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BH3-only proteins in apoptosis and beyond: an overview

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Abstract

BH3-only BCL-2 family proteins are effectors of canonical mitochondrial apoptosis. They discharge their pro-apoptotic functions through BH1-3 pro-apoptotic proteins such as BAX and BAK, while their activity is suppressed by BH1-4 anti-apoptotic BCL-2 family members. The precise mechanism by which BH3-only proteins mediate apoptosis remains unresolved. The existing data are consistent with three mutually non-exclusive models 1) displacement of BH1-3 proteins from complexes with BH1-4 proteins; 2) direct interaction with and conformational activation of BH1-3 proteins; and 3) membrane insertion and membrane remodeling. The BH3-only proteins appear to play critical roles in restraining cancer and inflammatory diseases such as rheumatoid arthritis. Molecules that mimic the effect of BH3-only proteins are being used in treatments against these diseases. The cell death activity of a subclass of BH3-only members (BNIP3 and BNIP3L) is linked to cardiomyocyte loss during heart failure. In addition to their established role in apoptosis, several BH3-only members also regulate diverse cellular functions in cell cycle regulation, DNA repair and metabolism. Several members are implicated in the induction of autophagy and autophagic cell death, possibly through unleashing of the BH3-only autophagic effector Beclin 1 from complexes with BCL-2/BCL-xL. The Chapters included in the current Oncogene Review issues provide in-depth discussions on various aspects of major BH3-only proteins.

Keywords

BH3-only; Apoptosis; Autophagy; Non-apoptotic functions; Cancer

Introduction

The BCL-2 family proteins are well-characterized regulators of apoptosis and are grouped into three sub-families based on the number of BH (BCL-2 Homology) domains they share (Fig. 1). The first sub-family includes the anti-apoptotic proteins BCL-2, BCL-xL, BCL-w, MCL-1, and A1/BFL-1. These proteins possess four BH domains -BH1-4. The next two groups are pro-apoptotic proteins possessing three BH (BH1-3) domains represented by BAX, BAK, and BOK and BH3-only proteins that, as their name implies, are characterized by the presence of only the BH3 domain. BCL-2 family proteins form a complex regulatory network that controls cell survival and death in response to different physiological and pathological stimuli. Our current understanding of the canonical apoptotic pathway in animal cells is that various cell death stimuli either transcriptionally or post-transcriptionally activate one or more of BH3-only effectors that integrate and transmit the death signal *via* the multi-domain BH1-3 pro-apoptotic proteins, BAX and BAK. The BH1-3 pro-apoptotic proteins undergo conformational activation leading to oligomerization and insertion in the outer mitochondrial membrane. This process is

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presumed to result in permeablization of outer mitochondrial membrane and egress of apoptogenic factors such as cytochrome c that activate the caspase cascade leading to cellular demise. This process is actively opposed by the BH1-4 anti-apoptotic members. The general mechanisms by which the BCL-2 family proteins regulate cell survival and death have been reviewed in a number of review articles. Since the discovery of the first member of the BH3-only family proteins (Boyd et al., 1995), a large number of this family's members have been identified and their roles in physiological and pathological cell elimination have been documented. Surprisingly, several members of this family, in addition to their function as the effectors of cell death, also play other non-cell death functions. This article provides an overview of the known BH3-only proteins and their functions. Comprehensive reviews on major BH3-only members, *viz*., BIK, EGl-1, BIM, BMF, NOXA, BID, BAD, BNIP3 and Beclin-1 and their physiological and pathological functions are reviewed in the Chapters included in the present Oncogene Review Issue.

Discovery of the BH3 domain and the first BH3-only BCL-2 family member

In 1995, a collaborative study between Tom Chittenden and Bob Lutz at Immunogen Inc. and the laboratory of G. Chinnadurai (Saint Louis University School of Medicine) that involved functional dissection of BAK (Chittenden et al., 1995) and the identification and cloning of the pro-apoptotic protein BIK (Bcl-2 Interacting Killer) that shared a conserved domain with other BCL-2 family proteins (Boyd et al., 1995) resulted in the first identification of the functions of the BH3 domain and the BH3-only proteins. These studies showed that: 1) the cell death activities of BAK and BIK were dependent on the conserved BH3 domain; 2) the BH3 domain of BAK was required and sufficient for heterodimerization with BCL-xL; and 3) the BH3 domain of BIK mediated interaction with cellular (BCL-2, BCL-xL) and viral (E1B-19K and EBV-BHRF1) anti-apoptosis proteins. In subsequent publications, the function of the BH3 domain was also identified by mutational analysis of BAX (Hunter et al., 1996; Zha et al., 1996a). The physiological relevance of the BH3-only members as death effector molecules in whole animal models was demonstrated by genetic studies in *C. elegans* (Conradt and Horvitz, 1998). This study identified the worm BH3-only member EGL-1 and demonstrated that it acted at the point of the cell death pathway where signals of cell death were integrated to activate the process of programmed cell death. The *egl-1* gene was shown to be required for most somatic and genotoxic stress-induced cell death in the worm (Gartner et al., 2000; Gumienny et al., 1999). The cell death activity of EGL-1 was linked to its interaction with CED-9, the worm ortholog of BCL-2 (Conradt and Horvitz, 1998) (see Chapter by Nehme and Conradt). The generation of a knockout mouse model that identified the function of BIM in mouse development and in cell death induced by external stimuli (Bouillet et al., 1999) further substantiated the role of a BH3-only member in a vertebrate. The genetic studies in *C. elegans* and in mouse suggested that the BH3-only proteins function as apical sentinels of different cell death signals and initiators of the canonical apoptotic program in animals (reviewed in (Bouillet and Strasser, 2002; Huang and Strasser, 2000)). The generation of mouse models deficient in several other BH3-only members such as *Noxa*, *Puma*, *Bnip3* and *Bnip3L* has established their role in cell death in response to physiological and pathological stimuli (see accompanying Chapters).

The BH3-only subfamily and siblings

After the discovery of BIK, the list of BH3-only members has increased substantially (Fig. 2). The well known members of this family such as BID (Wang et al., 1996), HRK/DP5 (Imaizumi et al., 1997;Inohara et al., 1997) and BIM/BOD (Hsu et al., 1998;O'Connor et al., 1998) were discovered in interaction cloning with cellular anti-apoptosis proteins BCL-2, BCL-xL and MCL-1. BIK was also identified in interaction cloning with adenovirus E1B-19K (Han et al., 1996). Proteins such as BNIP1, BNIP3 (Boyd et al., 1994) and BAD (Yang et al., 1995) were

subsequently included in the BH3-only list. Other members such as NOXA (Oda et al., 2000), PUMA/BBC3 (Han et al., 2001;Nakano and Vousden, 2001;Yu et al., 2001) and BMF (Puthalakath et al., 2001) were identified on the basis of differential mRNA expression in cells exposed to apoptotic stress or by interaction cloning. The autophagy effector Beclin 1 which was originally identified as a BCL-2 interacting protein (Liang et al., 1998) was also identified as a BH3-only member (Feng et al., 2007;Oberstein et al., 2007) (see Chapter by Sinha and Levine). Certain splice variants of multi-domain BCL-2 family members have also gained entry into this subfamily. They include MCL-1S (Bae et al., 2000;Bingle et al., 2000) and BCL-G_S (Guo et al., 2001). In *C. elegans*, a BH3-only member CED-13 (that shares a substantial homology with EGL-1 in the BH3 region) induced by the *cep-1/p53* gene in response to genotoxic stress has also been identified (Schumacher et al., 2005). Alternative splicing of the BH3-only members also appear to contribute to the diversity of these proteins and regulate their functions. For example, multiple splice isoforms were described for BIM (Marani et al., 2002;O'Connor et al., 1998;U et al., 2001), BID (Renshaw et al., 2004), NOXA (Wang and Sun, 2008), BNIP1 (Zhang et al., 1999), BMF (Morales et al., 2004). Some of these splice isoforms (like in BIM) retain the BH3-domain, while in other (like in BMF and NOXA) it is spliced out. In contrast to the worm and mammals, no BH3-only proteins have been identified in the annotation of *Drosophila* genome (Claveria and Torres, 2003). This raises an interesting question as to whether the functional niche of BH3-only proteins, if any, has been occupied by structurally different cell death regulators in *Drosophila*.

Several other proteins were reported to contain BH3-like domains and possess death-inducing activity and/or ability to interact with anti-apoptotic BCL-2 family proteins (Fig. 3). They include: p193 (Tsai et al., 2000); MAP-1 (Tan et al., 2001); Spike (Mund et al., 2003); SphK2 (Liu et al., 2003a); BRCC2 (Broustas et al., 2004); TG2 (Rodolfo et al., 2004); MULE/ARF-BP1 (Zhong et al., 2005); ApoL6 (Liu et al., 2005); ApoL1 (Wan et al., 2008); ERBB4/HER4 (Naresh et al., 2006); and ERBB2/HER-2 (Strohecker et al., 2008). Other proteins such as murine $ITM2B_S$ were reported to contain BH3-like domain and were shown to induce apoptosis (Fleischer et al., 2002). Relatively little is known about integration of these proteins in the apoptosis signaling pathways.

BH3 domain of BH3-only proteins – engagement with pro-survival proteins

It is well established that the BH3 domain of BH3-only proteins is essential for protein-protein interactions with BH1-4 BCL-2 family proteins. The interactions of BH3-only proteins with anti-apoptotic BCL-2 family proteins were readily detected by commonly used methods such as coimmunoprecipitation and two-hybrid interactions in yeast. The interactions were abolished by mutations within the BH3 domain. These results were substantiated by detailed structural studies. The first of such structural studies involved the analysis of a complex between a 16-amino acid peptide derived from the BH3 region of BAK and BCL-xL (Sattler et al., 1997). This study revealed that insertion of the BH3 α-helix of BAK into the hydrophobic cleft of BCL-xL (formed by its BH1, BH2 and BH3 domains) was a key event in heterodimerization between BCL-2-like cell death agonists and antagonists. Similar structural information has been gleaned from studies involving the following complexes between peptides containing the BH3 domain helixes of BH3-only proteins and different anti-apoptosis proteins: BAD:BCL-xL (Petros et al., 2000); BIM:BCL-xL (Liu et al., 2003b); BID:BCL-w (Denisov et al., 2006); Beclin 1:BCL-xL (Feng et al., 2007; Oberstein et al., 2007); hBIM:hMCL-1 (a truncated version containing BCL-2-like region); and mNOXA-B:hMCL1 (Czabotar et al., 2007). The structures of NOXA BH3 and PUMA BH3 in complex with MCL-1 (Day et al., 2008) were also demonstrated using different experimental approaches. Computer-aided modeling was used for the following: BID:BCL-xL complex (Chou et al., 1999; McDonnell et al., 1999), BAD:BCLxL and BIM:BCLxL complexes (Lama and Sankararamakrishnan, 2008). A rigorous structural, biochemical and functional study of

EGL-1 interaction with CED-9 also confirmed the critical aspects of such interactions (Yan et al., 2004).

The BH3 domain consists of 9–16 amino acids (Fig. 3). A phylogenic analysis of BH3 domain-containing proteins suggested that the core of BH3 domain is formed by a seven amino acid sequence motif LXXXGDE, where X is any amino acid (Lanave et al., 2004), although neither the Gly nor the Glu residues are strictly conserved. More recently, based on structural studies and sequence analyses a 13-residue consensus motif (Fig. 4) has been proposed (Day et al., 2008). Again, there is no strict conservation. The most conserved leucine and aspartic acid residues of the core BH3 domain appear to be more important for interactions with the pro-survival proteins. The leucine residue is buried in the protein-protein interface and packs against conserved residues of pro-survival protein, while the solvent-exposed aspartate forms ionic interaction with the conserved arginine residue in the BH1 domain of pro-survival protein (reviewed in (Hinds and Day, 2005). Additional important features of the BH3 domain: BH1-4 binding interface were recently reported to include the network of van der Waals, hydrophobic and hydrophilic interactions (Day et al., 2008;Lama and Sankararamakrishnan, 2008).

Based on the diversity of BH3-only proteins it would be important to know whether these proteins would differ in their binding selectivity to anti-apoptotic proteins. Indeed, initial *in vitro* binding assays demonstrated some preferences for interaction of the BH3 peptides from different BH3-only proteins with various anti-apoptotic proteins (Kuwana et al., 2005; Letai et al., 2002). A comprehensive *in vitro* analysis using recombinant anti-apoptotic proteins (human BCL-2 ΔC22, BCL-w C29S, A128EΔC29, BCL-xLΔC24 and mouse MCL-1ΔN151ΔC23 and A1ΔC20) and synthetic BH3-peptides of BH3-only proteins revealed a marked difference in binding selectivity among them (Chen et al., 2005). Among eight BH3-peptides derived from human BAD, BID, BIK, BIM, HRK, NOXA, PUMA and mouse BMF proteins, only BIM and PUMA peptides had comparable strong affinities to all anti-apoptotic proteins tested. BAD and BMF peptides preferably bound to BCL-2, BCL-xL and BCL-w. NOXA peptide bound strongly to MCL-1 and A1, but not to others. BID, BIK, and HRK peptides interacted more strongly with BCL-xL, BCL-w and A1. It was likely that the observed selectivity relied on the differences in amino acids sequence within the BH3 domains, because mutations in BH3 domain were able to change the binding preferences. Thus, these binding studies demonstrated additional functional diversity among the BH3-only proteins and provided a mechanistic explanation for the differences in apoptotic signaling induced by BH3-only proteins (see below).

In contrast to the near universal and well documented feature of the BH3-only proteins - their ability to bind to anti-apoptotic multidomain BH1-4 proteins, the ability to interact with pro-apoptotic multidomain BH1-3 proteins is limited to only a few of them. For example, direct binding of BID to BAX and BAK (Wang et al., 1996) and binding of BIM to BAX (Harada et al., 2004) have been reported. Another example appears to be the BH3-only-like protein tissue or type 2 transglutaminase (TGM2). TGM2 possesses a functional BH3 domain through which it was shown to interact with pro-apoptotic proteins BAX and BAK but not with anti-apoptotic BCL-2 and BCL-xL. Upon induction of cell death, mitochondrially localized TGM2 was shown to complex with proteins, including BAX (Rodolfo et al., 2004). Yeast two-hybrid and co-immunoprecipitation analyses revealed that the binding of BID and PUMA to BAX was *via* their BH3-domain and the firstα helix of BAX (Cartron et al., 2004). Recently, an NMR spectroscopic analysis revealed a novel BH3-interaction interaction site (comprising of helices α1 andα6) on BAX (Gavathiotis et al., 2008). A peptide containing the BH3 domain of BIM (in the form of ‘stabilized α-helix of BCL-2 domain’, SAHB) bound to the α1-α6 binding site BAX (Gavathiotis et al., 2008). Importantly, conformational activation of BAX was initiated at this novel site by BIM SAHB binding. Interestingly, the predicted structure of the BIM-BH3-BAX complex showed an interaction topography that was similar to that of BIM BH3

with anti-apoptotic BCL-xL, MCL1 and A1 that were previously determined by X-ray crystallography. Thus, the work of Gavathiotis et al. was the first direct structural study of a complex between the BH3 domain of a BH3-only protein and BAX. However, it is still uncertain whether the same interaction occurs in the context of full length proteins, and whether binding to BAK proceeds through a similar mechanism. Therefore, the functional significance of interaction of certain BH3-only proteins with BH1-3 pro-apoptotic proteins remains to be fully addressed.

Diversity in BH3 functions

After the identification and functional characterization of the BH3 domain in BIK (Boyd et al., 1995) and BAK (Chittenden et al., 1995), as well as in multiple BH3-only proteins in subsequent studies, it became clear that the BH3 domain represented the minimal “death domain” (reviewed in (Kelekar and Thompson, 1998)). Importantly, numerous studies have shown that mutations in the BH3 domain of BH3-only proteins resulted in simultaneous abrogation of their binding to pro-survival BCL-2 family proteins and their pro-apoptotic activity (Boyd et al., 1995; Inohara et al., 1997; O’Connor et al., 1998; Puthalakath et al., 2001; Wang et al., 1996; Zha et al., 1997). Thus, the interaction of the BH3-domain with anti-apoptotic multi-BH-domain proteins is crucial for cell death activity of BH3-only proteins. Is this sufficient for entire pro-apoptotic activity of all BH3-only proteins? It appears that the answer may vary depending on the context of the individual BH3-only member.

The first evidence for functional diversity within the BH3 domain came from an *in vitro* functional analysis of the BH3-domain-containing peptides derived from different BH3-only proteins to induce mitochondrial release of cytochrome c in purified mouse mitochondria (Letai et al., 2002). This study provided evidence for two distinct subclasses of BH3 peptides: one called “activators”, which included the BH3 domain of BID and BIM, that can directly activate BAK to oligomerize and induce mitochondrial release of cytochrome c; the other subclass called “sensitizers”, and is represented by BID, BIK and NOXA. This subclass did not directly activate BAK nor induce cytochrome c release, but instead bound to anti-apoptotic BCL-2 to displace BID-like peptides. Importantly, this study demonstrated a level of cooperation between BH3-only peptides. Transduction of the sensitizer BH3-peptide (BAD-BH3) and the activator peptide (BID-BH3) had a synergistic effect in killing cancer cells (Letai et al., 2002). Similar results were obtained using synthetic liposomes loaded with an expanded set of BH3 peptides and recombinant BAX protein (Kuwana et al., 2005). Taken together, the *in vitro* studies based on binding of BH3 peptides to multidomain proteins provided the experimental basis for different models on the mode of action of BH3-only proteins (see below). However, the limitation of such models is that they are based on BH3-peptides rather than native BH3-only proteins. Some aspects of these models based on BH3 peptides appear to contradict with certain results obtained with expression of BH3-only proteins from expression vectors. A detailed analysis of the pro-apoptotic activity of a panel of BIK mutants revealed that a C-terminal region (aa 120–134) was required in addition to the BH3-domain for efficient cell death activity of BIK (Elangovan and Chinnadurai, 1997). A recombinant version of BIK was shown to efficiently induce cytochrome c release in isolated mitochondria (Shimizu and Tsujimoto, 2000). The killing activity of NOXA BH3 domain was functional only when targeted to mitochondria *via* the C-terminal domain of the protein (Seo et al., 2003). It is possible that intact BIK and NOXA proteins might be potentially as effective as BID and BIM in inducing permeabilization of the outer mitochondrial membrane (MOMP). Thus, additional sequence elements in various BH3-only members may enhance the activity of BH3 domain and the BH3 domain-directed activity may be dependent on the whole protein.

Another important question is whether BH3-only proteins are the only proteins involved in binding to the anti-apoptotic BCL-2 family members and thereby inducing apoptosis. Several

reports argue that some cellular proteins lacking a BH3-domain are capable of binding to anti-apoptotic proteins and inducing apoptosis. The orphan nuclear receptor Nur77 was shown to translocate from the nucleus to the cytosol and interact with BCL-2 to induce apoptosis (Cao et al., 2004; Lin et al., 2004). Phosphorylation of K-RAS by protein kinase C promoted its rapid dissociation from the plasma membrane and association with mitochondria, where phospho-K-RAS bound to BCL-xL and induced apoptosis (Bivona et al., 2006). Calpain-mediated cleavage of Atg5 (an autophagy effector) resulted in translocation of truncated Atg5 from the cytosol to mitochondria, association with BCL-xL and apoptotic cell death (Yousefi et al., 2006). A transcription-independent pathway of p53-dependent apoptosis was shown to include physical interaction between mitochondrial p53 and anti- (BCL-2, BCL-xL) and pro-apoptotic (BAX, BAK) members of the BCL-2 family proteins (Chipuk et al., 2004; Leu et al., 2004; Mihara et al., 2003). It was recently suggested that mitochondrial p53 functionally resembled a 'super' BH3-only protein because it acted both as an 'enabler' and as an 'activator' BH3-only protein (Wolff et al., 2008). Therefore, there are probably diverse groups of proteins that are structurally different from BH3-only proteins that can execute the same or equivalent functions.

Multiple modes of regulation of the pro-apoptotic activity of BH3-only proteins

Transcriptional activation and silencing

The activity of BH3-only proteins is stringently regulated at multiple levels. The steady-state levels of BH3-only proteins are regulated both at transcriptional and at post-transcriptional levels. Under normal unstressed conditions, the level of many BH3-only proteins is very low or undetectable by common methods. Different apoptotic stimuli were reported to activate transcription of several BH3-only members through specific transcription factors. The transcription factor E2F-1 was shown to directly up-regulate expression of *Puma*, *Noxa*, *Bim*, and *Hrk/Dp5* (Hershko and Ginsberg, 2004) and *Bik* (Real et al., 2006; Subramanian et al., 2007). *Puma* and *Noxa* were shown to be transcriptionally activated by p53 in response to DNA damage and other cell death signals (Han et al., 2001; Michalak et al., 2008; Nakano and Vousden, 2001; Oda et al., 2000; Shibue et al., 2006; Villunger et al., 2003; Yu et al., 2001). Transcriptional activation of *Bik* by estrogen starvation or anti-estrogen exposure in estrogen-dependent breast cancer cell lines was also reported to depend on an activity of p53 that did not involve DNA binding to canonical p53-dinding sites (Hur et al., 2006; Hur et al., 2004). The forkhead box transcription factor FOXO3a (FKHRL1) was shown to up-regulate *Bim* expression in hematopoetic cells (Dijkers et al., 2000), neurons (Gilley et al., 2003) and in paclitaxel-treated breast cancer cells (Sunters et al., 2003). FOXO3a also up-regulated *Puma* expression in response to cytokine/growth factors withdrawal (You et al., 2006). *Dp5/Hrk* was shown to be a direct target for c-Jun during potassium deprivation-induced apoptosis in cerebellar granule neurons (Ma et al., 2007). *Noxa* (Kim et al., 2004) and *Bnip3* (Bruick, 2000; Sowter et al., 2001) were activated by HIF-1α in response to hypoxia. In contrast to the apoptotic stimuli, pro-survival signals were shown to promote transcriptional silencing of BH3-only genes. The transcriptional repressor DREAM that is activated by IL-3 was shown to bind to a silencer sequence in the 3′ untranslated region of the *Hrk* gene and abrogate its expression in interleukin-3 (IL-3)-dependent hematopoietic progenitor cells (Sanz et al., 2001). Under non-stress conditions, the expression of *Bnip3* was shown to be transcriptionally repressed by the p65 subunit of NF- B in cardiomyocytes (Shaw et al., 2008) or by the repressive form of E2F, E2F4, in fetal liver cells (Tracy et al., 2007). As described in the accompanying Chapters, the transcription BH3-only members such as *Bnip3* (Okami et al., 2004), *Noxa* (Yamashita et al., 2008) and *Bik* (Dai et al., 2006; Pompeia et al., 2004) were shown to be epigenetically silenced by promoter methylation and histone acetylation in different cellular contexts.

Stability and translation of mRNA

The posttranscriptional regulation of mRNA levels of the BH3-only members is the least studied of the regulatory mechanisms that control their activity. An example of such a regulation was noted in the control of stability of *Hrk* mRNA (Inohara et al., 1997). An AUUUA sequence motif in the 3′ untranslated region of *Hrk* was shown to contribute to mRNA destabilization. The stability of *Bim* mRNA was also reported to be regulated by an AU-rich element in the 3′ untranslated region (Matsui et al., 2007). The micro RNA (miR) cluster miR-17-92 was reported to inhibit translation of *Bim* mRNA (Xiao et al., 2008). Transgenic expression of the miR-17-92 resulted in phenotypes similar to those seen in $Bim^{-/-}$ mice (see Chapter by Penon, Egle and Villunger).

Protein stability

A number of studies that examined the effects of various proteasome inhibitors reported substantial increases in the levels of BH3-only proteins such as BIK (Hur et al., 2004; Marshansky et al., 2001; Zhu et al., 2005), BIM (Nikrad et al., 2005) and NOXA (Fernandez et al., 2005). Direct evidence linking the proteasomal pathway in down-regulation of the steady-state levels of BH3-only proteins is limited. A direct role for ubiquitilation-dependent regulation of BIM came from studies on apoptosis regulation in osteoclsts (Akiyama et al., 2003). It was shown that following growth factors withdrawal, reduced ubiquitylation and proteasomal degradation of BIM resulted in a rapid and sustained increase in the level of BIM. Importantly, c-Cbl family E3 ubiquitin ligases were involved in ubiquitilation and proteasomel degradation of BIM in osteoclasts. It appears that the regulation at the level of protein stability is required to prevent unscheduled apoptosis in cells where the BH3-only members are constitutively transcribed. For example, in some breast cancer cell lines that expressed relatively high levels of *Bik* mRNA, BIK protein was undetectable and treatment with proteasome inhibitors increased BIK accumulation (Hur et al., 2004).

Post-translational modifications

Phosphorylation of BH3-only proteins has been reported to either positively or negatively influence their apoptotic activity. Phosphorylation of BAD was shown to abolish its pro-apoptotic activity (Zha et al., 1996b; Zhou et al., 2000), and dephosphorylation of BAD by Ca^{2+}-sensitive phosphatases such as calcineurin led to apoptosis (Wang et al., 1999). Analysis of the regulation of BIM activity by phosphorylation (reviewed in (Ley et al., 2005)) revealed opposing effects of phosphorylation on its activity depending on the site(s) of modification and the kinase(s) involved. A more recent study using transgenic mice constructed with a series of mutant alleles with phosphorylation-defective BIM proteins clearly demonstrated that phosphorylation of BIM on different sites contributed to the sensitivity of cellular apoptotic responses (Hubner et al., 2008). Mutation of Thr112 caused decreased binding of BIM to BCL-2 and increased cell survival. In contrast, mutation of the Ser55, 65, and 73 sites caused increased apoptosis due to reduced proteasomal degradation of BIM. Phosphorylation of human BIK was shown to increase the pro-apoptotic activity through enhanced interaction with anti-apoptotic proteins (Verma et al., 2001). A phosphomimetic version of BIK (designated BikDD) was used to as an anti-cancer therapeutic gene to kill pancreatic cancer cells in an orthotopic mouse model (Xie et al., 2007).

The cell-cycle-checkpoint and DNA damage repair protein RAD9 was shown to interact with BCL-2 and BCL-xL through its BH3 domain and promote apoptosis after DNA damage (Komatsu et al., 2000). Exposure to DNA-damaging agents was shown to activate c-Abl tyrosine kinase that bound to the C-terminal region of RAD9 *via* the SH3 domain and phosphorylated the RAD9 BH3 domain at Tyr28. Phosphorylation of RAD9 induced binding to BCL-xL and apoptosis (Yoshida et al., 2002). Mutation of the phosphorylation site

(Tyr28→Phe) completely inhibited c-Abl-mediated phosphorylation, abrogated binding to BCL-xL and attenuated the apoptotic activity (Yoshida et al., 2002).

Conformational activation

As most BH3-only proteins appear to be constitutively pro-apoptotic when overexpressed, there are only limited examples of conformational activation of these proteins. An interesting example is the BH3-only-like protein, modulator of apoptosis-1 (MAP-1) that is involved in receptor-mediated apoptosis (Baksh et al., 2005). The BH3 domain of MAP-1 appeared to be hidden inside the inactive molecule through an intramolecular interaction. The death receptor signaling was shown to promote intermolecular electrostatic interactions involving the C-terminus of the tumor suppressor RASSF1A and a basic stretch within MAP-1 resulting in the opening of MAP-1. The 'open' form of MAP-1 was show to associate with BAX and induce BAX conformational change (Baksh et al., 2005). There are several BH3-only-like proteins without significant cell death activity under normal assay conditions. It is possible that some of these proteins may need appropriate conformational activation to unleash their apoptotic activity.

The BH3-only protein BID (which appears to be structurally related to BAX, see Chapter by Billen, Shamas-Din and Andrews) was shown to be non-functional as a full-length protein. Upon death factor receptor signaling, BID was shown to be cleaved by caspase-8 resulting in a N-terminally truncated form (tBID) (Li et al., 1998; Luo et al., 1998). tBID was shown to be targeted to mitochondria and amplify death receptor-induced cell death *via* the mitochondrial pathway. Thus, the activation of BID appears to represent a unique mode of post-translational activation.

Subcellular localization and sequestration

Several BH3-only proteins, including BIK, BIM, BNIP3, and HRK have predicted transmembrane domains (Fig. 2), that are implicated in targeting them to intracellular membranes (including the ER and/or mitochondria). PUMA was shown to be targeted to mitochondria by a C-terminal hydrophobic domain (Nakano and Vousden, 2001;Yu et al., 2003). Other BH3-only members such as BAD, BID and NOXA do not contain obvious membrane targeting sequences. NOXA was shown to possess a unique mitochondrial targeting domain in the C-terminal region (Seo et al., 2003). The mitochondrial targeting of tBID was shown to be facilitated by post-translational (rather than co-translational) N-myristoylation upon exposure of a glycine residue after cleavage with caspase-8 (Zha et al., 2000) as well as by two α helices (Garcia-Saez et al., 2004). Several of the BH3-only proteins have been reported to mediate efficient apoptosis independent of such membrane targeting sequence, at least under *in vitro* assay conditions that involved transfection of expression vectors. This raises a question whether interaction with multidomain BCL-2 family proteins may also play a role in membrane localization. Mutants of BIM that either lacked the entire BH3-domain (O'Connor et al., 1998) or point mutants that carry mutations rendering them unable to bind to BCL-2 (Weber et al., 2007) were found to localize in intracellular membranes, suggesting that BH3-only proteins may autonomously localize to membranes. Thus, it appears that the membrane localization activity of BH3-only members is an intrinsic property intended to increase their local concentration in intra-cytoplasmic membranes.

In some instances the BH3-only proteins have been shown to be sequestered in inactive protein complexes. In healthy cells BIM was shown to be sequestered in the microtubule-associated dynein motor complex (Puthalakath et al., 1999). Apoptotic signals induced BIM dissociation from the complex and interaction with BCL-2. In a number of lung cancers with the worst prognosis, BNIP3 was reported to be localized in the nucleus (Giatromanolaki et al., 2004). Similar nuclear localization was also reported in the majority of primary glioblastoma

multiforme samples (Burton et al., 2006) suggesting that functional sequestration of BNIP3 may play a role in the progression of solid tumors.

The above examples suggest that the activity of BH3-only proteins is strictly regulated in time and in place by multiple mechanisms to assure proper response to cell death/survival signals. Additional examples and discussion may be found in previous reviews on this subject (Fernandez-Luna, 2008; Puthalakath and Strasser, 2002; Shibue and Taniguchi, 2006; Willis and Adams, 2005).

Role in apoptosis: bind to neutralize or activate, insert to disturb

In the canonical mitochondrial apoptotic pathway, BH3-only proteins are considered to be apical sentinels that connect the various death stimuli with the multidomain BCL-2 family proteins to mediate apoptosis. But the precise mode of action of BH3-only proteins remains an intensely debated issue. At present there are two models – 'direct binding and activation of BAX/BAK' and 'neutralization of anti-apoptotic BCL-2 family proteins and displacement of BAX/BAK'. These models have been discussed in several reviews (Chipuk and Green, 2008; Hacker and Weber, 2007; Shibue and Taniguchi, 2006; Willis and Adams, 2005) and in the accompanying Chapter by Giam, Huang and Bouillet (also see Fig. 5; indicated by bold arrows 1 and 2). Both of these models are based on the assumption that the key role of BH3-only proteins in apoptosis is the interaction through their BH3-domains with multidomain BCL-2 family proteins. In the first model, the priority and significance in mediating the irreversible decision to die is dependent on binding to multidomain pro-apoptotic proteins BAX and BAK. The second model is based on the near universal property of BH3-only proteins to interact with the anti-apoptotic BCL-2 family members. Both models agree that BAX and BAK are activated as the result of binding. According to the direct activation model, BAX and BAK are postulated to be conformationally activated as a result of binding with activator BH3-only proteins (such as tBID and BIM). In the second model, BAK is postulated to be released from complexes with BCL-xL and MCL-1. However, the displacement model does not adequately address the mode of BAX activation (see below). The two models predict that BH3-only proteins differ in their apoptotic potential. Moreover, according to both models BIM is a more potent killer than BAD. However, the rationale behind such supposition is different for the two models. According to the first model, BIM is more apoptotic because it is able to directly bind and activate BAX/BAK whereas the "sensitizer" BAD cannot bind to BAX/BAK and requires an additional "activator" BH3-only protein for apoptosis to proceed. By the second model, BIM is more active because it binds to all five anti-apoptotic BCL-2 proteins (BCL-2, BCL-xL, BCL-w, MCL-1 and A1), where as BAD is able to bind and neutralize only three of them (BCL-2, BCL-xL and BCL-w).

Both of these models have limitations. The major concern about the direct activation model is that at present there is no direct structural evidence for complex formation between native activator BH3-only proteins and BAX/BAK. The second model cannot explain activation of BAX, because in normal cells BAX is presumed to exist mainly as a monomeric cytosolic protein and not in complex with anti-apoptotic proteins. Hence it is believed that BAX may not require displacement for activation. It appears that this issue may need further investigation to determine whether a fraction BAX is in complex with BCL-2/BCL-xL. It should be noted that BAX was originally discovered in complex with BCL-2 (Oltvai et al., 1993). Recently, BIK (which mediates its pro-apoptotic solely through BAX in human epithelial cells) was shown to activate BAX by a displacement mechanism (Gillissen et al., 2007). A caveat to both models is that they assume all BH3-only members function identically in different cell types. There is evidence to suggest that cell death activity of some BH3-only members is dependent on the cell type. Therefore, caution should be exercised in universal interpretation of the data on protein interactions and cell death activities.

In this context, it is necessary to consider additional features of BH3-only proteins that might be important for understanding their role in apoptosis – ability to interact with lipids (see Fig. 5, indicated by thin arrow, 3). Such an activity may contribute to the potency of activator BH3-only members BID and BIM. It has been suggested that the hydrophobic cleft at the protein surface of BID could be involved in binding to amphipathic molecules (Chou et al., 1999). Interestingly, the homologous cleft in BCL-xL was shown to bind with lysophosphatidylcholine (Losonczi et al., 2000). It was suggested that such binding mimicked natural interactions with membranes and their lipids, suggesting the natural ligand for the exposed hydrophobic cleft in BID and BCL-xL might be a lipid-like molecule (Cristea and Degli Esposti, 2004). Dynamic interactions of BID with membrane lipids, especially cardiolipin (reviewed in (Cristea and Degli Esposti, 2004)) and a channel-forming activity of BID in synthetic lipid bilayers have been reported (Schendel et al., 1999). A BH3-independent membrane destabilizing property of tBID has also been described (Kluck et al., 1999;Kudla et al., 2000). By analogy with BAX, recombinant tBID was reported to form oligomers in the presence of non-ionic detergents and mitochondrial lipids (Grinberg et al., 2002). tBID was reported to mediate remodeling of mitochondrial cristae resulting in relocation of cytochrome c from cristae stores to the intermembrane space, independent of interaction with BAX (Scorrano et al., 2002). Although the mechanism of cristae remodeling by tBID is unknown, it was speculated that tBID might participate in lipid-modifying multiprotein complexes that regulate changes of mitochondrial ultrastructure (Karbowski and Youle, 2003). Recent data on BIM also highlight the importance of mitochondrial membrane insertion for the cell-death activity (Weber et al., 2007). First, an analysis of interaction of BIM with anti-apoptotic BCL-2 proteins showed that the amount of BCL-2, BCL-xL, and MCL-1 co-precipitating with BIM-S appeared to be similar in healthy cells and in apoptotic cells with induced BIM-S expression. Second, examination of BIM-S mutants revealed that apoptosis induction correlated with mitochondrial localization but not with the ability to bind BCL-2. These results suggested that the pro-apoptotic activity of BIM-S was dependent more on insertion into mitochondrial membrane rather than on interaction with anti-apoptotic proteins. In the case of BNIP3, the trans-membrane domain (which can form autonomous stable detergent-resistant dimers) was reported to be the primary determinant for its cell death activity through mitochondrial membrane insertion and induction of mitochondrial membrane potential loss (Vande Velde et al., 2000) (see accompanying Chapter by Chinnadurai, Vijayalingam and Gibson)

Thus, it appears that the majority of the published data support the notion that anti-apoptotic BCL-2 family members block the apoptotic activity of BH3-only proteins by direct binding and sequestering them. But these data do not mean that the opposite is true *i.e*., the BH3-only proteins induce apoptosis primarily through binding to anti-apoptotic BCL-2-like proteins. Similarly, activation of BAX/BAK by direct binding with BH3-only proteins appears to be limited to BH3-only proteins such as BID and BIM. Since BID appears to be structurally similar to BAX, interpretations that it may function like other BH3-only members should also be tempered. Issues such as the potential interactions of BH3-only proteins with intracellular membranes/organelles (mainly mitochondria) should be considered.

Non-apoptotic functions of BH3-only proteins

Growing evidence suggests that BH3-only proteins are also involved in other vital cellular functions besides their role in apoptosis (Hetz and Glimcher, 2008). The best studied example is BAD (see accompanying Chapter by Danial). BAD was reported to play a role in regulation of cell cycle progression through dimerization with BCL-xL to bypass G0/G1 arrest (Chattopadhyay et al., 2001). In a different study, overexpression of BAD was found to enhance cell cycle progression and interleukin 2 production after T cell activation as a consequence of Akt/BAD regulation in primary T cells (Mok et al., 1999). BAD was also shown to reside in a functional holoenzyme complex containing glucokinase involved in glucose-driven

mitochondrial respiration (Danial et al., 2003). In addition, BAD was shown to play an important role in glucose-stimulated insulin secretion by pancreatic β cells (Danial et al., 2008). This function of BAD was specifically dependent upon the phosphorylation of its BH3 domain.

Although conflicting results on the role of BID in DNA-damage response have been reported (Kamer et al., 2005; Zinkel et al., 2005), others have reported no such effect (Kaufmann et al., 2007). As pointed out earlier, BID has been implicated in lipid transfer between mitochondria and other cellular membranes, suggesting that BID may be involved in the transport and recycling of mitochondrial phospholipids (Esposti et al., 2001). BNIP1 (also known as vesicle transport protein SEC20) was shown to participate in the organization and biogenesis of the ER by mediating membrane fusion as a component of the SNARE complex (Nakajima et al., 2004). Interestingly, the BH3 domain of BNIP1 was important for the binding to alpha-SNAP, an adaptor that serves as a link between the chaperone ATPase NSF and SNAREs (Nakajima et al., 2004). The role of RAD9 (which possesses a 3′→5′ double stranded DNA exonuclease activity (Bessho and Sancar, 2000)) as a component of the 9-1-1 cell-cycle checkpoint response complex in DNA repair is well established (Lieberman et al., 1996; St Onge et al., 1999). TGM2 (Tissue or type 2 transglutaminase) is a multifunctional enzyme belonging to the transglutaminase family (Lorand and Graham, 2003). Its primary enzymatic activity involves post-translational modification of proteins by establishing ε(γ-glutamyl)lysine and N, N-bis (γ-glutamyl)polyamine isopeptide linkages. This activity mediates cross-linking of proteins and conjugation of polyamines to proteins. Missense mutations in the *Tgm2* gene encoding transglutaminase 2 were found in patients with early-onset type 2 diabetes (Porzio et al., 2007).

Role in autophagy and autophagic death

Beclin 1 was identified as a BCL-2 interacting protein (Liang et al., 1998). It is also known that Beclin 1 is a critical effector of autophagy in mammalian cells (Kihara et al., 2001; Zeng et al., 2006). The structure of BCL-xL – Beclin 1 complex demonstrated that Beclin 1 contained a BH3 domain through which it interacted with BCL-xL (Feng et al., 2007; Oberstein et al., 2007) and vBCL-2s (Ku et al., 2008; Sinha et al., 2008) (see Chapter by Sinha and Levine). Although these findings raised the possibility that Beclin 1 might have a pro-apoptotic role, no such activity has yet been associated with Beclin 1. However, interaction of Beclin 1 with cellular and viral BCL-2s was shown to inhibit autophagy (Pattingre et al., 2005). The BH3-only protein BNIP3 was associated with autophagic cell death induced by certain anticancer agents (Daido et al., 2004). Other BH3-only proteins BAD, EGL-1 and BIK as well as the BH3 mimetic ABT737 were also reported to induce autophagic death (Maiuri et al., 2007b; Rashmi et al., 2008). This activity of BAD, EGL-1, ABT737 (Maiuri et al., 2007b) or BNIP3 (Zhang et al., 2008) was linked to their ability to dislodge Beclin 1 from a complex with BCL-xL or BCL-2. Further studies are required to elucidate the role of BH3-only members in autophagy under normal physiological conditions. Studies with *Bnip3L*$^{-/-}$ mice revealed that BNIP3L was required for normal maturation erythrocytes (Diwan et al., 2007a). This activity of BNIP3L was linked to targeted autophagic elimination of mitochondria (Sandoval et al., 2008; Schweers et al., 2007). Knock-down of BAD in mammalian cells reduced starvation-induced autophagy while overexpression increased the autophagic response (Maiuri et al., 2007a). In *C. elegans*, a gain-of-function mutant of *egl-1* induced autophagy, while a deletion diminished such a response (Maiuri et al., 2007a). Thus, the BH1-4 and BH3-only proteins have emerged as important regulators of autophagy and autophagic cell death in mammalian cells. It is possible that several BH3-only proteins may serve as sensors and initiators of autophagy.

Role in tumorigenesis

As BH3-only proteins play pivotal role in apoptosis following cellular damage, it is likely that these proteins are critical components of the tumor suppression pathways. Loss of expression of BH3-only genes may promote tumorigenesis due to lack of apoptosis and accumulation of cells with tumorigenic potential. There is strong evidence that several BH3-only members function as tumor suppressors (reviewed by (Fernandez-Luna, 2008; Karst and Li, 2007)). Gene disruption in mice revealed the role for some BH3-only proteins in suppressing tumor development, but not for others. *Puma*$^{-/-}$ and *Noxa*$^{-/-}$ mice exhibited no developmental abnormalities (Shibue et al., 2003; Villunger et al., 2003). *Bid*-deficient mice did not display a phenotype initially (Yin et al., 1999), but subsequently developed a myeloid hyperplasia that progressed to a malignancy resembling chronic myelomonocytic leukemia (Zinkel et al., 2003). These results suggested that BID might function as a tumor suppressor in myeloid cells. *Bad*$^{-/-}$ mice spontaneously developed diffuse B cell lymphoma (Ranger et al., 2003). Loss of a single or both alleles of *Bim* in mice was shown to promote B cell lymphoma development under conditions of oncogenic c-Myc expression (Egle et al., 2004). Thus, studies with mutant mouse models have suggested tumor suppressive functions for some of the BH3-only members.

Additional insights about the role of BH3-only proteins in tumorigenesis arise from genetic studies of human cancers. Different studies have indicated that loss of expression of several BH3-only proteins might contribute to human cancer. Frequent deletions encompassing the *Puma* locus in chromosome 19q13.3 were reported in gliomas, neuroblastomas, and in certain B cell lymphomas (reviewed in (Karst and Li, 2007)). The chromosomal locus containing *Bmf* (15q14/15) was also proposed to harbor a tumor suppressor gene, since loss of this region was associated with advanced carcinomas of the breast, lung and colon (Schmutte et al., 1999; Wick et al., 1996). Loss of BIK expression, associated with allelic loss and/or methylation, was reported in renal cell carcinomas (Sturm et al., 2006). Chromosomal deletions in 22q13 harboring the *Bik* gene were reported in gliomas (Bredel et al., 2005), colorectal cancers (Castells et al., 1999) and in head and neck cancers (Reis et al., 2002). Mutations in the *Bik* gene were found in B-cell lymphomas (Arena et al., 2003). Among a large number apoptosis regulating genes examined in a gene expression profiling study, only expression of *Bik* mRNA was highly activated in human lung, prostate and renal carcinoma cell lines that were treated with inhibitors of DNA methyltransferase and histone deacetylases, suggesting silencing of *Bik* expression in these cells (Dai et al., 2006). Inactivation of the *Hrk* gene by methylation was reported in gastric and colorectal cancers (Obata et al., 2003) and glioblastomas (Nakamura et al., 2005). It was also suggested that reduced expression of HRK may serve as one important molecular mechanism in the progression to secondary glioblastoma (Nakamura et al., 2005).

Low rates of inactivating *Bid* mutations were observed in gastric cancers (Lee et al., 2004). *Bim* mutations in human cancers have not been reported (Karst and Li, 2007). But, loss of the chromosomal region 2q13, containing the *Bim* gene, was observed in mantle cell lymphomas (Tagawa et al., 2005). Analysis of major subtypes of human B-cell lymphomas found no mutation in the *Bad* gene, indicating that mutational inactivation of BAD plays no role in the pathogenesis of these malignances (Schmitz et al., 2006). However, suppressed expression of *Bad* through methylation was found in a multiple myeloma cell line (Pompeia et al., 2004). The *Noxa* gene was rarely mutated in human carcinomas (Lee et al., 2003), but *Noxa* was found to be mutated and preferentially silenced in diffuse large B cell lymphomas (Mestre-Escorihuela et al., 2007). Together, these studies suggest that loss of expression of many BH3-only proteins through gene deletion or epigenetic silencing may contribute to tumor formation. Interestingly, inactivating mutations in BH3-only genes were rarely observed.

In contrast to the results summarized above, there are several reports that seem to suggest that some BH3-only proteins may not fit the tumor suppressor mold. A large study of BID protein expression in prostate, ovarian, colorectal, and brain cancers, and B cell non-Hodgkin's lymphomas demonstrated increased expression in tumor tissues (Krajewska et al., 2002). An immunohistochemical analysis of BID expression in hepatocellular carcinomas revealed that BID expression was often stronger in nuclei of the epithelial cells in tissues from liver metastases than in the normal liver tissues, and the nuclei of metastatic cells showed considerable mitotic activity, indicating that they were in active proliferation (Chen et al., 2001). Tumorigenesis induced by diethylnitrosamine was considerably retarded in *Bid-null* mice (Bai et al., 2005). Further analysis demonstrated that there were significantly fewer proliferating cells in diethylnitrosamine-treated $Bid^{-/-}$ livers. Although the mechanism underlying such an activity of BID in cell proliferation is not known, it is possible that apoptosis incompetent full-length BID may play such a role. As pointed in the accompanying Chapter on BIK, overexpression of *Bik* in sporadic breast cancers (Garcia et al., 2005) and in non-small cell lung tumors from patients with high-risk cancer recurrences was reported (Lu et al., 2006). Since overexpression of *Bik* also accompanied other genes such as *Bcl-2* and pro-inflammatory genes, it is possible that inflammatory response to chronic *Bik*/*Bcl-2* expression may promote tumorigenesis. Similarly, high expression of *Bnip3* was also reported in breast and lung cancers with poor prognosis. Since *Bnip3* expression was enhanced in the necrotic areas of the tumor, tumor necrosis resulting from *Bnip3* overexpression may promote tumor progression. Initial overexpression of BH3-only members may facilitate selection of aggressive tumor cells during tumor progression, ultimately leading to silencing of these genes in advanced cancers.

Role in cytotoxicity to anticancer drugs and in the development of cancer therapeutics

Multiple studies have demonstrated the pivotal role of BH3-only proteins in the cytotoxic response to anticancer agents. In addition, chemoresistance of many cancers was attributed to the loss or lower level of expression of BH3-only proteins. PUMA expression in many cancer cell lines was strongly and rapidly upregulated in response to DNA-damaging agents, such as adriamycin, 5-fluorouracil, etoposide, and ionizing radiation (Han et al., 2001; Nakano and Vousden, 2001; Yu et al., 2001). In addition, thymocytes and myeloid progenitors from $Puma^{-/-}$ mice were resistant to apoptosis induced by various cytotoxic stimuli, including anticancer drugs (Jeffers et al., 2003; Villunger et al., 2003). Dramatic up-regulation of NOXA, BIM, and BIK was reported in cancer cells treated with the proteasome inhibitor bortezomib (Velcade) (Fernandez et al., 2005; Nikrad et al., 2005; Qin et al., 2005; Zhu et al., 2005). Rapid accumulation of BIM was also noted in cancer cells treated with the anti-microtubule drug paclitaxel and $Bim^{-/-}$ cells were resistant to the drug (Tan et al., 2005). Cytotoxicity of glucocorticoid to lymphoid tumor cell lines was also linked to induction of BIM expression (Bachmann et al., 2005; Wang et al., 2003).

Based on the well-established feature of BH3-peptides of BH3-only proteins to bind to anti-apoptotic BCL-2 family proteins to induce apoptosis, it was hypothesized that BH3 peptides and non-peptide small-molecules that mimic BH3-peptide could be exploited as cancer drugs. Indeed, a search for non-peptide inhibitors of BCL-2, BCL-xL and MCL-1 resulted in the identification of several candidates (Enyedy et al., 2001; Kitada et al., 2003; Ponassi et al., 2008; Tang et al., 2007; Tzung et al., 2001). Intensive investigations on one of the compounds, ABT-737, demonstrated that as a single agent, ABT-737 effectively killed cells from several tumor types and caused regression of certain tumors in mice (Oltersdorf et al., 2005). Molecular analysis revealed that ABT-737 was similar to the BAD BH3-peptide in the binding preferences to BCL-2 family members - it targeted BCL-2, BCL-xL and BCL-w but not MCL-1 or A1 (Oltersdorf et al., 2005; van Delft et al., 2006). Another BH3 mimetic, GX15-070 bound to all

five anti-apoptotic proteins (Nguyen et al., 2007; Shore and Viallet, 2005). GX15-070 is currently in multiple phase I and phase II clinical trials against solid tumors and hematologic malignancies (Fernandez-Luna, 2008; O'Brien et al., 2008). Importantly, design of new BH3-mimetics is being intensively pursued as a novel strategy for the development of new anti-cancer drugs. More comprehensive discussion on various BH3 peptidomimetics could be found in recent reviews (Fennell and Chacko, 2008; Vogler et al., 2008) and in the accompanying Chapter by Ni Chonghaile and Letai.

Role in rheumatoid arthritis and in heart failure

Rheumatoid arthritis (RA) is characterized by the excessive accumulation of inflammatory cells in joints. The increase in cellularity in RA joints appears to be results of reduced apoptosis. The fibroblast-like synoviocytes were shown to exhibit up-regulation of BCL-2 family anti-apoptosis proteins (Liu et al., 2006). BH3-only members BIM and BID were shown to protect against RA in the mouse model (Scatizzi et al., 2006; Scatizzi et al., 2007). BNIP3 was also up-regulated in synoviocytes from patients with RA, but its cell death activity appeared to be blocked (Kammouni et al., 2007). Considering the protective role of BIM and BID, in an accompanying Chapter Hutcheson and Perlman propose the use of BH3-mimetics for treatment of RA.

Heart failure as a result of ischemic and non-ischemic injuries is known to result in cardiomyocyte death. Based on studies with rodent models, two BH3-only members, BNIP3 and BNIP3L have been shown to play prominent roles in cardiomyocyte death during these injuries (reviewed by (Dorn and Diwan, 2008; Shaw and Kirshenbaum, 2008)). Expression of the dominant negative forms of BNIP3 and BNIP3L (lacking the respective trans-membrane domain) inhibited myocyte death (Regula et al., 2002; Yussman et al., 2002). Transduction of a cell permeable version of dominant negative BNIP3 (tagged with the HIV-1 Tat peptide) into whole heart conferred protection against ischemia/perfusion injury and improved cardiac function (Hamacher-Brady et al., 2007). Studies with the *Bnip3*$^{-/-}$ mouse model indicated that myocyte death following ischemia/perfusion was a delayed response (Diwan et al., 2007b). In an accompanying Chapter, Dorn and Kirshenbaum suggest that this delay could be exploited for rescue of myocytes from death using strategies to inhibit BNIP3 and BNIP3L.

Conclusions

Intense investigations on BH3-only family proteins during the past decade have been highly rewarding. This work has established their critical role in the initiation of apoptosis in animal cells. Although there has been a better understanding of their role in the apoptotic process, substantial aspects of their mechanism of action remain unresolved. In addition to their primary role as initiators of apoptosis, emerging evidence indicates that many of them also have a multitude of non-apoptotic functions. How their apoptotic *vs* non-apoptotic functions are differentially regulated and integrated will be an important area of future investigation. Studies on human cancers have revealed that they exert a restraining role during tumor progression. Anti-cancer drugs that stabilize the BH3-only proteins or transcriptionally activate their expression have shown significant promise against human cancers. Similarly, several small molecules that mimic the effect BH3-only proteins are showing promise as anti-cancer agents. It is anticipated that a second generation of such drugs will be forthcoming. These drugs may also find applications in treating inflammatory diseases such as arthritis. In contrast to these two classes of drugs that are intended to stimulate the activities of BH3-only molecules or mimic their effect to promote apoptosis, it could be envisioned that a third category of drugs that inhibit the activity of specific BH3-only molecules such as BNIP3 might be of great value in preventing pathological cell death as in heart failure. Such drugs might find applications in inhibiting BH3-only molecules in certain cancers where their expression is linked to poor

prognosis. The availability of animal models for many BH3-only members offers excellent opportunity for further investigations on these important molecules.

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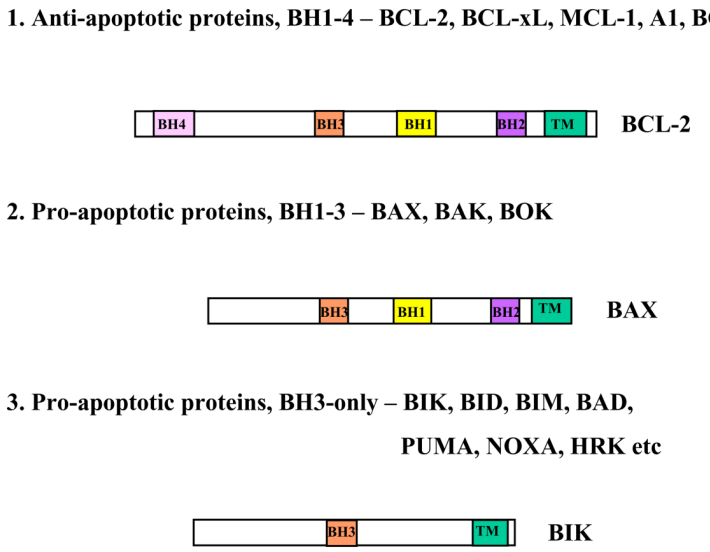

Figure 1.
BCL-2 family proteins. Domain structures of representative members of each subfamily are shown.

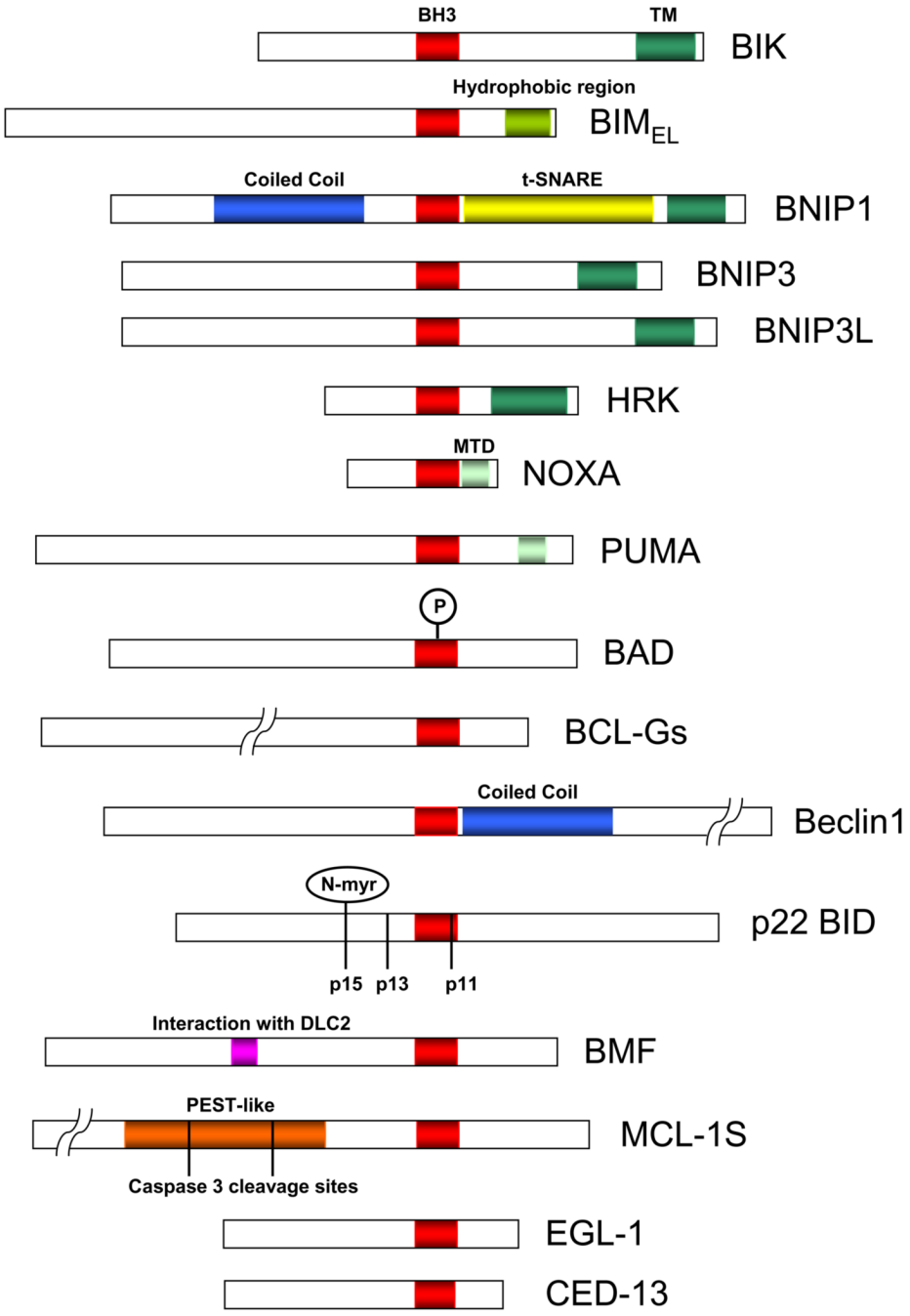

Figure 2.

Domain structure of major mammalian and worm BH3-only proteins. In addition to the BH3 domain, the C-terminal trans-membrane domain (TM), and other membrane targeting hydrophobic sequences are indicated. MTD, indicates mitochondrial targeting domain. The caspase cleavage sites in BID and MCL-1S and the phosphorylation site in the BH3 domain of BAD are indicated.

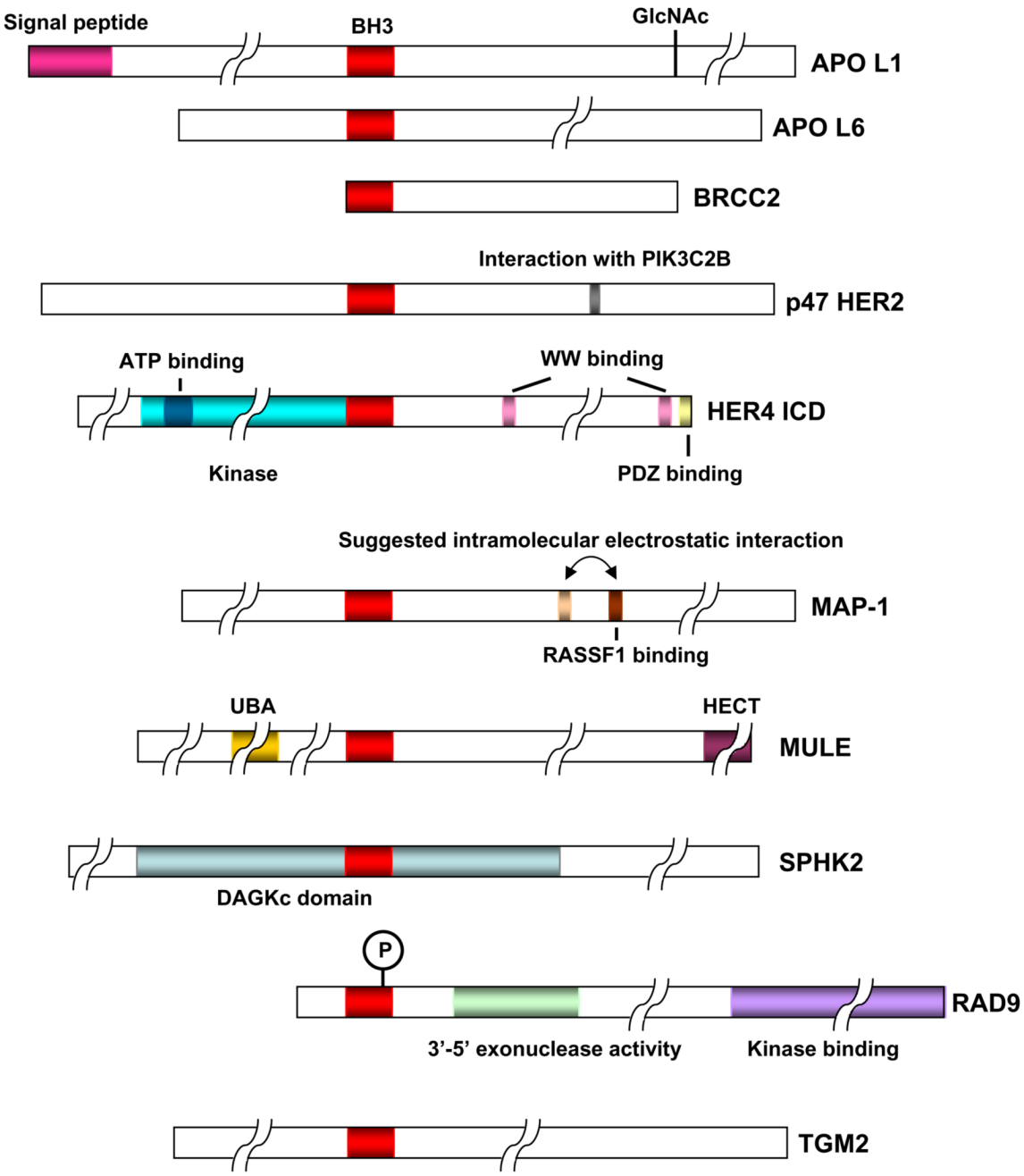

Figure 3.
Domain structure of some BH3-only-like proteins. The BH3 domain and other functional domain(s) in the respective proteins are indicated.

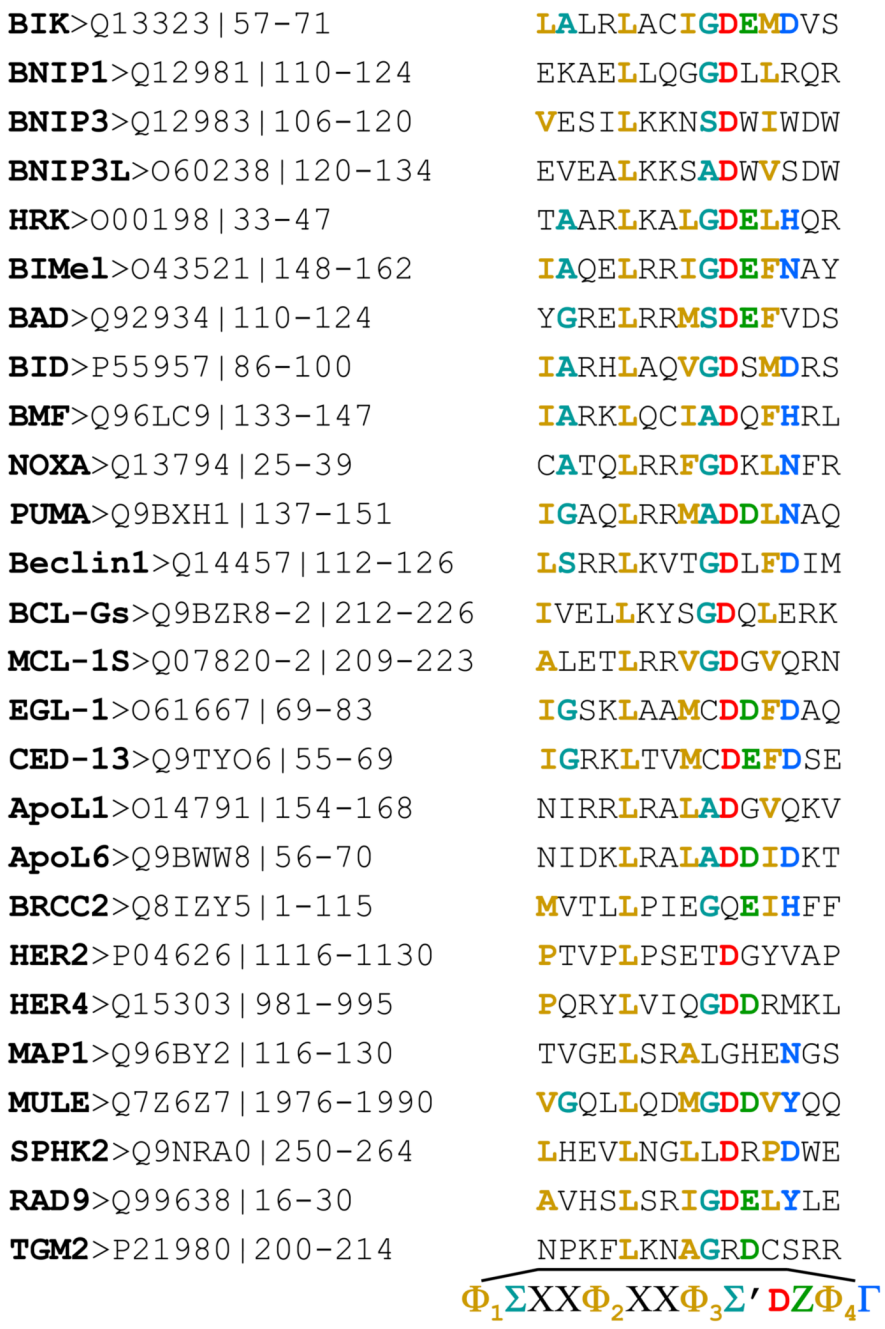

Figure 4.

Amino acid sequences of the BH3 domains. The sequences of various BH3-only and BH3-only-like proteins, their accession numbers and amino acid coordinates are indicated. The consensus sequence of BH3 domain is shown in the bottom, Φ represents a hydrophobic residue (Φ_2 is usually leucine); Σ is a small residue (G, A, S), Z is usually an acidic residue; and Γ is a hydrophilic residue (N, H, D or Y) (Day et al., 2008).

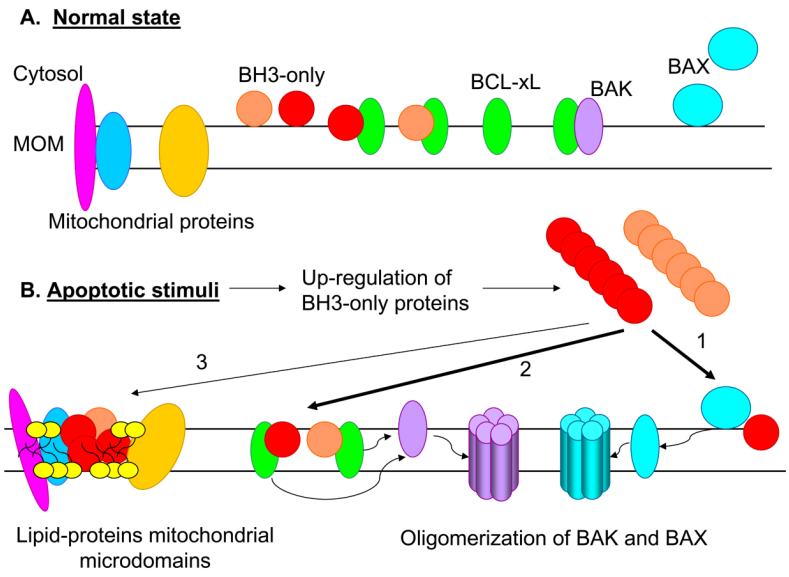

Figure 5.

Models for the apoptotic activity of BH3-only proteins. **A**. In normal state, BH3-only proteins are present in very low levels in the cytosol or in mitochondrial membrane, most likely in complex with anti-apoptotic BCL-2 family proteins. BAK is shown to be located in mitochondrial membrane in inactive complexes with BH1-4 anti-apoptotic proteins, where as BAX is shown to be in the cytosol or loosely attached to the mitochondrial membrane. **B.** Different apoptotic stimuli induce significant up-regulation of the BH3-only proteins through transcriptional and post-transcriptional mechanisms. BH3-only proteins then transmit the apoptotic signals to BAX and BAK proteins either through “direct binding/and activation” (1), or “neutralization of BH1-4 proteins and displacement of BAK/BAX” (2). In addition, BH3-only proteins is also shown to induce mitochondrial membrane remodeling/formation of altered microdomains through mitochondrial outer membrane (MOM) insertion and interaction with membrane lipids (*e.g.*, cardiolipin – yellow heads) and/or with other mitochondrial proteins (3). Two classes of BH3-only proteins (that bind with multi domain anti-apoptotic and pro-apoptotic proteins or only with anti-apoptotic proteins) are indicated in red and orange.