) and

migrates to mitochondria^{47,48}; it is probably attracted by the cardiolipin-rich ‘contact sites’ between the outer and inner mitochondrial membranes⁶². If Bak (or Bax) is present⁶³, Bid then very rapidly (within a minute) triggers cytochrome *c* release⁶⁴ and apoptosis. Bid might act by inducing Bax and Bak to oligomerize and form pores in the membrane, but the oligomers do not contain Bid⁶³, which seems to form homotrimers in the membrane⁶⁵. The resemblance between Bid and the pore-forming subunit of some bacterial toxins^{59,60} indicates that it might nucleate channel formation by Bax and Bak.

The Bax family. Bax and Bak are widely distributed, whereas the little-studied protein **Bok** is more prevalent in reproductive tissues. Inactivation of *Bax* affected apoptosis only slightly and disruption of *Bak* had no discernible effect, but inactivation of both genes dramatically impaired apoptosis in many tissues^{43,66,67}. So, the presence of either Bax or Bak seems to be essential for apoptosis in many cell types.

Bax and Bak are thought to function mainly at the mitochondrion^{10,15}, but their potential roles elsewhere (for example, the ER) merit attention. Bax is a cytosolic monomer in healthy cells, but it changes conformation during apoptosis, integrates into the outer mitochondrial membrane and oligomerizes^{68–71}. The three-dimensional structure of monomeric Bax²⁸ closely resembles that of its pro-survival relatives (FIG. 3). Intriguingly, the Bax hydrophobic carboxy-terminal helix occludes its BH1/2/3 hydrophobic groove (FIG. 3c). As the carboxyl terminus is essential for targeting to mitochondria⁷², the tail presumably flips out after the cell receives stress signals²⁸. Even in healthy cells, Bak is an oligomeric integral mitochondrial membrane protein, but it too changes conformation during apoptosis and might form larger aggregates^{63,69,70,73}. How the homooligomers form is unclear. Perhaps the Bax-like proteins can assume both a ‘BH3 donor’ and a ‘BH3 acceptor’ conformer within the membrane environment. Alternatively, some molecules might anchor in the

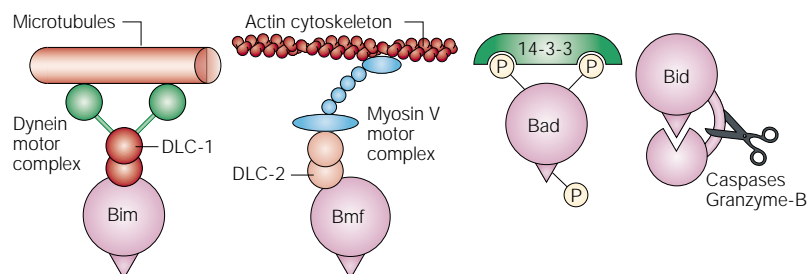


Figure 4 | Diverse modes of post-translational regulation of BH3-only proteins. In healthy cells, BH3-only proteins are held in check by a variety of strategies. Bim and Bmf are sequestered to the microtubules or actin cytoskeleton, respectively, via interaction with a dynein light chain (DLC)^{44,45}. Phosphorylated Bad is bound by 14-3-3 scaffold proteins⁴⁶. Bid is synthesized as a precursor, which requires proteolytic cleavage to be fully active^{47,48}. The ‘beak’ in each represents the BH3 domain.

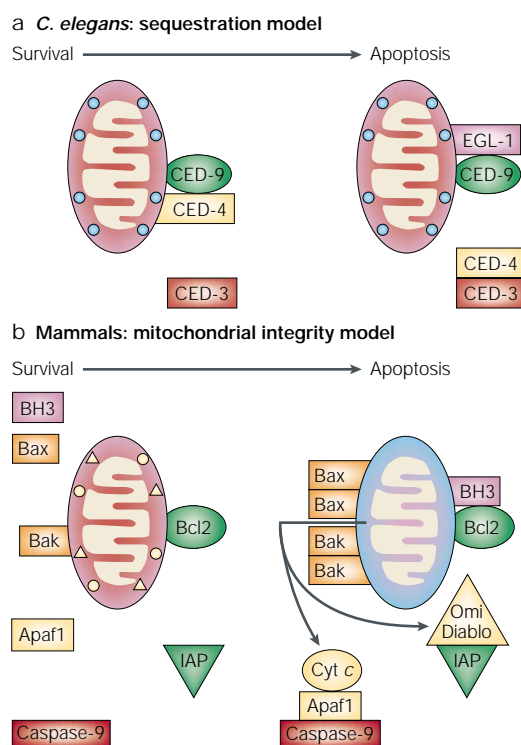


Figure 5 | Two models for Bcl2 survival activity.
a | *Caenorhabditis elegans*: sequestration of a caspase activator. The Bcl2 homologue CED-9 binds the adaptor protein CED-4 and prevents it from activating the CED-3 caspase until the BH3-only protein EGL-1 binds to CED-9 and displaces CED-4. **b** | Mammals: protection of mitochondrial integrity (see text). Bcl2 and its anti-apoptotic homologues guard mitochondrial membrane integrity until neutralized by a BH3-only protein. Bax and Bak then form homo-oligomers within the mitochondrial membrane, resulting in the release of cytochrome *c*, which activates Apaf1, allowing it to bind to and activate caspase-9. Other pro-apoptotic molecules that exit the mitochondria include Omi and Diablo, which antagonize inhibitor of apoptosis proteins (IAPs). Protein complexes are shown as juxtaposed boxes or triangles. Apaf1, apoptotic protease-activating factor 1; cyt *c*, cytochrome *c*.

membrane via the carboxyl terminus and enable others to assemble on them as 'daisy chains' via intermolecular association of grooves and extruded tails.

Bax and Bak oligomers are widely believed to provoke or contribute to the permeabilization of the outer mitochondrial membrane, allowing efflux of apoptogenic proteins¹⁰ (see below). The mechanism, however, is controversial^{15,74,75}. One model, which is based on the structural resemblance of Bcl2 family members and diphtheria toxin²⁴, is that Bax and Bak form channels. Consistent with this hypothesis is the fact that Bax oligomers can form pores in liposomes⁷⁶ that allow passage of cytochrome *c*^{77,78}, and that mitochondria from apoptotic cells contain a novel channel⁷⁹. Alternatively, Bax might interact with components of the existing permeability transition pore — for example, the voltage-dependent anion channel (VDAC) — to create a larger channel^{74,75}, but several studies have found no evidence for such complexes^{15,70,71}.

Liaising for life or death. In lymphocytes, at least, induction of apoptosis by diverse signals (for example, cytokine deprivation) requires Bim⁵⁴, as well as Bax or Bak^{66,67}. As Bim does not bind to Bax or Bak⁸⁰, it must act by preventing the pro-survival proteins from inhibiting the activation of Bax and Bak. How the Bcl2-like proteins antagonize the Bax-like proteins, however, remains unknown. Direct interaction might not occur physiologically, because it is only observed in certain non-ionic detergents⁶⁸. Moreover, although high concentrations of the pro-survival proteins prevent Bax oligomerization and channel-forming activity⁷⁰, cross-linking reveals no Bcl2–Bax complexes⁷¹.

Direct or indirect control of caspase activation? The ongoing debate about how the Bcl2 family controls apoptosis hinges on whether its pro-survival members control caspase activation directly^{8,12} or only indirectly, by controlling mitochondrial integrity^{9,10,14}. In other words, does caspase activation occur independently of mitochondrial disruption or only as a consequence of it?

For *C. elegans*, a direct sequestration model is strongly favoured¹⁶ (FIG. 5a): CED-9, the worm Bcl2, binds the adaptor CED-4 and prevents it from activating the CED-3 caspase until the BH3-only protein EGL-1 displaces CED-4 (REF. 41). Accordingly, CED-4 and CED-9 co-localize on mitochondria in healthy cells, but in dying cells CED-4 moves to the nuclear membrane⁸¹. Moreover, CED-9 survival activity is enhanced by a mutation that reduces its affinity for EGL-1 (REFS 82,83).

The ability of human BCL2 to support survival in the worm^{17,84} indicated that Bcl2-like proteins might sequester Apaf1, the first mammalian homologue of CED-4 (REF. 85). Unlike CED-4, however, Apaf1 is cytosolic⁸⁶ and, contrary to earlier reports, is not bound by any Bcl2-like protein (or Bax)⁸⁷. Instead, Apaf1 activity is restrained by its large carboxy-terminal WD40 REPEAT DOMAIN and is unleashed by cytochrome *c*^{20,88}. These observations, plus the ability of Bcl2 to prevent cytochrome *c* release^{89,90}, have given rise to the widespread view that the sole function of Bcl2-like proteins is to guard mitochondrial integrity (FIG. 5b)^{9,10,14}, thereby keeping enclosed a plethora of 'killers'^{19,75}. In addition to cytochrome *c*, these include Diablo/Smac and Omi/HtrA2, which antagonize the inhibitor of apoptosis proteins (IAPs) that inhibit processed caspases; the flavoprotein apoptosis-inducing factor (AIF), which is implicated in chromatin condensation and large-scale DNA degradation; endonuclease G, which might aid the CAD (caspase-activated DNase) nuclease in nucleosomal DNA fragmentation; and even, in some cells, a small proportion of some procaspase molecules⁹¹.

Despite these findings, a central role for mitochondrial disruption in apoptosis is difficult to reconcile with the lack of any evidence for the involvement of cytochrome *c* in cell death in *C. elegans*. Furthermore, although *Drosophila* has an essential Apaf1 orthologue with WD40 repeats (DARK), apoptosis in the fly does not seem to require cytochrome *c*^{92,93}.

WD40 REPEAT DOMAIN

A conserved protein domain that is approximately 40 residues long and that has a characteristic tryptophan–aspartate motif. In the case of the caspase-activator Apaf1, two groups of WD40 repeats in the carboxy-terminal region are thought to keep the protein inactive until cytochrome *c* engages the repeats.

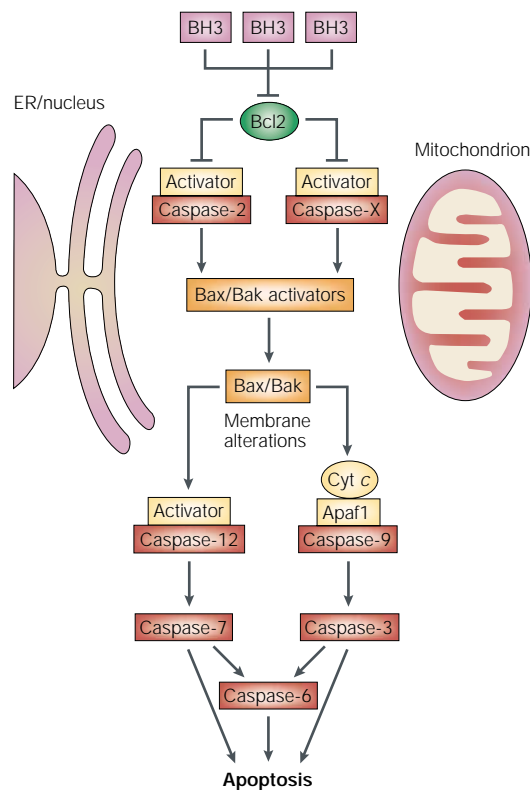


Figure 6 | **Caspase inhibitor model for Bcl2 function.** In this speculative model, Bcl2 pro-survival proteins, acting at the mitochondrion and the endoplasmic reticulum (ER)/nuclear membrane, control the activation of several upstream initiator caspases, perhaps by sequestering their activators. These caspases, in turn, process proteins (for example, Bid) that activate Bax and Bak. Oligomerization of Bax and Bak then produces damage to the organelles that amplifies the proteolytic cascade. Apaf1, apoptotic protease-activating factor 1; cyt c, cytochrome c.

The mitochondrial guardian model (FIG. 5b) readily explains how Bcl2 might control activation of Apaf1 and caspase-9 and, through them, caspase-3, but can this axis account for all stress-induced apoptosis? That possibility seemed to be supported by initial gene-inactivation reports: mice lacking either Apaf1 (REFS 94,95) or caspase-9 (REFS 96,97) often died before birth and had enlarged brains, and apoptosis of several cell types was impaired *in vitro*⁹⁵⁻⁹⁷. Nevertheless, recent evidence rules out an essential role for Apaf1 and caspase-9 in stress-induced death. Unlike Bcl2 overexpression, the absence of Apaf1 or caspase-9 does not increase lymphocyte numbers *in vivo*, and lymphocytes and embryonic fibroblasts die at normal rates in response to diverse insults against which Bcl2 protects (V. Marsden, J.M. Adams, and A. Strasser, unpublished observations). Even post-mitotic neurons that lack Apaf1 die normally⁹⁸, and some *Apaf1*^{-/-} mice become healthy adults⁹⁹. Furthermore, the deletion of thymocytes with self-reactivity requires Bim⁵⁵ but not Apaf1 (REF. 100); fibroblasts lacking both Bax and Bak are more resistant to cytotoxic insults, including overexpression of BH3-only proteins, than those lacking Apaf1 or caspase-9

(REF. 43); and Bcl2 can protect embryonic stem cells that lack Apaf1 (REF. 101). Finally, the absence of cytochrome c only attenuates apoptosis¹⁰². So, the cytochrome-c-Apaf1-caspase-9 'apoptosome' is not indispensable for stress-induced apoptosis. Rather, it acts as a caspase amplification system that is more important in certain cell types (for example, neuronal precursors) than others (for example, lymphocytes).

It could still be argued that activation of the relevant initiator caspase(s) requires mitochondrial disruption, because certain synthetic caspase inhibitors (typically, z-VAD-fmk) have blocked cell death but not cytochrome c release^{89,103}. In other studies, however, such inhibitors have also blocked cytochrome c release^{104,105}. Indeed, caspase-dependent apoptosis can occur without cytochrome c release^{106,107}, whereas certain cells can remain viable for days after disruption of the mitochondrial outer membrane¹⁰⁸. So, a mitochondrial breach is neither necessary nor sufficient for apoptosis, and it could often be triggered by caspases rather than being required for caspase activation¹⁰⁹.

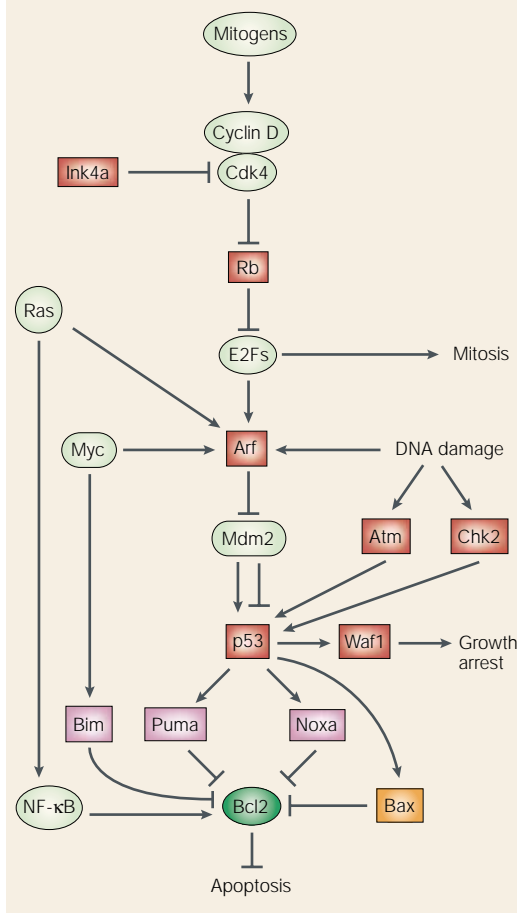
Bcl2 might regulate multiple initiator caspases. Speculatively, Bcl2 might control the activation of several initiator caspases that act upstream or independently of any mitochondrial breach (for example, caspase-2 and -X in FIG. 6). For instance, Bcl2 can control apoptosis from the ER¹¹⁰⁻¹¹², and caspase-12, which can process other caspases in the absence of Apaf1 or cytochrome c¹¹³, is activated by ER-regulated stress¹¹⁴ and by serum deprivation¹¹⁵. Caspase-2 is another plausible initiator, because elimination of its mRNA by RNA INTERFERENCE (RNAi)¹¹⁶ in certain cell lines inhibits apoptosis, release of cytochrome c and Diablo, and recruitment of Bax to mitochondria¹⁰⁹. However, as mice that lack caspases 1, 2, 8, 11 or 12 develop normally, with only slight defects — if any — in stress-induced apoptosis¹¹⁷, we speculate that several of these caspases redundantly trigger the caspase cascade. Their activation might involve oligomerization by cognate proteins that bear both a caspase recruitment domain (CARD) and a CED-4-like nucleotide binding domain¹¹⁸. If so, the pro-survival clan might sequester such caspase activators (FIG. 6), just as CED-9 directly constrains CED-4 (FIG. 5a).

The Bcl2-like proteins presumably also function at the mitochondrion to prevent Bax/Bak oligomerization. In the absence of convincing evidence for physical interaction of these opposing factions under physiological conditions (see above), indirect models must be considered. First, if Bcl2 helps to gate a mitochondrial pore, as some (controversial) findings indicate⁷⁴, engagement of Bcl2 by a BH3-only protein might allow the release of small molecules that provoke a conformational change in Bax/Bak. Second, if Bcl2 and Bax/Bak compete for an unknown target on mitochondria, the ligation of Bcl2 might free it, allowing Bax to bind and nucleate pore formation. Third, if Bcl2 sequesters caspase activators (see above), their release from Bcl2 might allow an activated caspase to mediate Bax translocation, perhaps via cleavage of a Bid-like protein or an outer mitochondrial membrane

RNA INTERFERENCE
A technique in which double-stranded RNA, or synthetic double-stranded RNA oligonucleotides about 21 nucleotides long, is used to silence expression of a gene of the same sequence. Ribonucleases in the cell use the introduced RNA as a guide to target and cleave the mRNA transcribed from that gene.

Box 2 | Bcl2 and the Rb/Arf/p53 network

Inactivation of the retinoblastoma (Rb) pathway — for example, by loss of cell-cycle inhibitor Ink4a, which can prevent cyclin-D-Cdk4 from phosphorylating Rb — unleashes the transcription factor E2f1, which increases expression of Arf, a protein that is encoded by the same locus as Ink4a (REF 136). Arf, which is also a transcriptional target of Myc, sequesters Mdm2, a negative regulator of p53. Raised p53 levels can either impose growth arrest, typically by inducing the Waf1 cell-cycle inhibitor, or promote apoptosis through targets such as Bax, Puma and Noxa. The apoptotic targets seem to also require the p53 relative p63 or p73 (REF. 152). Circles/ovals denote oncogene products; rectangles denote known or likely tumour suppressors. For more detail, see REFS 4–6,136. ATM, ataxia telangiectasia mutated; Chk2, checkpoint 2; NF-κB, nuclear factor-κB.



protein, as suggested for caspase-2 (REF 119). The Bax/Bak oligomers might not only produce pores in the mitochondrial outer membrane; they could also perturb the ER/nuclear membrane. For example, they might promote the release from the ER of calcium ions, which could contribute to caspase activation either via calpain¹²⁰ or by effects on mitochondria. Alternatively, Bax/Bak oligomers might serve, somehow, as a platform for activation of some upstream caspases.

Bcl2 family and tumorigenesis

The evidence from human tumours that cancer generally requires impaired apoptosis is not yet overwhelming, but the hypothesis is strongly supported by experimental models. In particular, the oncogenic potential of elevated Bcl2 has been clearly shown in several transgenic mouse models. *Bcl2* transgenes that mimic the *BCL2* translocation gave rise to B-lymphoid tumours, and their stochastic onset implied a need for acquired mutation(s)^{29,121,154}. Myc aided the transformation of *Bcl2*-expressing cells *in vitro*², and co-expression of *Bcl2* and *Myc* transgenes dramatically accelerated lymphomagenesis¹²², revealing a previously unsuspected strong cooperation between mutations that enforce proliferation and those that inhibit apoptosis. This synergy also occurs in breast¹²³ and pancreatic β-cell tumours^{124,125}. It presumably reflects the ability of Bcl2 to counter the apoptosis elicited by Myc under suboptimal growth conditions, and the ability of Myc to override the retardation of cell-cycle entry by Bcl2 (REFS 3,5). Partnerships are not limited to *Myc*: *Bcl2* can also synergize with the chimeric promyelocytic leukaemia-retinoic-acid receptor-α (*PML-RARα*) to induce acute promyelocytic leukaemia¹²⁶. Although it has been inferred that enforced proliferation plus impaired apoptosis might suffice for fully fledged malignancy^{5,125}, in most cell types, bypassing of senescence minimally requires the elimination of p53 function⁴.

All the *Bcl2* pro-survival family members are likely to be oncogenes. *Bcl-x_L*, for example, has been implicated in mouse myeloid and T-cell leukaemias¹²⁷. Conversely, members of both pro-apoptotic subfamilies are probably tumour suppressors. Bax or Bak is mutated in some human gastric and colorectal cancers^{128,129}, as well as in leukaemias¹³⁰, and loss of Bax increases tumorigenicity^{131–133}. Absence of both Bax and Bak can enhance transformation, beyond loss of either alone⁴². Bim also seems to be a tumour suppressor: absence of even one *Bim* allele accelerates *Myc*-induced lymphomagenesis (A. Egle and S. Cory, unpublished observations). The p53 targets, Noxa and Puma, might mediate p53 apoptotic function, and Bmf might inhibit metastasis⁴⁵.

Many oncogenic mutations probably impair apoptosis indirectly, by affecting signal-transduction pathways that promote or repress expression of Bcl2 family members (BOX 2). For example, mutations that increase the activity of nuclear factor-κB (NF-κB) transcription factors (for example, *Ras*) can enhance the expression of pro-survival family members^{134,135}. Conversely, mutations that inactivate the retinoblastoma (*RB*) tumour-suppressor pathway or promote *Myc* activation upregulate apoptosis inducers such as p53, the targets of which include Bax, Puma and Noxa¹³⁶ (BOX 2). Given that the *Rb/Myc/p53* circuitry is shorted in almost all tumours⁴, some dysregulation of the Bcl2 family during oncogenesis might be almost universal.

Targeting the apoptotic machinery for therapy Most cytotoxic agents, irrespective of their primary targets, are now thought to kill cells predominantly by triggering their apoptosis programme^{6,7}. Supporting

that notion, overexpression of Bcl2 renders tumour cells refractory to diverse therapeutic drugs and radiation, *in vivo* as well as *in vitro*^{137,138}, and selection for drug resistance in cancer cells is often accompanied by upregulation of Bcl2 (REF. 139). Moreover, elimination of Bax from human colorectal cancer cells abolished their apoptotic response to non-steroidal anti-inflammatory drugs¹⁴⁰.

So, the prospect of directly switching on the apoptotic machinery is gaining widespread interest¹⁴¹. One promising approach is to engage death receptors, such as that for TNF-related apoptosis-inducing ligand (TRAIL), because tumorigenesis often spares that arm of the apoptotic response and normal cells are surprisingly refractory^{21,141} (FIG. 1). Other approaches target Bcl2 — for example, Phase III clinical trials with antisense *Bcl2* deoxyoligonucleotides are underway¹⁴¹. Although such studies might establish the value of compromising *Bcl2* function, antisense oligonucleotides have a chequered history, and RNAi¹¹⁶ using small RNA duplexes, delivered as synthetic oligonucleotides or expressed from vectors, seems more promising.

BH3 mimetics. An exciting approach for manipulating Bcl2 function is to mimic the binding of a BH3 peptide to its groove. The dramatic rescue of the degenerative defects in *Bcl2*^{-/-} mice by loss of a single *Bim* allele⁵⁷ indicates that degenerative diseases might be retarded by drugs that modulate the action of BH3-only proteins. Conversely, small molecules that mimic the BH3 domain and neutralize Bcl2-like function might well be effective against cancer or autoimmune diseases. Several reports have already described small organic molecules that bind to Bcl2 *in vitro* (albeit with low affinity) and compromise cell viability^{142–144}, and the potential of such approaches has been discussed^{145,146}.

Why might a BH3 mimetic be more effective than many conventional anticancer drugs? As most genotoxic drugs act primarily through p53 to induce apoptosis (BOX 2), p53 mutation gives tumour cells a decided advantage over normal cells. By targeting Bcl2 directly, the BH3 mimetic would bypass that roadblock. Normal cells must tolerate reduced Bcl2 levels, because mice that lack one *Bcl2* allele, or an allele of any pro-survival relative, are completely healthy. The tumour cell might be more vulnerable because of

oncogenic changes such as Myc activation, which reduces Bcl2 and Bcl-x_L expression¹⁴⁷ and might prime that of certain BH3-only proteins such as Bim. Moreover, BH3 mimetics that target specific family members — for example, Bcl2 and not Bcl-x_L — would allow therapy to be tailored to the dominant pro-survival molecule in that tumour, increasing the therapeutic index.

Puzzles and prospects

The decisive first step towards apoptosis occurs when sentinel BH3-only proteins respond to developmental cues or damage to particular cellular compartments, but how they register those signals needs clarification. Once unleashed (FIG. 4), the BH3-only proteins engage the Bcl2-like anti-apoptosis proteins (FIG. 3b), but how that neutralizes their pro-survival function remains uncertain. A central unresolved issue is the nature of the immediate effectors of Bcl2 function. Rather than acting merely as a guardian of the mitochondrion (FIG. 5b), Bcl2 might act primarily by constraining the activation of several initiator caspases (FIG. 6). RNAi offers new opportunities for testing this (unorthodox) model, and identifying the initiators and their activators. Another puzzle is how Bax and Bak are activated — does this involve caspase activation, for example, by cleavage of Bid-like proteins, or do Bax and Bak instead contribute to caspase activation? Although caspase-mediated apoptosis apparently can occur without disruption of the outer mitochondrial membrane, that step often provides the *coup de grâce* for the cell by allowing several pro-apoptotic molecules, including cytochrome *c*, to escape to the cytosol and augment the caspase cascade. The disruption is often attributed to pores formed by Bax or Bak oligomers, but convincing *in vivo* evidence for these pores is still lacking.

Impairment of apoptosis is a central step in tumorigenesis and many BH3-only proteins are likely to be tumour suppressors. Determining which BH3-only proteins are activated by specific anticancer agents could lead to a more rational basis for cytotoxic therapy. Finally, even though impaired apoptosis might seem to render the tumour cell invulnerable, it could instead prove to be its 'Achilles' heel', because pharmacological manipulation of the Bcl2 family and other apoptosis regulators is likely to open up new therapeutic opportunities.

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