Chapter 9
Evolution of the BCL-2-Regulated Apoptotic Pathway

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Abstract The mitochondrion descends from a bacterium that, about two billion years ago, became endosymbiotic. This organelle represents a Pandora's box whose opening triggers cytochrome-c release and apoptosis of cells from multicellular animals, which evolved much later, about six hundred million years ago. BCL-2 proteins, which are critical apoptosis regulators, were recruited at a certain time point in evolution to either lock or unlock this mitochondrial Pandora's box. Hence, particularly intriguing is the issue of when and how the "BCL-2 proteins–mitochondria–apoptosis" triptych emerged. This chapter explains what it takes from an evolutionary perspective to evolve a BCL-2-regulated apoptotic pathway, by focusing on the events occurring upstream of mitochondria.

9.1 Introduction

It is in the form of cells that life has continued over generations for billions of years. Most of the time, these building blocks of life are defined as self-replicating elements, overlooking the fact that cells endowed with the ability to self-destruct were described in all branches of the tree of life (Bozhkov and Lam 2011; Dwyer and Winkler 2013; Kerr et al. 1972; Madeo et al. 1997). In multicellular animals (metazoans), organismal success and complexity are built upon the silent destruction and rapid removal of cells through a genetically encoded cell death process called apoptosis. This form of active (or programmed) cell death functions to sculpt shapes, optimize functions and eliminate damaged, superfluous, or harmful cells from the body, thus playing crucial roles in animal development and homeostasis.

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Adding to its importance as a physiological phenomenon, apoptosis dysregulation is involved in a wide range of diseases such as cancer (Czabotar et al. 2014; Elmore 2007). Studies on vertebrate cells have revealed the existence of two major apoptotic pathways: the extrinsic pathway initiated by the ligation of death receptors by extracellular ligands at the surface of target cells and the intrinsic (mitochondrial or BCL-2-regulated) pathway, which can be stimulated by a plethora of signals (e.g., DNA damage, endoplasmic reticulum stress, hypoxia, growth factor deprivation, and developmental cues) (Czabotar et al. 2014; Tait and Green 2013).

The BCL-2-regulated apoptotic pathway is initiated through transcriptional and/or post-transcriptional activation of so-called BH3-only proteins, which form a disparate group of proteins traditionally considered as sensors of cellular stress and damage (Doerflinger et al. 2015; Shamas-Din et al. 2011). In response to distinct upstream signaling events, some of these death effectors (i.e., BIM, PUMA, tBID) serve as ligands to activate the pro-apoptotic BCL-2 family members BAX and BAK through direct interaction, while all of them can activate BAX/BAK indirectly by binding to and inhibiting the prosurvival BCL-2 homologous proteins. Once activated through a complex multi-step process, BAX and BAK are thought to homo-oligomerize and form (or participate to) pores in the mitochondrial outer membrane (Tait and Green 2013; Volkmann et al. 2014; Westphal et al. 2014). These oligomeric pores cause the release of mitochondrial intermembrane space proteins, including cytochrome-c (cyt-c), in the cytosol (in a process termed MOMP, for mitochondrial outer membrane permeabilization). Leaked cyt-c then triggers the activation of a family of death proteases called caspases through a well-defined post-mitochondrial pathway (which will not be reviewed here). Prosurvival BCL-2 proteins can inhibit BAX-BAK activity through one or more possible mechanisms: sequestration of the "direct activator" BH3-only proteins (Llambi et al. 2011) or local inhibition of BAX (and BAK) at the mitochondrial outer membrane level (via inhibitory complex formation, oligomer disassembly, and/or retrotranslocation to the cytosol) (Billen et al. 2008a; Edlich et al. 2011; Subburaj et al. 2015). The "sensitizer" BH3-only proteins can neutralize the prosurvival BCL-2 proteins through direct binding, thus releasing the direct activators to promote BAX-BAK activation and apoptosis. An important concept that emerges from this mechanistic description is that the BCL-2-regulated pathway appears to be organized in a hierarchical manner, from cellular sentinels (BH3-only proteins) to the BCL-2/BAX apoptotic switch controlling cytosolic release of mitochondrial apoptogenic factors (Fig. 9.1). The next sections will address evolutionary perspectives on all three categories of constituents, with a particular emphasis on BCL-2 homologous proteins [aka BCL-2 family members, see https://bcl2db.ibcp.fr/BCL2DB/BCL2DBNomenclature for an explanation of nomenclature (Aouacheria 2014; Rech de Laval et al. 2014)].

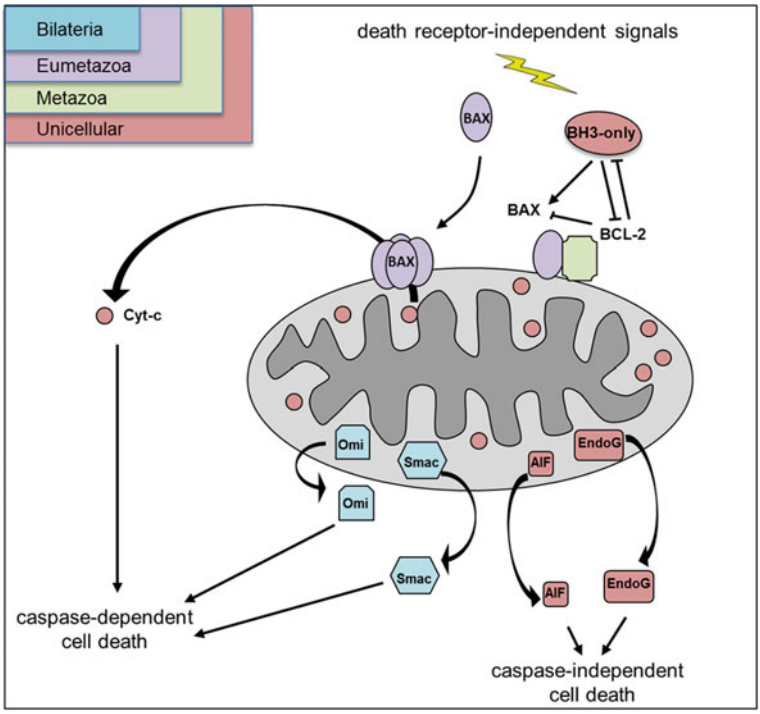


Fig. 9.1 Simplified representation of the mitochondrial apoptotic pathway. Mitochondrial outer membrane permeabilization (MOMP) constitutes the pivotal event in the mitochondrial or BCL-2-regulated intrinsic death pathway and results in the release of cytochrome-c (cyt-c) and other mitochondrial apoptogenic factors from the mitochondrial intermembrane space to the cytosol. Once in the cytoplasm, cyt-c activates a family of death proteases called caspases that leads to the cleavage of a myriad of cellular substrates, causing cell demise. This pathway is initiated by activation of BH3-only proteins which serve as ligands to activate proapoptotic BCL-2 family members (e.g., BAX) through direct interaction or by binding to and inhibiting prosurvival BCL-2 homologous proteins (like BCL-2). Once activated, BAX homo-oligomerizes and forms pores in the mitochondrial outer membrane which cause the release of apoptogenic factors. Among these apoptogenic proteins, cytochrome-c, Omi/HtrA2, and SMAC/Diablo promote caspase-dependent cell death, whereas AIF and endoG induce caspase-independent cell death. Gene products are colored by their phyletic distribution (*inset*, see text for details)

9.2 Mitochondrial Intermembrane Space Proteins

The output of MOMP corresponds to the cytosolic release of mitochondrial apoptogenic proteins normally sequestered within the intermembrane space. The following five death-promoting factors have received significant characterization: cyt-c, apoptosis-inducing factor (AIF), second mitochondrial activator of caspases (Smac)/Diablo, Omi/HtrA2 and endonuclease G (endoG). During apoptosis, cyt-c directly induces caspase activation whereas Smac/Diablo and Omi/HtrA2 neutralize

the inhibition of caspase activation (Lorenzo and Susin 2004; Saelens et al. 2004). AIF and endoG translocates to the nucleus to trigger caspase-independent DNA fragmentation (Arnoult et al. 2003; Cregan et al. 2004). All of these mitochondrial factors are encoded in the nucleus. Cyt-c and AIF have the widest phylogenetic distribution as they are found both in prokaryotes (Archaea, bacteria) and eukaryotes including protists, plants, fungi, and animals. Omi/HtrA2 and endoG also display a wide phylogenetic pattern and are present in all kingdoms of life except Archaea. Based on this, it seems reasonable to infer that these four mitochondrial apoptogenic proteins represent endosymbiotic acquisitions from the mitochondrial ancestor. In contrast, Smac/Diablo orthologues are only present in vertebrate species, suggesting a late phylogenetic origin. Interestingly, most of these mitochondrial intermembrane space proteins can act as "pencils–erasers": cyt-c, for instance, has a vital daily job in respiration (as an essential electron carrier) and becomes cytotoxic only when its gets to the cytosol (Garrido and Kroemer 2004). AIF was also suggested to exert vital normal functions (possibly pertaining to its oxidoreductase activity) (Porter and Urbano 2006; Vahsen et al. 2004; Sorrentino et al. 2015). EndoG may be involved in DNA recombination and repair in addition to proliferation (Buttner et al. 2007; Huang et al. 2006). These examples illustrate an important but often neglected aspect of many apoptotic players: their polyfunctional nature. Pleiotropy is backed by a peculiar subcellular compartmentation, i.e., sequestration of conditionally toxic proteins in a normally non-accessible subcellular compartment (the mitochondrial intermembrane space). The molecular determinants underlying the apoptotic and non-apoptotic functions have been deciphered for cyt-c and AIF (Cheung et al. 2006; Hao et al. 2005) but await characterization for the other mitochondrial factors.

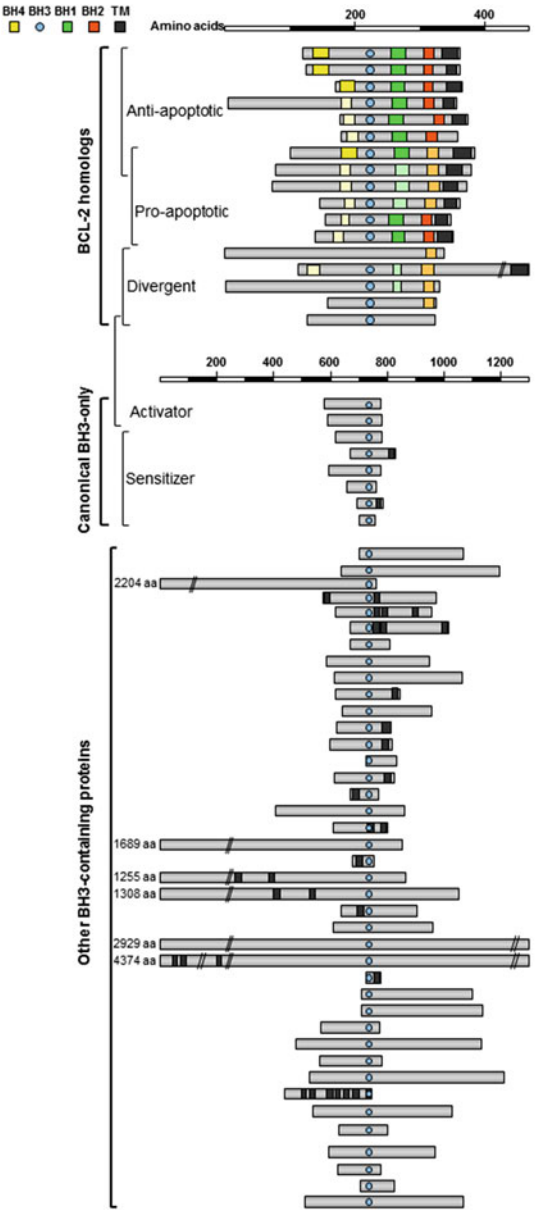
9.3 BCL-2 Homologous Proteins

BCL-2 family proteins control apoptosis upstream of the release of mitochondrial apoptogenic proteins and subsequent activation of caspases. This family of proteins comprises anti-apoptotic BCL-2 and pro-apoptotic BAX and their respective homologs. Our previous phylogenomic studies have revealed that BCL-2 homologous genes were restricted to metazoan species (and some animal viruses) and absent in fully sequenced genomes from Archaea, Eubacteria, Viridiplantae, Fungi, and other unicellular Eukaryota (Aouacheria et al. 2005), suggesting that this family arose in the metazoan stem. Logically, BCL-2 homologs are not found in the fully sequenced genomes of the choanoflagellate *Monosiga brevicollis* and the filasterean *Capsaspora owczarzaki* (King et al. 2008; Suga et al. 2013). Therefore, BCL-2 homologs most probably evolved only about 600 or 700 MYA and do not trace their origin back to the mitochondrial ancestor, their appearance being concomitant to the emergence of metazoan multicellularity.

Previous analysis indicated that gene duplication (for instance in marine invertebrates and fishes) and loss (e.g., in nematodes) played a prominent role in the

evolution of the BCL-2 family and contributed to the generation of lineage-specific diversity. Six representatives are present in the demosponge *Amphimedon queenslandica* (Srivastava et al. 2010), four in the placozoan *Trichoplax adhaerens* (Srivastava et al. 2008), nine in the cnidarian *Hydra vulgaris* (Lasi et al. 2010), ten in the zebrafish Danio rerio (Kratz et al. 2006), and fourteen in the humans (Aouacheria et al. 2005), whereas the worm *Caenorhabditis elegans* has a unique BCL-2-like gene (called CED-9) (Hengartner and Horvitz 1994) and the fruit fly (Drosophila melanogaster) only a pair of homologs (known as Buffy and Debcl) (Clavier et al. 2015). Thus, the BCL-2 gene complement of extant metazoans is not a mere function of organismal complexity but include differential gene expansion and loss across lineages. As a result, BCL-2 homologous genes are present in multiple paralogs showing substantial sequence divergence and BH (BCL-2 Homology) motif arrangements (Aouacheria et al. 2005; Aouacheria et al. 2013; Guillemin et al. 2009) (see Fig. 9.2). Phylogenetic reconstruction indicates that, in vertebrates, BCL-2 homologs segregate into three major clades: BCL-2-like, BAX-like, and BID-like members (Aouacheria et al. 2013). BCL-2-like and BAX-like members correspond to prosurvival and pro-apoptotic proteins, respectively, while BID-like members form a divergent group of proteins with diverse activities toward apoptosis. Within this last group, BPR/BCL2L12 is an anti-apoptotic protein (shown to reside in the nucleocytoplasmic compartment rather than mitochondria) (Stegh and DePinho 2011), BFK/BCL2L15 is poorly characterized but may constitute a pro-apoptotic protein (Coultas et al. 2003), and BCL-G/BCL2L14 appears to be neutral against apoptosis (Tischner and Villunger 2012). BCL-2 family members have been reported in multiple invertebrate species, including sponges, cnidarians, echinoderms, and mollusks (see Table 9.1), and many more are predicted [e.g., in *Trichoplax adhaerens* (Srivastava et al. 2008), *Ciona intestinalis* (Terajima et al. 2003), *Bombyx mori* (Zhang et al. 2010), *Apis mellifera* (Dallacqua and Bitondi 2014), and *Octopus vulgaris* (Castellanos-Martinez et al. 2014)]. Unfortunately, only a small proportion of these proteins have been fully characterized in an experimental way. Although there have been numerous published phylogenetic studies, none of them has addressed the full diversity of BCL-2 family members in invertebrates and a robust, comprehensive phylogenetic analysis is not yet available.

Since most bcl-2 family genes and proteins share commonalities in structure (e.g., an intron dividing the BH2 motif, and a similar "helical bundle" tridimensional fold—see Fig. 9.3), it appears likely that the diversity of metazoan BCL-2 genes was generated from a single precursor. The origin and functions of this ancestral BCL-2 protein are unknown. The early discovery that BCL-2 homologous proteins bear structural resemblance (analogy) to microbial toxins like colicins or the translocation domain of diphtheria toxin (Muchmore et al. 1996) has led to the speculation that they might have been acquired by horizontal gene transfer from the bacterial world. However, a set of viral proteins structurally related to BCL-2 (but functionally divergent) were recently characterized (Graham et al. 2008; Neidel et al. 2015), suggesting that the hypothesis of a viral origin for the founder gene should also be considered (Fig. 9.3). Whatever their origins, it seems reasonable to



Protein	Gene	Acc. °
BCL2	Bcl-2	P10415
BCL2L1	Bcl-x$_L$, Bcl2l1	Q07817
BCL2L2	Bcl-w, Bcl2l2	Q92843
MCL1	Mcl-1, Bcl2l3	Q07820
BCL2L10	Bcl2l10, Bcl-B, Nrh, Diva, Boo	Q9HD36
BCL2A1	Bcl2a1, Bfl1, Bcl2l5	Q16548
CED-9	CED-9 (Ce)	P41958
BUFFY	Buffy, dBorg2 (Dm)	Q8T8Y5
DEBCL	Debcl, dBorg1 (Dm)	Q9V9C8
BOK	Bok, Bcl2l9, Mtd	Q9UMX3
BAX	Bax, Bcl2l4	Q07812
BAK1	Bak, Bcl2l7	Q16611
BCL2L12	Bcl2l12, Bpr	Q9HB09
BCL2L13	Bcl2l13, Bcl-rambo, Mil1	Q9BXK5
BCL2L14	Bcl2l14, Bcl-G	Q9BZR8
BCL2L15	Bcl2l15, Bfk	Q5TBC7
BID	Bid	P55957
BCL2L11	Bim, Bod, Bcl2l11	O43521
BBC3	Puma	Q9BXH1
BAD	Bad, Bbc2, Bcl2l8	Q92934
BIK	Bik, Nbk, Blk	Q13323
BMF	Bmf	Q96LC9
EGL-1	EGL-1 (Ce)	O61667
HRK	Hrk, Dp5	O00198
PMAIP1	Noxa, Apr	Q13794
M	AF2240-M (NDV)	Q9WBL3
F0	F (NDV)	Q9DLD4
L	L (NDV)	Q6X1B6
APOL1	Apol1	O14791
APOL2	Apol2	Q9BQE5
APOL6	Apol6	Q9BWW8
ATG12	Atg12	O94817
AVEN	Aven	Q9NQS1
BECN1	Beclin-1	Q14457
BNIP1	Bnip1	Q12981
BNIP2	Bnip2	Q12982
BNIP3	Bnip3	Q12983
BNIP3L	Bnip3l, Nix	O60238
BLID	Brcc2	Q8IZY5
BNIP3	CEBNIP3 (Ce)	Q09969
CED-13	CED-13 (Ce)	Q9TY06
CLU	Clusterin	P10909
CORE	Core (HCV)	Q00269
CUL7	Cullin-7, p193 (Mm)	Q8VE73
E	E (SARS-CoV)	P59637
ERBB2	Erbb2, Her2	P04626
ERBB4	Erbb4, Her4	Q15303
ITM2B	Itm2bs	Q9Y287
MOAP1	Map-1	Q96BY2
MCF	Mcf (Pl)	Q8KT65
HUWE1	Mule	Q7Z6Z7
PXT1	Pxt1 (Mm)	Q8K459
RAD9A	Rad9a, hRad9	Q99638
RAD9	Rad9sp (Sp)	P26306
HEPB2	Soul	Q9Y5Z4
SPHK2	Sphk2	Q9NRA0
CHMP5	Spike	Q9NZZ3
TGM2	Tg2, TGase	P21980
YNL305C	Ynl305cp, Ybh3p, Bxi1p (Sc)	C8ZGL9
VIRF-1	vIRF-1 (HHV8)	P88946
BLM-s	Ccdc132	Q0GET0
BOP	Bop	Q7L3V2
HBX	X (HBV)	Q65Z75
HBSP	P (HBV)	B5TZH4
Casp8p41	Casp8	Q14790

◀ **Fig. 9.2** BH motif composition in BCL-2 homologous proteins and BH3-containing proteins. Schematic representation and BH motif composition of BCL-2 homologous proteins (including BCL-2-like, BAX-like and BID-like subgroups), canonical BH3-only proteins and other reported BH3-containing proteins (with UniProtKB accession numbers). *Light shades* depicted BH motif is uncertain. Total amino acid (aa) number is indicated for proteins that were not drawn to scale. Abbreviations for non-human proteins: *Ce Caenorhabditis elegans*; *Dm Drosophila melanogaster*; *NDV* Newcastle disease virus; *Mm* Mus musculus; *HCV* hepatitis C virus; *Pl Photorhabdus luminescens*; *Sp Schizosaccharomyces pombe*; *SARS-CoV* human SARS coronavirus; *Sc Saccharomyces cerevisiae*; *HHV8* Human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus)

infer that the appearance of BCL-2 proteins might have been instrumental to the emergence of metazoan multicellularity, through the recruitment of mitochondria to a cell death program enabling tissue differentiation and homeostasis. However, the opposite assertion could also be true, namely recruitment of BCL-2 family proteins into a mitochondrial death program as a consequence of animal multicellularity. This issue is particularly interesting because in addition to their daily job in apoptosis, BCL-2 proteins have been shown to exert physiological, non-apoptotic functions such as regulation of mitochondrial dynamics, metabolism, DNA damage response, calcium homeostasis, general autophagy, and mitophagy (Pattingre et al. 2005; Alavian et al. 2011; Autret and Martin 2009; Chen et al. 2011; Chen and Pervaiz 2007; Gross 2006; Hardwick and Soane 2013; Hollville et al. 2014; Karbowski et al. 2006; Laulier and Lopez 2012; Murakawa et al. 2015; Perciavalle et al. 2012; Pinton and Rizzuto 2006; Wang et al. 2013). These findings suggesting that the function of BCL-2 proteins is pleiotropically linked to prosurvival traits in extant metazoan species raise the possibility that the ancestral function of BCL-2 proteins was unrelated to apoptosis regulation and that these proteins were exapted from an ancestor with an originally different function.

Evolutionary information is scarce about the beginnings of paralog divergence in the family and about how the repertoire of BCL-2 family genes evolved in the different metazoan lineages. Evidence of conserved colinearity (i.e., relict linkage) was gathered for the divergent BCL-2 homologs BID and BCL2L13 in vertebrate genomes (Aouacheria et al. 2005), providing information about the time when the cross talk between the intrinsic and extrinsic apoptosis pathways—which is mediated by BID—evolved. An early duplication involving these genes at the invertebrate-to-vertebrate transition, followed by two further duplications gave rise to the BID-like clade characterized by the absence of the C-terminal transmembrane segment (TM) (Aouacheria et al. 2013), illustrating the fact that many gene (sub) family expansions probably originally occur as tandem or proximal duplications (Charon et al. 2012; Fan et al. 2008; Srivastava et al. 2008). In the case of BCL2A1/BFL-1, a prosurvival BCL-2 homolog which appears to be found only in mammals, the exon encoding this TM segment has been replaced by a heterologous sequence, possibly as a result of duplication and shuffling events (Ko et al. 2007). BID, BCL2L13, and BCL2A1 correspond to phylogenetically recent innovations in metazoans, but other BCL-2 family genes are of more ancient origin such as proapoptotic BAK and prosurvival BCL2L1 (BCL-xL), for which close or

Table 9.1 Reported invertebrate BCL-2 proteins

Species	Reference	Gene/protein (Acc. °)	Motif composition (as published)	Function
Echinodermata				
Strongylocentrotus purpuratus	Robertson et al. (2006)	SPU_024469 SPU_006124 SPU_021416 SPU_001916 SPU_016028 SPU_014028 SPU_010641 SPU_010786 SPU_017154	BH4 TM TM BH3, TM BH3, TM BH3, TM	
Arthropoda				
Apis mellifera	Dallacqua and Bitondi (2014)	Ambuffy (A0A088ACI8)	BH1-4, TM	
Bombyx mori	Pan et al. (2014)	Bmbuffy (E9JEG2)	BH1-3, TM	Anti
Drosophila melanogaster	Quinn et al. (2003) Colussi et al. (2000)	Buffy (Q8T8Y5) Debcl (Q9V9C8)	BH1-3, TM BH1-3, TM	Anti Pro
Mollusca				
Ruditapes philippinarum	Lee et al. (2013)	RpBCL-2A (KC506418) RpBCL-2B (KC506419)	BH1-4, TM BH1-3, no TM	
Mytilus galloprovincialis	Estevez-Calvar et al. (2013)	Bcl2 (KC545829) Bax (KC545830)	BH1-4, TM BH1-3, TM	
Chlamys farreri	Qi et al. (2015)	CfBcl-2 (KJ611244) CfBax (KJ620057)	CfBcl-2: BH4, BH3, BH1, BH2, no TM CfBax: BH3, BH1, BH2, no TM	
Crassostrea hongkongensis	Xiang et al. (2015)	ChBax (KM262836) ChBak (KM262837)	BH3, BH1, BH2, TM	
Nematoda				
Caenorhabditis elegans	Hengartner and Horvitz (1994)	P41958	BH1-4, TM	Anti

(continued)

Table 9.1 (continued)

Species	Reference	Gene/protein (Acc. °)	Motif composition (as published)	Function
Platyhelminthes				
Schmidtea mediterranea	Bender et al. (2012)	Smed-Bak-1 (JN621808) Smed-Bak-2 (JN621809) Smed-bak-3 (JN621810) Smed-bok-2 (JN621814) Smed-bcl2-1 (FJ807655) Smed-bcl2-3 (JN621816)		Pro
Schistosoma mansoni	Lee et al. (2011)	sjA/smA sjB /smB sjBcl2/2 smBcl2/2 sjBcl2/1 smBcl2/1 sjC /smC sjD	BH1-4, TM BH1-4, TM BH1-4, TM BH1-4, no TM BH1 BH1 BH3 BH3	Anti Pro Pro
Cnidaria				
Aiptasia pallida	Dunn et al. (2006)	ABHP (DQ211980)	BH1, BH2, no TM	
Stylophora pistillata	Kvitt et al. (2011)	StyBcl-2-like (EU715319)	BH1-4, TM	
Hydra magnipapillata	Lasi et al. (2010)	HyBak-like 1 (EF104645) HyBak-like 2 (EU035760) HyBcl-2-like 1 (EF104646) HyBcl-2-like 2 (EF104647) HyBcl-2-like 3 (EU035765) HyBcl-2-like 4 (EU035764) HyBcl-2-like 5 (EU035763) HyBcl-2-like -6 (EU035762) HyBcl-2-like 7 (EU035761) HyBH3-only 1 (hma2.230679)	BH1-3, TM BH1-3, TM BH1-4, TM BH1-4, TM BH1-4, TM BH1-4, TM BH1-4, TM BH1-4, TM BH1-4, TM BH3 BH3 BH3 BH3	Pro Pro Anti Anti Anti Anti Slightly pro Anti Anti ND Pro Neutral Neutral

(continued)

Table 9.1 (continued)

Species	Reference	Gene/protein (Acc. °)	Motif composition (as published)	Function
		HyBH3-only 2 (hma2.221399) HyBH3-only 3 (hma2.218794) HyBH3-only 4 (hma2.224514)		
Porifera				
Geodia cydonium	Wiens et al. (2001)	BHP2-GC (AJ293508)	BH1, BH2, TM	Anti
Geodia cydonium *Suberites domuncula*	Wiens et al. (2000)	BHP1_GC (CAB97129) BHP1_SD (CAB97205)	BH1, BH2, TM	
Lubomirskia baicalensis	Wiens et al. (2006)	BAK-2_LUBAI (CAJ12144) BCL-2a_LUBAI (CAJ12145)	BH3, BH2, TM BH1-4, TM	

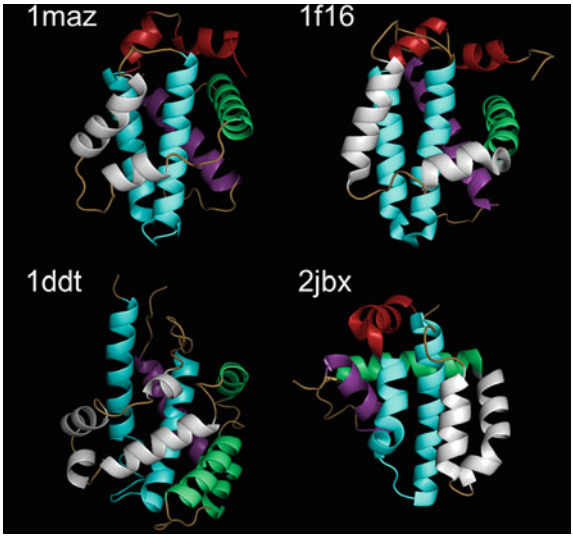


Fig. 9.3 Structural similarity between BCL-2 homologs and microbial proteins. Ribbon diagrams of anti-apoptotic protein BCL-xL (PDB code: 1maz), proapoptotic protein BAX (1f16), diphtheria toxin translocation domain (1ddt), and myxoma virus antiapoptotic protein M11L (2jbx). These proteins form a compact α-helical bundle with a pair of central helices (*in cyan*) surrounded by other (mainly amphipathic) helices. The figure was made with PyMol

divergent homologs, respectively, can be found in early-branching metazoans (Srivastava et al. 2010; Wiens et al. 2001; Wiens et al. 2000).

Particularly, intriguing is the issue of how opposite activities evolved in proteins that share a similar 3D structure, as do BCL-2-type and BAX-type proteins. The precise molecular determinants that underpin the extreme functional divergence

between structural homologs of the BCL-2 family are not completely understood. Distinct regions of the BCL-2 domain were shown to be involved in the functional dichotomy between pro- and anti-apoptotic members: the BH4 region (which is often located in the first α-helix) (Borner et al. 1994; Lee et al. 1996), the BH3 motif (Lee et al. 2014), and the α5-α6 helical hairpin motif (often referred to as a "pore-forming" domain) (Bleicken et al. 2013; Guillemin et al. 2010). However, subtle differences are scattered along the entire protein domain of pro- and anti-apoptotic BCL-2 proteins and it is expected that various sites may contribute to their antagonist actions on cell survival. A prime difference between BAX-type and BCL-2-type proteins might be related to the ability of proapoptotic homologs to self-assemble by forming dimers and higher order oligomers (Subburaj et al. 2015; Westphal et al. 2014), and of the prosurvival ones to inhibit these association processes in the context of the mitochondrial membrane by behaving like chain terminators, i.e., dominant negative forms (Reed 2006; Westphal et al. 2014). If this scenario is correct, then what distinguishes both types of proteins should be looked for in contact interfaces (with partners and/or lipids) in addition to isolated regions, bearing in mind that those interactions can be "cloudy" and involve distant residues. An alternative view may be that the separation between BCL-2-like and BAX-like family members has been overstated and that some kind of dualistic thinking is at work that somehow hides the partly artificial nature of the pro- versus anti-apoptotic dichotomy. This alternative scenario is not without support from a variety of experimental data, including the demonstration that (i) most prosurvival BCL-2 family proteins can be converted into death factors following proteolytic cleavage (Cheng et al. 1997; Clem et al. 1998; Kucharczak et al. 2005; Michels et al. 2004; Xue and Horvitz 1997); (ii) proapoptotic isoforms can be produced by alternative splicing of prosurvival bcl-2-like genes (Bae et al. 2000; Boise et al. 1993); (iii) pro-apoptotic BAK or BAK proteins can behave as prosurvival factors in specific cellular contexts or cell types (Kiefer et al. 1995; Lewis et al. 1999); and (iv) a number of BCL-2 family members were characterized both as pro- and anti-apoptotic factors (e.g., BCL2L10, BOK, and Bcl-rambo/BCL2L13) (Lee et al. 2001; Song et al. 1999; Aouacheria et al. 2001; Inohara et al. 1998; Ke et al. 2001; Zhang et al. 2001; Jensen et al. 2014). Hence, the scission between prosurvival and proapoptotic BCL-2 family members might be less definitive and clear than currently assumed.

9.4 BH3-Only Proteins

BCL2 homologous proteins act as receptors for BH3-only proteins, which are structurally unrelated proapoptotic molecules. In response to death signals, BH3-only proteins either inhibit the BCL-2-like apoptosis inhibitors or activate the BAX-like death activators. These proteins therefore form a signal-processing layer that connects onto the BCL-2/BAX core machinery the various inputs telling the cell either to survive or to commit suicide. Synthetic peptides encompassing the

BH3 motif of various BH3-only proteins were shown to bind with high affinities to a hydrophobic groove at the surface of prosurvival BCL-2 homologous proteins (Petros et al. 2000). Following this finding, BH3-mimetic drugs were developed that rapidly entered clinical trials as anticancer agents (Davids and Letai 2012). Given their key roles, the discovery of novel BH3-only proteins has represented and continues to represent a critical endeavor in the cell death field. Historically, this protein group contained nine non-homologous proteins discovered in the "1990s and early 2000s" (BIM, BMF, PUMA, NOXA, BAD, HRK, BIK, EGL1, and BID, herein termed "canonical" BH3-only proteins), which were sometimes erroneously appended to the protein family of BCL-2 homologs. In fact, only BID qualifies both as a BH3-only protein, as it contains a single BH3 motif, and as a BCL-2 homologous protein, because it shares a similar 3D structure with BCL-2 and BAX (Billen et al. 2008b; Chou et al. 1999; McDonnell et al. 1999). Current models of apoptosis regulation and a majority of review articles exclusively focus on the proapoptotic activity of these nine BH3-only proteins, ignoring the fact that the number of claimed BH3-only proteins has dramatically increased to reach a total of $\sim$40 (Aouacheria et al. 2015; Aouacheria et al. 2013) (Fig. 9.2). Contrary to the other BH motifs that were only detected in BCL-2 homologous proteins, BH3 motifs are now found in a gamut of folded (e.g., BCL-2) and unstructured protein domains [note that, except BID, all BH3-only proteins are intrinsically disordered proteins (Barrera-Vilarmau et al. 2011; Craxton et al. 2012; Hinds et al. 2007; Rogers et al. 2013; Yan et al. 2004)], bringing the grand total number of reported BH3 sequences to more than 60 unique instances. As a result, the evolutionary histories of BH3 motifs are singular, inherently coupled to the evolution of the proteins that harbor them, and therefore difficult to disentangle collectively. Depending on the case, evolution of BH3 motifs can be attributed to homologous processes (e.g., duplication divergence of BCL-2 family genes) or homoplastic mechanisms (e.g., random coincidence or convergence, as in the case of the E3 ubiquitin ligase MULE and the insecticidal toxin Mcf1, among many other putative instances). Interestingly, inspection of gene structures suggests that transfer events (e.g., exon shuffling) could also be involved, as illustrated by the relatively high similarity of the BCL-2 homolog BAK and the BH3-only gene BIK in their BH3 regions (Aouacheria et al. 2015).

The reason that explains this complicated situation has its root in the very nature of the BH3 motif, whose sequence signatures are diverse and of low complexity (i.e., very predictable) (Aouacheria et al. 2013). Following on this observation, we recently advanced the argument that the BH3 motif meets the criteria for classification as a short linear motif (SLiM) or a molecular recognition element/feature (MoRE/MoRF) involved in protein–protein interactions between structured domains (e.g., globular domains of the BCL-2 type) and between structured domains and intrinsically disordered proteins (as exemplified by the interaction between canonical BH3-only proteins and BCL-2-like or BAX-like proteins). Rather than considering the BH3 as an apoptotic motif per se, this novel conceptual framework poses this motif as a versatile and evolutionary plastic module associated with binding events in various branches of the tree of life, within metazoans

but also probably outside the animal kingdom as well. Future experiments will have to (i) assess the prevalence of BH3 motifs in proteins from non-metazoan species, (ii) unravel the identity of their putative receptors, and (iii) determine their possible roles in the biology of the cognate organisms.

9.5 Conclusion

To sum up, the BCL-2-regulated apoptotic pathway (a metazoan synapomorphy) emerged as the result of the interplay between an eukaryotic organelle (the mitochondrion) sequestrating proteins which have both vital and proapoptotic roles, a membranotropic structural domain (the BCL-2 globular fold) able to convey opposite activities toward cell survival and cell death, and a short and evolutionary plastic module (BH3) mediating protein–protein interactions. It is likely that acquisition of a proto-bcl-2 gene occurred only once during the evolution of the first multi-celled animals, followed by vertical evolutionary descent, lineage-specific diversification, and gene losses, contributing to the numerous morphological and lifestyle features of animals. Although sequences are "documents of evolutionary history" [in reference to Zuckerkandl and Pauling (1965)], it is hard to figure out in any real way whether the repertoire of molecules involved in the control of active cell death was "simple" or "complex" in the last common ancestor of modern-day animal species. Yet, as they are descendants of lineages that diverged early in the history of multicellular animals, the study of basal metazoan species can offer useful clues, e.g., about the presence of a BH3-dependent mitochondrial apoptotic pathway in their ancestors, or about the possible non-apoptotic function(s) of the BCL-2 ancestral protein. Whether metazoan BCL-2 homologous proteins emerged as stress-signaling molecules, or as switches connecting and controlling the execution of the various pathways involved in cell survival and death (including apoptosis, autophagy and programmed necrosis), or as key players serving biochemical functions distinct from cell death regulation remains an open question.

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