The Role of the Bcl-2 Family in Proliferation and Apoptosis and in Mediating the Development of Lymphatic Diseases

by

Marcie A. Eipper-Mains

B.A. Biology
Cornell University, 2001

SUBMITTED TO THE DEPARTMENT OF BIOLOGY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE IN BIOLOGY
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

SEPTEMBER 2004

Signature of Author: _____
Department of Biology
September 14, 2004

Certified by: _____
Jaqueline A. Lees
Professor of Biology
for Luk Van Parijs
Associate Professor in Immunology
Thesis Supervisor

Accepted by: _____
Stephen P. Bell
Professor of Biology
Chairman, Committee for Graduate Students

The Role of the Bcl-2 Family in Proliferation and Apoptosis and in Mediating the Development of Lymphatic Diseases

Introduction

The development of the immune system is a highly dynamic process, characterized by quickly and frequently changing cell types and numbers. The orchestration of cell growth and proliferation and also of cell death is a necessarily complex process, taking cues from a wide variety of sources. The nematode *Caenorhabditus elegans* has provided an elegant and simple model of the control of programmed cell death, or apoptosis, in metazoans. Apoptosis in mammals is regulated by pathways related to but more intricate than metazoans. Several key features define the onset of apoptosis in any given cell; these include DNA fragmentation and chromatin condensation, "blebbing" of the plasma membrane, and subsequent phagocytosis of the resulting cell fragments by adjacent cells (Kerr *et al.* 1972 and Wyllie *et al.* 1980).

In terms of the immune system, apoptosis can serve a spectrum of roles. A developing immune cell is subjected to processes of both positive and negative selection; a cell that fails either of these tests will be eliminated. Negative selection eliminates cells that recognize antigens native to the organism, to prevent the potential development of an autoimmune response. Positive selection ensures that an immune cell indeed serves a function in the organism, and eliminates unnecessary cells. In the absence of the appropriate nutrients and growth factors, immune cells are also killed (Vaux and Strasser 1996 and Hengartner 2000).

Programmed cell death in mammals is coordinately controlled by two pathways; these are the activation of the death receptor and the initiation of the mitochondrial

damage pathway. Ligation of the death receptor *Fas* by *Fas*-ligand (*FasL*) causes the formation of *DISC*, the death-inducing signaling complex. *DISC* instructs several adaptor proteins to bind to the death receptor proteins through homotypic interaction between their respective death domains, in turn signaling the activation of caspases, which are aspartate-specific cysteine proteases (Chinnaiyan *et al.* 1995 and Kischkel *et al.* 1995). Caspases are activated in a cascade from initiator caspases through effector caspases, ultimately resulting in proteolysis of essential cellular proteins and apoptosis (Thornberry and Lazebnik 1998). The other main means of controlling apoptosis is through the *Bcl-2* protein family, which consists of both pro- and anti-apoptotic members sharing a variety of structural features (Adams and Cory 1998; Figure 1). The primary anti-apoptotic proteins include *bcl-2, A1/Bfl-1, bcl-w, bcl-xL, bcl-b,* and *mcl-1*. These anti-apoptotic proteins share three or four *BH*-domains, or *bcl-2-homology* domains. From the amino to carboxy terminus of *bcl-2*, there are four such domains, named in order *BH4, BH3, BH1,* and *BH2* (Figure 2). The pro-apoptotic proteins can be sub-divided into two categories, those with two to three *BH* domains and those with only one, the *BH3* domain. The former category includes *bax, bak,* and *bcl-xS*, while the latter category includes *bad, bid, bik, bim, Noxa,* and *PUMA*.

The *Bcl-2*-regulated cell death pathway is remarkably similar to the pathway controlling programmed cell death in *C. elegans*. Apoptosis in *C. elegans* is initiated by induction of transcription of pro-apoptotic *EGL-1*; *EGL-1* binds to and inhibits the anti-apoptotic protein *CED-9*. Inhibition of *CED-9* permits *CED-4* to activate *CED-3*, the effector protein that commences apoptosis (Ellis *et al.* 1991 and Bouillet *et al.* 2002). The mammalian *bcl-2*-regulated apoptosis pathway is homologous to most steps of the *C.*

elegans pathway (Figure 3). In mammals, *BH3*-only proteins such as *Bim* are required to initiate apoptosis and perform a function analogous to *EGL-1*. Mammalian *Apaf-1* performs a function markedly similar to *C. elegans CED-4*, effecting the activation of downstream caspases (Hausmann *et al.* 2000).

At the structural level, there is a clear relationship between the *C. elegans* and mammalian apoptosis pathways, as well as instances of divergence between the two. *Bcl-2* shows homology to *CED-9*, and can be substituted to partially perform the same function *in vivo*. Intriguingly, *CED-9* has homology both to several anti-apoptotic proteins, such as *bcl-2* and *bcl-xL* but also to pro-apoptotic *bax*, suggesting that *CED-9* may be able to change conformation and switch between pro- and anti-apoptotic states (Hengartner and Horvitz 1994). Despite their similar structure and function, *Apaf-1* differs from *CED-4* in that it does not bind to anti-apoptotic *bcl-2* while *CED-4* does bind to anti-apoptotic *CED-9*. *Apaf-1* contains several *WD40* repeating sequences, and it must be bound to *cytochrome c* to activate the effector protein *caspase 9* (Hausmann *et al.* 2000).

Anti-apoptotic members of the *bcl-2* protein family share both structural and functional properties. In addition to each containing three or four *BH* domains (Figure 2), all of these proteins are localized primarily to the mitochondrial membrane. The function of these proteins is likely to preserve the integrity of the membane against assault from pro-apoptotic proteins (Sorenson 2004). *Bax* and *bak* are pro-apoptotic proteins containing several *BH* domains each; these proteins form a heterodimeric cluster that has been suggested to act as a pore in the mitochondrial membrane. This pore is hypothesized to allow *cytochrome c* release from the mitochondrion. *Apaf-1* can bind to

the released *cytochrome c* and subsequently activate *caspase 9*, beginning the proteolytic cascade culminating in phagocytosis of the cell (Marsden and Strasser 2003, Green 2003, and Craig 1995).

In this review, I will discuss the different properties and features of six anti-apoptotic members of the *bcl-2* protein family: *bcl-2, bcl-xL, bcl-b, bcl-w, A1/Bfl-1,* and *mcl-1*. These proteins are markedly similar in structure and function; the differences between them lie primarily in their different expression patterns, from the timing of expression to the specific tissues in which they act. I will not discuss the pro-apoptotic members of the *bcl-2* family beyond their relevance to my discussion to the anti-apoptotic family members. For each protein, I will discuss research pertaining to cellular and molecular biology as well as our understanding of the contribution of each protein to the pathology of disease and the relevant murine and human genetic models.

Bcl-2

Cellular and Molecular Biology

Translocation of *bcl-2* and its subsequent mis-regulation is the basis for the development of more than half of all cases of human follicular lymphomas. B-cell lymphoma arises from the t(14;18) translocation that places a segment of chromosome 14 adjacent to a segment of chromosome 18. The region of chromosome 14 begins at the 5' end of an immunoglobulin heavy-chain joining segment, and the region of chromosome 18 includes the *Bcl-2* genetic locus (Tsujimoto *et al.* 1985b). In one experiment, researchers made DNA probes for the breakpoint flanking regions; these probes were able to detect approximately 60% of the translocation incidences. Band q21 of

chromosome 18 was identified within a region roughly 2.1 kb in length; this region produced a roughly 6 kb RNA transcript (Tsujimoto *et al.* 1985a). The site of recombination in chromosome 14 at the Ig heavy-chain joining segment is where diversity (D) regions are found, indicating that t(14;18) is due to an error in VDJ joining. This likely occurs at the pre-B cell step of differentiation, where the recombinase incorrectly joins a section of chromosome 18 to chromosome 14 instead of rejoining two sections of chromosome 14 (Tsujimoto *et al.* 1985b). To compare the expression pattern of *Bcl-2* in normal cell lines and cell lines carrying the t(14;18) translocation, researchers compared the transcripts generated. Three overlapping mRNAs are produced, and the shorter two transcripts encode *bcl-2α* and *bcl-2β*. Follicular lymphomas with or without a *bcl-2* rearrangement show the same *bcl-2* protein products as do normal cell lines, indicating that the expression pattern of *bcl-2* must be mis-regulated in B-cell lymphomas (Tsujimoto and Croce 1986).

Greater than 60% of all human follicular lymphomas contain the t(14;18) (q32;q21) translocation. The breakpoints on chromosome 18 are clustered within a small 4.3 kb region, and on chromosome 14 the breakpoint is near an immune regulatory element. The result of this combination is that the chromosome 14 Immunoglobin enhancer region is placed in close proximity to 18q21, which contains the *bcl-2* locus (Bakhshi *et al.* 1985). The breakpoint in chromosome 14 occurs in joining region 4 (J4) of the nonfunctional immunoglobin heavy chain allele. It is thus likely that D-J recombination enzymes are involved in the mechanism of translocation (Cleary and Sklar 1985). The break in the Ig heavy chain locus on chromosome 14 is an interruption of the normal V(D)J recombination process. By contrast, the break on chromosome 18 does not

occur within a region that typically experiences breakage. The region is confined to approximately 150 base pairs, a region known as the Major breakpoint region (Mbr). Researchers reproduced characteristic features of the Mbr on an episome that propagated in human cells. This region took on an atypical non-B-DNA structure; the result is a fragile region that is easily cleaved by the RAG complex. The RAG complex is the enzyme complex that normally cleaves DNA at V, D, or J segments (Raghavan *et al.* 2004).

Bcl-2 overexpression studies have yielded valuable insights into its function. When a human lymphoblastoid B cell line was infected with Epstein-Barr virus (EBV) containing the SV40 promoter and enhancer region driving a *Bcl-2* construct, the resulting overproduction of *Bcl-2* gave the infected cells a growth advantage. These cells did not, however, become tumorigenic in athymic nude mice, suggesting that additional tumor-promoting conditions are required for tumor development. These researchers also used a *Bcl-2* construct with its own promoter plus the Ig heavy chain enhancer, to mimic the t(14;18) configuration seen in B-cell lymphomas. This construct conferred the same growth advantage as the SV40 enhancer and promoter. This indicates that *Bcl-2* overproduction plays a direct role in the pathogenesis of follicular lymphoma (Tsujimoto 1986). *Bcl-2* is usually only expressed in proliferating B cells; it is quiescent in resting B cells and down-regulated in differentiated B cells. B-cell lymphoma results from abnormal expression of *bcl-2* due to the t(14;18) translocation. When *bcl-2* was introduced by retroviral gene transfer into human B-lymphoblastoid cell lines (LCLs), the overexpression of *bcl-2* alone was not sufficient to initiate tumorigenicity. The expression of *bcl-2* did, however, confer a 3-4-fold growth advantage to single cells

growing on soft agar. In immunodeficient mice with exogenous expression of the *MYC* oncogene, *bcl-2* overexpression complemented the transforming effects of *MYC*, resulting in increased frequency and shortened latency of tumor induction (Nunez *et al.* 1989). Some gene transfer experiments looking at both *bcl-2*α and *bcl-2*β have indicated that *bcl-2* alone has oncogenic potential, but the majority of experiments indicate that cooperation with another oncogene such as *MYC* is necessary (Reed *et al.* 1988).

The abnormal expression pattern of *Bcl-2* in cases of B-cell lymphoma is due solely to the translocated *bcl-2* allele and not to the normal allele. Normal *bcl-2* is expressed in a wide variety of haematopoietic lineages, including both B and T cells. mRNA levels are high during pre-B cell development and then are usually down regulated during cell maturation. Resting B cells show down-regulation of *bcl-2* while activated B cells show up-regulation. In cases of t(14;18) translocation, which occurs during the pre-B cell developmental stage, *bcl-2* mRNA levels are log-folds higher than normal cells. S1 protection assays showed that these transcripts are all *Bcl-2-Ig* fusions and thus originate from the translocated and not the normal allele (Graninger *et al.* 1987).

To elucidate the role played by this elevated *bcl-2* protein level, researchers performed immunolocalization studies. *Bcl-2* appears to be an inner mitochondrial protein with a relative molecular mass of 25K. When a variety of haematopoietic cell types were subjected to growth factor withdrawal, transfection with *bcl-2* enhanced survival with respect to controls. The elevated protein levels resulting from t(14;18) increase cell survival rates but do not increase the rate of cell cycling (Hockenbery *et al.* 1990). In another series of growth factor withdrawal studies, researchers studied a cytotoxic T cell line (CTLL2). These cells require the growth factor interleukin 2 (IL-2);

in its absence, the cells undergo apoptosis, indicating that IL-2 must mediate the transcription of genes necessary for survival. Researchers transfected CTLL2 cells with *bcl-2* under the metallothionein promoter and then subjected the cells to IL-2 withdrawal. The cells did survive but did not cycle; rather, the cells arrested either at G0/G1 or G2/M. This result indicates that apoptosis upon IL-2 withdrawal results from suppression of IL-2 mediated expression of *bcl-2* (Deng and Podack 1993).

Although the protein products from the translocated *bcl-2* allele in t(14;18) are identical to those from the endogenous allele, the expression patterns of the two loci differ dramatically. The basis for this difference may lie in the upstream CRE sites of the two loci. The CRE site is a cAMP responsive element, and increased levels of cAMP typically enhance CRE-binding (CREB) protein binding at the site. The CRE sites upstream of endogenous *bcl-2* are silent due to positional effects, such that CREB proteins cannot bind to the site. In the translocated allele, CREB proteins can bind and act as positive regulatory agents. Treatment with phorbol 12-myristate 13-acetate (PMA) leads to increased levels of phosphorylated CREB proteins and consequently to greatly increased levels of *bcl-2* expression. Moreover, mutation of the CRE site in the translocated allele abolishes the induction of *bcl-2* expression by PMA (Ji *et al.* 1996). In the thymus, *bcl-2* is found primarily in mature T cells of the medulla. When researchers experimentally redirected *bcl-2* expression to cortical thymocytes, immature CD4+8+ thymocytes were protected from glucocorticoid-induced, anti-CD3-induced, and radiation-induced apoptosis. In addition, there were increased levels of CD3hi and CD4-8+ thymocytes. Negative selection by clonal deletion of T cells recognizing endogenous superantigens occurred normally (Sentman *et al.* 1991). Developing T lymphocytes, by

in large, die at an early age. To investigate whether *bcl-2* expression could moderate this process, researchers used an Eμ-*bcl-2* transgene within the T lymphoid compartment. These T cells were resistant to lymphotoxic agents and showed prolonged viability as well as spontaneous differentiation *in vitro*. Total T cell numbers were unaffected, but fewer activated T cells were killed, such that autoreactive T cells appeared in the thymus (Strasser *et al.* 1991).

At a molecular level, the function of *bcl-2* remains a subject of speculation. One study using GFP to localize the protein kinase *Raf-1* suggested that *bcl-2* targets *Raf-1* to the mitochondria. Here, *Raf-1* phosphorylates the pro-apoptotic protein *Bad* and other protein substrates to prevent apoptosis. If *Raf-1* is erroneously targeted to the plasma membrane, it phosphorylates *ERK-1* and *ERK-2* instead of *Bad*, and cannot protect the cell from apoptosis (Wang *et al.* 1996). An interesting question is raised by an experiment performed on cells lacking mitochondrial DNA. In these cells, apoptosis can still be induced, and overexpression of *bcl-2* can protect these cells, indicating that the function of *bcl-2* does not involve the expression of mitochondrial genes. *Bcl-2* presumably still functions within the mitochondrial membrane in these cells but it cannot play a role in the respiratory chain response (Jacobson *et al.* 1993). Another line of experiments indicates that *bcl-2* may regulate a caspase activation program independent of the cytochrome *c/Apaf-1/casp-9* apoptosome. *Apaf-1* requires cytochrome *c* to activate *casp-9*; because *bcl-2* prevents mitochondrial cytochrome *c* release, it may thus prevent the apoptotic program initiated by *casp-9*. In cells without *Apaf-1* or *casp-9*, however, *bcl-2* overexpression continues to result in caspase activity, suggesting that *bcl-2*

amplifies a caspase cascade independent of the cytochrome *c/Apaf-1/casp-9* apoptosome (Marsden *et al.* 2002).

Disease and Models: Murine and Human

Researchers looking at a variety of lymphoid cancers used Southern blotting to examine the genetic arrangement of *bcl-2*. In 30% of follicular lymphomas and in 19% of diffuse lymphomas of follicle center cell lineage, *bcl-2* rearrangements were found; nearly all of these rearrangements also contained the Ig heavy chain from chromosome 14, indicating the t(14;18) translocation. In lymphomas not derived from follicle center cells, *bcl-2* was always found in the germline configuration (Aisenberg *et al.* 1988). In a survey of peripheral blood lymphocytes (PBLs) and autopsied spleens, researchers determined the background frequency of t(14;18)(q32;q21) using a nested PCR assay. At a frequency of between 1 and 853 translocations per million cells, the rearrangement occurred in 55% of PBLs and 35% of autopsied spleens. The frequency of translocation rose dramatically with age in both groups, as did the lymphoma risk. The risk of translocation in the oldest spleens was 40 times greater than the youngest spleens; PBLs from the oldest patients had a 13-fold greater frequency of translocation than the youngest patients. Clones containing t(14;18) are present quite commonly; these clones rise in frequency with age, increasing the risk of developing lymphoma (Liu *et al.* 1994). Rearrangement of *bcl-2* has been linked to a poor prognosis in response to chemotherapy as compared to patients without *bcl-2* rearrangements (Yunis *et al.* 1989).

Bcl-2 may play a role in the etiology of neurodegenerative diseases. When researchers used immunohistochemistry to study *bcl-2* localization in aged brains, it was

found primarily in lipofuscin and autophagic vacuoles of neurons, glial, and vascular cells. This localization pattern indicates that *bcl-2* expression may be a general cellular response to increased levels of lipofuscin; oxidative stress leads to ROS-mediated damage resulting in lipofuscin expression (Migheli *et al.* 1994). Although the endogenous role of *bcl-2* in neurons remains unresolved, overexpression and gain-of-function studies provide insight into its potential mechanisms of activity. Support for a role for *bcl-2* in neurons comes from a study with transgenic mice expressing *bcl-2* under the neuron-specific enolase promoter. Researchers looked at sensory neurons isolated from dorsal root ganglia in these newborns; in wild-type mice, these cells should require nerve growth factor to survive in culture. Sensory neurons from *bcl-2* transgenic mice show greater survival; following sciatic nerve axotomy, motor neurons in these mice undergo a lower degree of apoptosis than wild-type mice. The number of neurons in both the central and peripheral nervous systems in the transgenic mice was 30% greater than wild-type mice (Farlie *et al.* 1995). In a related experiment, researchers used a transgene to drive overexpression of *bcl-2* and studied the induction of apoptosis in photoreceptors triggered by environmental or inherited factors. Overexpression of *bcl-2* increased photoreceptor survival in mice with defective opsin or cGMP phosphodiesterase that would otherwise lead to retinal degeneration. *Bcl-2* overexpression also muted the effects of radiation damage. Interestingly, the researchers also found that very high levels of *bcl-2* could also induce apoptosis in normal photoreceptors (Chen *et al.* 1996).

Naturally occurring cell death (NOCD) is a process that pares down the number of neurons in the developing nervous system. Researchers used either the enolase promoter or the phosphoglycerate kinase (PGK) promoter to drive neuron-specific *bcl-2*

expression in transgenic mice. As a result of *bcl-2* overexpression, there was a reduced rate of neuronal loss during NOCD; this resulted in hypertrophy of the nervous system. When the researchers performed occlusion of the middle cerebral artery and studied the subsequent permanent ischemia, the size of the resultant brain infarction was reduced by 50% in transgenic mice compared to wild-type mice (Martinou *et al.* 1994). In an experiment studying progressive motor neuropathy (PMN), researchers employed homozygous mutant *pmn/pmn* mice. When these mice were crossed with transgenic mice overexpressing *bcl-2*, the result was rescue of facial motoneurons, restored cell body size, and restored choline acetylcholinesterase expression patterns. *Bcl-2* overexpression did not, however, prevent the loss of myelinated axons in phrenic or facial motor nerves, and it did not increase the lifespan of the animals (Sagot *et al.* 1995). These experiments employing transgenes to express *bcl-2* must be interpreted carefully, as the results imply functions of *bcl-2* as a result of manipulated expression patterns.

Bcl-2 plays a role in the maintenance of B-cell memory. Long after the end of obvious cell division, antigen-binding B-cells persist; *bcl-2* blocks programmed cell death in these B-cells, thus maintaining immune responsiveness. Transgenic *bcl-2* mice show long-term persistence of immunoglobin-secreting cells and extended lifetime of memory B-cells (Nunez *et al.* 1991). The survival of mature B-cells was enhanced by transgenic overexpression of *bcl-2* in another study. These researchers used minigene constructs with a *bcl-2-Ig* fusion gene to mimic the t(14;18) translocation rearrangement. This minigene was placed into the germline of mice to assess the developmental effects. The resulting lymphoid pattern of expression led to an expanded follicular center cell population and the accumulation of white splenic hyperplastic follicles. After 15 weeks

of age, the researchers saw lymphadenopathy characterized by cellular infiltrates of polyclonal B220-positive, IgM/IgD-positive B-cells. *In vitro* survival assays showed an advantage for mature transgenic B-cells as compared to mature wild-type B-cells (McDonnell *et al.* 1989).

To study the potential role of *bcl-2* in heart tissue, researchers used the alpha-myosin heavy chain promoter to drive *bcl-2* expression in transgenic mice. Overexpression of *bcl-2* led to an increased percentage of cycling myocytes and a greater mitotic index at 24 days, 2 months, and 4 months of age. In the transgenic mice, the researchers found lower levels of the cell cycle inhibitors p21 (*WAF1*) and p16 (*INK4a*) as well as increased levels of the anti-apoptotic Mdm2-p53 complex. Levels of apoptosis were not affected by *bcl-2* expression, but the transgenic mice had more myocytes than wild-type in the left ventricle at all ages studied. The size of binucleated myocytes was lower in transgenic than wild-type mice, consistent with a higher rate of myocyte cycling (Limana *et al.* 2002).

Bcl-2 may cooperate with oncogenes to promote the development of tumorigenic clones. In one experiment, researchers used a retroviral vector containing the human *bcl-2* cDNA to infect bone marrow in either normal mice or mice that constitutively express *myc* in B-lineage cells through the Eμ-*myc* transgene. With respect to infected normal cells, *bcl-2* and *myc* cooperate in infected transgenic bone marrow cells to promote proliferation of B-cell precursors, some of which are tumorigenic. When the researchers looked at the effect of *bcl-2* overexpression in IL-3-dependent lymphoid and myeloid cell lines, they found that *bcl-2* promotes survival upon IL-3 withdrawal but that these cells subsequently arrest at G0 (Vaux *et al.* 1988).

Mice bearing knockouts in both *bcl-2α* and *bcl-2β* are small but viable; roughly half of these *bcl-2 -/-* mice are dead by six weeks of age. The number of CD8+ T lymphocytes is greatly reduced in these mice as compared to the other various haematopoietic lineages. When researchers studied the proliferative response of b*cl-2 -/-* lymphocytes to a variety of stimuli including anti-CD3, ConA, PMA and ionomycin, IL2, lipopolysaccharide, and anti-IgM antibodies, they found the identical response as in wild-type cells. The developmental effects seen in *bcl-2 -/-* mice occur primarily at sites of inductive interactions between epithelium and mesenchyme, resulting in small auricles, defects in renal tubules in the kidney, and the development of gray hair at 4 to 5 weeks of age. Despite the high endogenous level of *bcl-2* in the nervous system, intestines, and skin in normal mice, *bcl-2 -/-* mice do not show abnormalities in these tissues, suggesting a degree of functional redundancy between *bcl-2* and its homologs (Nakayama *et al.* 1994).

A1/Bfl-1

Cellular and Molecular Biology

A1/Bfl-1 is a member of the *bcl-2* family, functioning primarily as a mediator of the inflammatory response. *A1* is the murine *bcl-2* family member, and *bfl-1* is its human homologue. *A1* was first identified through a screen examining the genes expressed in response to granulocyte-macrophage colony-stimulating factor (GM-CSF); GM-CSF was previously shown to drive differentiation, activation, and proliferation in haematopoietic cell lineages. Expression of *A1* is found in T-helper lymphocytes, neutrophils, and macrophages; the expression is quite rapid but fleeting upon treatment with GM-CSF

(Lin *et al.* 1993). *Bfl-1* was identified as the human homologue of *A1* through a screen of cDNA clones from human fetal liver cells; there was a 72% amino acid identity between the two sequences. The strongest expression of *bfl-1* was detected in bone marrow tissue, with some detectable levels in other tissues. This initial detection of *bfl-1*showed a small but significant link between the overexpression of *bfl-1* and the development of stomach cancer (Choi *et al.* 1995). Using a screen of cytotoxic T-lymphocyte clones specific for minor histocompatibility antigens (mHAgs), *A1* was recently re-identified; *A1* encodes two such mHAgs. Researchers performed a two-point linkage analysis on these two clones and identified a 3.6 centimorgan interval containing at least 46 genes. Of these 46 genes, only *A1* was expressed in haematopoietic cells (Akatsuka *et al.* 2003).

When a cell is challenged by growth factor withdrawal or other apoptotic factors, *A1/Bfl-1* acts to protect the cell and prevent death. When the interleukin-3 dependent cell line 32D c13 is stably transfected with *A1/Bfl-1*, apoptosis upon IL-3 withdrawal is decreased with respect to non-transfected cells. This result is the same as reported for *bcl-2*. Although the *bcl-2*-transfected cell line accumulated a greater number of cells upon IL-3 withdrawal than the *A1/Bfl-1*-transfected cell line, only the *A1/Bfl-1*-transfected cell line was able to accumulate differentiated myeloid cells (Lin *et al.* 1996). The ability of *A1/Bfl-1* to prevent apoptosis induced by the tumor-suppressor protein p53 is similar to that of *bcl-2* and its family member *bcl-xL*. When researchers studied the transforming capacity of *A1/Bfl-1*, they looked at rodent epithelial cells transformed with the *E1a* oncogene. These cells were transformed more readily when both *A1/Bfl-1* and *E1a* were present than *E1a* alone (D'Sa-Eipper *et al.* 1996). Expression of *A1/Bfl-1* via a transgene was able to block apoptosis in *c-rel* -/- mice. In these *Rel*-deficient mice,

antigen receptor ligation leads to apoptosis. Given that constitutive expression *A1Bfl-1* via a transgene was able to rescue B-cells from these mice, *Rel* may be a regulator of *A1/Bfl-1* activity (Grumont *et al.* 1999). Additional studies of *c-rel -/-* mice have gleaned insight into the regulation of *A1/bfl-1* through NF-κB. The *c-rel* subunit of NF-κB binds to the 5' regulatory region of *A1/Bfl-1* and nucleates the region, drawing in transcription factors *AP-1, C/EBPβ,* and others. *C-rel -/-* mice consequently cannot induce *A1/Bfl-1* transcription in response to NF-κB-activating stimuli. *In vivo* T-cell activation requires the recruitment of *c-rel, c-Jun, C/EBPβ, HMG-1C,* and *SWI/SNF* chromatin remodeling factor to the *A1/Bfl-1* 5' regulatory region as well as hypoacetylation of histones *H3/H4*. This pattern is consistent with a requirement for *A1/Bfl-1* induction in the course of T-cell activation (Edelstein *et al.* 2003).

Studies of knockout mice suggest that *A1/Bfl-1* has an important endogenous role in preventing apoptosis. Developing peripheral blood neutrophils undergo spontaneous apoptosis on a regular basis. In wild-type mice, treatment with lipopolysaccharide (LPS) can rescue these cells from death; *in vivo*, transendothelial migration also inhibits neutrophils apoptosis. Peripheral blood neutrophils from mice bearing a knockout of *A1* subtype *a* (*A1-a -/-*) are not rescued from apoptosis by LPS treatment or via transendothelial migration. Cells from heterozygous *A1-a +/-* mice behaved similarly to cells from wild-type mice. Apoptosis induced by TNFα affected all three genotypes equally, suggesting that TNFα-induced apoptosis is mediated by a pathway distinct from *A1/Bfl-1* signaling (Hamasaki *et al.* 1998). These data suggest that *A1/Bfl-1* is important in preventing neutrophil apoptosis induced by some, but not all, cytotoxic stimuli.

An important feature of the inflammatory response is the induction of *A1/Bfl-1* expression. *A1/Bfl-1* expression is triggered in endothelial cells in response to pro-inflammatory stimuli. This induction of *A1/Bfl-1* expression leads to the inhibition of endothelial cell activation, and it turns off the transcription factor nuclear-factor-κB (NF-κB). Turning off NF-κB inhibits the expression of pro-inflammatory proteins, thus squelching the inflammatory response. The expression of *A1/Bfl-1* in response to pro-inflammatory stimuli acts to restore a quiescent phenotype to endothelial cells by inhibiting its own further expression as well as expression of pro-apoptotic and pro-inflammatory proteins (Stroka *et al.* 1999).

The expression pattern of *A1Bfl-1* is developmentally regulated in T cells. Both *A1/Bfl-1* mRNA and protein are detected early in the thymus. Very low levels are found in CD4-8- double-negative cells, and *A1/Bfl-1* is then up-regulated to very high levels in double-positive thymocytes. Single-positive thymocytes show a reduced level of *A1/Bfl-1*, and this level is reduced 25-fold in mature single positive CD4+ and CD8+ lymph node T cells. *In vitro* ligation of the T-cell receptor leads to an increase in *A1/Bfl-1* expression in both single- and double-positive thymocytes, suggesting that *A1/Bfl-1* may mediate the viability of double-positive thymocytes (Tomayko *et al.* 1999).

Cells immortalized by infection with the Epstein-Barr Virus (EBV) express abundant levels of *A1/Bfl-1*, and the regulation of *A1/Bfl1* expression may be mediated by another protein, *Lmp1*. A Burkitt's lymphoma cell line expressing the latent EBV proteins also showed up-regulation of *A1/Bfl-1*; *Lmp1* is included in the array of latent EBV proteins. To confirm this relationship, the researchers studied an EBV-negative Burkitt's lymphoma cell line expressing *Lmp1* under an inducible tetracycline system.

Induction of *Lmp1* expression drove a spike in *A1/Bfl-1* mRNA as well as increased stability of *A1/Bfl-1* mRNA. This induction of *A1/Bfl-1* by *Lmp1* was seen neither in the human Jurkat T cell line nor in the epithelial cell line C33A, both of which are not normally infected by EBV. The role of *A1/Bfl-1* in response to *Lmp1* remains unclear. In EBV-positive cells with a latent type I infection, however, ectopic *bfl-1* overexpression showed a protective effect against growth-factor withdrawal-induced apoptosis, suggesting that *bfl-1* may serve a general anti-apoptotic function in these cells to allow survival of the host cell (D'Souza *et al.* 2000).

A1/Bfl-1 may play a variety of roles over the developmental course of immune cells, in particular in maturing B cells. In mature B cells, one group of researchers studied the contribution of *A1Bfl-1* as well as *bcl-2* in the transition of B cells from transitional type 2 (T2) to follicular (FO) cells. B cells with a deficiency of phospholipase-Cγ2 (PLCγ2) are unable to make the transition from T2 to FO. PLCγ2-deletion also leads to B-cell receptor-I (BCR-) induced apoptosis at much higher levels than those observed in cells without the deficiency. PLCγ2-deficient B-cells have very low levels of *bcl-2* protein and are unable to induce *A1/Bfl-1* expression; splenic B-cell subpopulations can be rescued from BCR-induced apoptosis through constitutive overexpression of *bcl-2*. *Bcl-2* overexpression partially rescues FO B-cells from apoptosis, while *A1/Bfl-1* overexpression completely rescues FO B-cells and partially restores the number of cells present. The differing activities of *bcl-2* and *A1/Bfl-1* indicate that *bcl-2* is active in all splenic B-cell subpopulations and that *A1/Bfl-1* is present in all mature FO B-cells (Wen *et al.* 2003). Another group of researchers recently looked at the role of *A1/Bfl-1* in activated naïve T-cells by examining the gene

expression pattern following activation of splenocytes from naïve mice. There was a strong peak in *A1/Bfl-1* expression at two to six hours on the first day of activation; by contrast, *bcl-2* mRNA levels were markedly down-regulated in the same time frame. Transgenic overexpression of *A1-a* via the T-cell-specific *lck* distal promoter resulted in lower levels of apoptosis following activation by either anti-CD3/anti-CD28 or Concanavalin A (ConA). A notable difference between the activities of *A1/Bfl-1* and *bcl-2* was that transgenic overexpression of either gene reduced levels of apoptosis in both resting and activated T-cells, but these T-cells were only able to cycle in response to transgenic overexpression of *A1/Bfl-1*. *Bcl-2* inhibits entry into S-phase of the cell cycle in activated T-cells while *A1/Bfl-1* does not. There were twice as many T-cells present five days post-activation in cells expressing transgenic *A1/Bfl-1* than in cells expressing transgenic *bcl-2* (Gonzalez *et al.* 2003).

A1/Bfl-1 may play a key role in determining whether a B-cell survives to become a long-lived peripheral B-cell; approximately 5% of the initial pool survives this transition. Both positive and negative selection processes in the bone marrow and periphery occur to weed out B-cells, primarily at late stages of differentiation. Newly formed B-cells express surface IgM as well as CD24, CD45, and surface IgD; by contrast, mature B-cells express only surface IgM. A group of researchers used semi-quantitative reverse-transcriptase PCR to study *bcl-2, Bax, and A1/Bfl-1* levels in both immature and mature peripheral B-cells. Although levels of *bcl-2* and *Bax* were constant between the two groups, there was a notable increase in *A1/Bfl-1* expression in mature B cells as compared to both pro- and pre-B-cell stages. Thus, expression of *A1* may

correlate with recruitment of B-cells to the long-lived peripheral B-cell pool (Tomayko and Cancro 1998).

Disease and Models: Murine and Human

A1/Bfl-1 has a clear and demonstrated role in the inflammatory response. At the site of inflammation, expression of *A1* is induced in myeloid leukocytes. To induce an inflammatory response in mice, researchers used an intraperitoneal infection with a virulent strain of *Toxoplasma gondii*. This infection provoked a peak in *A1* mRNA and protein levels in direct correlation with the infectious dose of *T. gondii*; notably, *bcl-2* levels did not change in response to this challenge. When researchers examined the inflammatory exudates, they detected *A1* in all neutrophils and roughly half of the macrophages. The pro-apoptotic *bcl-2* family member, *Bax*, was also induced concurrently with *A1* in macrophages, suggesting interplay between *Bax* and *A1* determining the apoptotic fate of a cell (Orlofsky *et al.* 1999). Expression of *A1* is 300-fold higher in inflammatory macrophages than in resident peritoneal macrophages. By contrast, the related isoforms *A1-b* is expressed at 3-fold higher levels and *A1-d* at 10-fold higher levels. Because of this pronounced expression pattern difference, researchers looked at mice with a deficiency of *A1-a*. Upon infection, there is a lower level of peritoneal leukocytosis and a small increase in the survival rate of these mice. This difference may be due to the increased levels of apoptosis the researchers observed in inflammatory neutrophils (Orlofsky *et al.* 2002).

A1/Bfl-1 expression plays a critical role in the response of mast cells to activation in response to an allergic reaction. Over the course of an allergic reaction, the activation

of the high-affinity IgE receptor (FcεRI) leads to activation and degranulation of mast cells, allowing these cells to mediate the reaction. Immediately following activation, mast cells begin to express both *A1/Bfl-1* mRNA and protein. Mice bearing *A1*-deficient mast cells are still able to release granule mediators but they possess fewer total mast cells, and these mast cells are not able to survive the allergic activation. Researchers studying the regulation of *A1/Bfl-1* found that its induction is mediated by calcium. Treatment of wild-type mast cells with the calcium chelator EDTA prevents *A1/Bfl-1* expression, whereas treatment with the calcium ionophore ionomycin induces *A1/Bfl-1* expression. The mast cell secretagogue compound 48/80 does not induce *A1/Bfl-1* expression, indicating that *A1/Bfl-1* induction is not a general mast cell response (Xiang *et al.* 2001).

When *A1* is overexpressed in both the B- and T-cell lineages via a transgene under the control of the Eμ enhancer element and the various cell numbers are assessed in adult mice, there are greater numbers of early B cells and thymocytes in the transgenic mice than in wild-type mice. Transgenic overexpression of *A1* hindered the pro- to pre-B-cell transition, resulting in the accumulation of more pro- than pre-B-cells. In mice with severe combined immune deficiency (SCID), an autoimmune disorder, transgenic overexpression of *bcl-2* restored pre-B-cell development; *A1* expression did not. While *A1* was able to protect lymphocytes *in vitro*, its protective effect was limited to specific stages and specific cell lineages *in vivo*, confirming the importance of the developmental regulation of *A1* expression (Chuang *et al.* 2002).

Given that *bcl-2* has a demonstrated protective effect in the mouse brain, researchers studied the potential role of *A1/Bfl-1* in the brain. The *db* gene provides a

mouse model for the study of ischemic damage; homozygous mutant *db/db* mice debelop diabetes at a very young age. Female diabetic (*db/db*) mice suffering ischemic brain damage due to hyperglycemia or hypoxia show lower levels of damage than their male littermates. Because of this difference, researchers hypothesized that estrogen might mediate the expression of anti-apoptotic genes. When female *db/db* and normoglycemic mice were subjected to ovariectomy and subsequently treated with either estrogen or vehicle, estrogen treatment reduced the level of damage in response to hypoxia and ischemia (H-I). In these normoglycemic mice, estrogen treatment induced *A1/Bfl-1* expression rapidly in microglia and macrophages, and this expression appeared to have a protective effect. In the *db/db* mice, expression of *A1/Bfl-1* was induced at 48 hours following H-I in microglia and macrophages, but there was no protective effect, suggesting that the timing of *A1/Bfl-1* expression was critical to the anti-apoptotic effects (Zhang *et al.* 2004).

Bcl-xL

Cellular and Molecular Biology

Another member of the *bcl-2* gene family, *bcl-x* was initially discovered through a screen of a variety of different human lymphoma tissues. *Bcl-x* exists primarily as two different species when expressed; these are *bcl-xL* and *bcl-xS*. These two isoforms are the product of alternative splicing of the *bcl-x* RNA transcript to produce a larger mRNA, the *bcl-xL* transcript, and a shorter mRNA, the *bcl-xS* transcript. *Bcl-xL* is similar in sequence and structure to *bcl-2*, and its shows similar anti-apoptotic activity when expressed in cells subjected to growth factor withdrawal. *In vivo*, *bcl-xL* is expressed

mostly in long-lived post-mitotic cells, such as those found in brain tissue. By contrast, *bcl-xS* acts to antagonize *bcl-2* in growth factor-starved cells and contributes to the cell death process. *In vivo*, it is found primarily in cells with a high rate of turnover (Boise *et al.* 1993). Because scientists knew that *bcl-2 -/-* mice were viable despite the development of lymphopenia and polycystic renal disease, they suspected some degree of functional redundancy with other members of the *bcl-2* gene family. The human and murine homologs of *bcl-xL* share a 97% amino acid identity and both share BH1, BH2, and BH3 domains with *bcl-2*. *Bcl-xL* prevents cell death upon growth factor withdrawal, and it localizes to the mitochondrial periphery. Researchers noted that *bcl-xL* levels are higher than *bcl-2* levels early in the course of development. In adult mice, levels of *bcl-xL* are highest in bone marrow, brain, thymus, and kidney tissues (Gonzalez-Garcia *et al.* 1994).

In addition to the *–xL* and *–xS* splice variants of *bcl-x*, several other species have been identified, although their physiological significance remains only poorly understood. One such variant is *bcl-xδ TM*; this species is missing the carboxyl terminal transmembrane domain found in *bcl-xL*, and consequently it is a soluble cytosolic protein. Researchers know that splenocytes experimentally stimulated with anti-*CD3* or lipopolysaccharide express *bcl-xL* both *in vivo* and *in vitro*. When *Hela* cells are transfected with immature *bcl-x* RNA, splicing directs *bcl-xS* and *bcl-xL* to the mitochondrial membrane and *bcl-xδ TM* to the cytosol. Overexpression of either *bcl-xL* or *bcl-xδ TM* can rescue *IL-3* dependent cells from apoptosis due to growth factor withdrawal (Fang *et al.* 1994). Two additional splice variants are *bcl-xα* and *bcl-xβ*, which have been identified in human, mouse, and rat cells. While *bcl-xα* is structurally

similar to *bcl-xL* and is spliced in a manner similar to *bcl-2*, *bcl-xβ* undergoes a different splicing process. *Bcl-xβ* loses the 5' untranslated region during the splicing process, such that the resulting open reading frame covers the splice sites for both *bcl-xS* and *bcl-xL*. This species has a wide expression pattern and a poorly defined function, but it is known to bind to the pro-apoptotic family member *bax*; *bcl-xL* also binds to *bax* (Ban *et al.* 1998). When *bcl-xβ* is strongly overexpressed in an *IL-3*-dependent promyeloid cell line, the cells undergo DNA fragmentation and begin the process of programmed cell death (Shiraiwa *et al.* 1996). A sixth species of *bcl-x* is *bcl-xγ*; this isoform has 47 C-terminal amino acids that are different from the other isoforms. *Bcl-xγ* is expressed primarily in thymocytes, where it interacts with the T-cell receptor (TCR) and major histocompatibility (MHC) products. In mature T-cells, the role of *bcl-xγ* may be ligation of the TCR. Overexpression of *bcl-xγ* in T-cells inhibits activation-induced cell death (AICD); by contrast, inhibition of *bcl-xγ* by transfection of antisense cDNA increases levels of AICD. T-cells that express *bcl-xγ* following *CD3* ligation survive, whereas cells that fail to express *bcl-xγ* undergo programmed cell death (Yang *et al.* 1997).

The cellular activity of *bcl-xL* appears to be similar to that of *bcl-2*; both proteins are primarily localized to the mitochondrial membrane, where they act to prevent the release of *cytochrome c*, prevent rupture of the outer mitochondrial membrane and prevent swelling of the inner membrane. When researchers overexpressed *bcl-xL*, they found a lower resting mitochondrial membrane potential, which prevented cellular damage induced by growth factor withdrawal or treatment with toxins such as staurosporine. *Bcl-xL* overexpression also prevented swelling of the mitochondria in response to drugs that inhibit oxidative phosphorylation. These results indicate that *bcl-*

xL helps to maintain both the osmotic and electrical homeostasis of a cell (Vander Heiden *et al.* 1997). To investigate how *bcl-xL* regulates the release of *cyt c* and mitochondrial membrane permeability, another group of researchers studied the membrane of liposomes. Using liposomes with a voltage-dependent anion channel (VDAC), a mitochondrial porin channel, these researchers studied the effect of expressing *bcl-xL, bax,* or *bak. Bax* and *bak* both enhanced VDAC opening, resulting in both enhanced VDAC opening, resulting in *cyt c* release, while *bcl-xL* inhibited VDAC opening and *cyt c* release even when *bax* or *bak* were also expressed (Shimizu *et al.*1999).

Because T-cells are the primary site of *bcl-xL* expression, researchers investigated its expression pattern using immunoblotting techniques in resting and activated T-cells and also in thymocytes. In addition, they tested the levels of *bcl-2* and *bax* in the same cells. Interestingly, they found that the patterns of *bcl-2* and *bax* were coordinated; levels of both were higher in splenic T-cells than in thymocytes, and these levels increased even more following T-cell activation. Expression of *bcl-xL*, however, was not detected in splenic T-cells but was found in very high levels in thymocytes and activated T-cells. When these researchers looked at the *IL-2*-dependent cell line *CTLL-2*, they noted that when these cells began to die upon *IL-2* withdrawal, *bcl-2* levels were unaffected while *bcl-xL* levels dropped significantly. Transfection of either *bcl-2* or *bcl-xL* could rescue these cells from apoptosis upon *IL-2* withdrawal; while all control non-transfected cells were dead after 48 hours, cells transfected with either *bcl-2* or *bcl-xL* were 70-90% viable. These results suggest that *bcl-xL* acts to prevent *IL-2* withdrawal-induced cell death and that *bcl-2* can perform the same function if necessary (Broome *et al.* 1995). Additional evidence for the contribution of *IL-2* to *bcl-xL* expression comes from an

experiment designed to study *CD28* co-stimulation of the T-cell receptor. *CD28* co-stimulation enhances T-cell survival rates and promotes additional *IL-2* production. This additional *IL-2* sharply stimulates *bcl-xL* expression but not *bcl-2* expression. These resulting high levels of *bcl-xL* are sufficient to prevent cell death induced by *IL-2* withdrawal, *Fas* cross-linking, or TCR cross-linking (Boise *et al.* 1995).

Given the demonstrated role of *bcl-xL* in preventing cell death caused by *IL-2* withdrawal, researchers became interested in the potential relationship of other growth factors to *bcl-xL* expression. *IL-3* and *IGF-1* both inhibit apoptosis in *Baf-3* cells, and at the same time drive an increase in the expression of *bcl-xL* at both the mRNA and protein levels; these growth factors also initiate a separate anti-apoptotic pathway that operates independently of protein synthesis. These researchers were able to demonstrate that *IL-3* activates the *PI3-kinase-AKT* pathway, which results in the phosphorylation of *bad* and the up-regulation of *bcl-xL* mRNA (Leverrier *et al.* 1999). A different group of researchers approached this question by studying *IL-3*-dependent *FL5.12* prolymphocytic cells in culture. These cells were transfected with *bax*, *bcl-xL*, or an empty control vector; after 24 hours, *bcl-xL* transfectants showed a significantly higher rate or survival than the control, and the *bax* transfectants showed a much lower rate of survival. When these cells were subjected to *IL-3* withdrawal, the control and *bax* transfectants began to die very rapidly; the *bcl-xL* transfectants showed no signs of apoptosis at all time points measured (Bojes *et al.* 1997). The *IC.DP* cell line has also proven useful in the study of *IL-3*-withdrawal and apoptosis. In this cell line, activation of the *v-Abl* protein tyrosine kinase (*PTK*) renders the cells resistant to apoptosis upon *IL-3* withdrawal. *V-Abl PTK* activation causes protein kinase C (*PKC*) to localize to the nucleus; inhibition of the

localization of *PCK* to the nucleus restores apoptosis in response to *IL-3* withdrawal. *V-Abl PTK* activation induces a two-fold increase in *bcl-xL* mRNA within six hours and a two-fold increase in protein within 24 hours. When *IC.DP* cells were treated with calphostin C, a *PKC* inhibitor, induction of *bcl-xL* was prevented and apoptosis occurred; the levels of *bax, bad,* and *bcl-2* were unaffected in all experimental manipulations. This result suggests that *bcl-xL* expression is a target of *IL-3* and that *bcl-xL* is responsible for the anti-apoptotic effect associated with *IL-3* production (Chen *et al.* 1997).

IGF-1 is another *PI3-kinase-AKT* pathway-activating growth factor, and its expression drives the up-regulation of *bcl-xL.* An experimentally useful cell line is the *PC12* cell line, which relies upon *IGF-1* to prevent apoptosis following serum deprivation. When serum-deprived *PC12* cells are incubated with *IGF-1*, within 3 to 6 hours there is a sharp increase in *bcl-xL* mRNA and a two-fold total increase in *bcl-xL* levels after 24 hours of incubation. This result suggests that the incubation with *IGF-1* stimulated *bcl-xL* expression and that this expression prevented apoptosis caused by serum deprivation (Parrizas and LeRoith 1997).

Beyond its anti-apoptotic activity, *bcl-xL* may play a significant role in cellular differentiation. A group of researchers looked at the *HCD-57* cell line; these murine erythroid progenitor cells depend on erythropoietin (*Epo*) to survive. *Epo* is a hormone that regulates red blood cell production by maintaining erythroid progenitor cell viability. When *HCD-57* cells are cultured without *Epo*, expression of *bcl-xL* and *bcl-2* is halted while *bax* expression is not affected, and the cells begin to die. This cell death can be rescued by transfection with either *bcl-xL* or *bcl-2* without adding back *Epo*, confirming that both genes can prevent apoptosis. In *bcl-xL* or *bcl-2* transfectants, the endogenous

levels of *bcl-xL* and *bcl-2* are down-regulated upon *Epo* withdrawal even though viability is maintained by the expression of the transfected genes. Cells transfected with *bcl-xL* in the absence of *Epo* are able to undergo differentiation into mature erythroid cells, indicating that *bcl-xL* and not *Epo* is the signal that drives differentiation (Silva *et al.* 1996).

The regulation of apoptosis involves the interaction of pro- and anti-apoptotic members of the *bcl-2* gene family. *Bcl-xL* interacts directly with pro-apoptotic *bad* to form a heterodimer; when there is more *bad* protein present than *bcl-xL* protein, the cell will undergo apoptosis. Deletion analysis of the various structural domains of *bad* revealed the minimal domain necessary to interact with *bcl-xL*; this region contains a 26 amino acid peptide with strong similarity to the BH3 domains of *bax* and *bak*. This *bad* BH3 domain was shown to be both necessary and sufficient to dimerize with *bcl-xL*. When these researchers made versions of *bcl-xL* with mutations in the hydrophobic BH3-binding pocket, the resulting protein could not bind to *bad*. Interestingly, several mutants that could not bind to *bad* still showed anti-apoptotic activity in cells. This result suggests that *bad* may promote apoptosis by binding to and essentially inactivating *bcl-xL* to negate its anti-apoptotic activity (Kelekar *et al.* 1997). *Bcl-xL* contains several regions that may regulate cell survival. Experiments using site-specific mutagenesis revealed that the BH1 and BH2 regions of *bcl-xL* contain residues necessary to inhibit apoptosis; these residues were not the same residues as those shown to be necessary in *bcl-2*. Mutant versions of *bcl-xL* protein that interrupt its interaction with *bax* or *bak* were still able to prevent apoptosis about 70-80% as well as wild-type *bcl-xL*, again suggesting that the

anti-apoptotic activity of *bcl-xL* does not depend on its interaction with pro-apoptotic proteins (Cheng *et al.* 1996).

Disease and Models: Murine and Human

The relationship between aberrant *bcl-xL* expression and disease was first elucidated in a study of human lymphoma tissues. Researchers used RT-PCR to screen 50 cases of non-Hodgkins lymphomas (NHLs) and Hodgkins disease (HD) tissues to detect *bcl-x* mRNA. Expression of *bcl-x* was found in nearly all NHLs and all HDs; next the researchers compared the species of *bcl-x* detected to find the ratio of *bcl-xL* to *bcl-xS*. *Bcl-xL* was always found as the dominant species, and the level of *bcl-xS* was variable; there was no correlation between the expression profile of *bcl-2* and the *bcl-xL/bcl-xS* ratio (Xerri *et al.* 1996). In another screen, researchers looked at bladder cancer tissues samples and compared them to normal samples using RT-PCR to detect *bcl-2* and *bcl-xL*. *Bcl-2* was not found in the normal samples, and was found in elevated levels in most (66%) low-stage and all high-stage tumors. *Bax* was found in most (62%) normal samples, and only 14-16% of low- or high-stage tumors. *Bcl-x* was found only in the *–xL* and not the *–xS* isoforms, and its expression pattern complemented that of *bcl-2*, such that any given tumor sample expressed either *bcl-xL* or *bcl-2* but not both. This result suggests that the functional redundancy of *bcl-xL* and *bcl-2* allows the overexpression of either gene to contribute to the pathogenesis of bladder cancer (Gazzaniga *et al.* 1998).

Given the demonstrated role of *bcl-xL* in cells of the haematopoietic lineage, it is a likely candidate for contributing to haematopoietic malignancies. Experimentally,

researchers made cells containing an insertion in the *bcl-x* gene resulting in increased expression levels; these elevated levels of *bcl-xL* protein correlated with the development of myeloid and T-cell malignancies. Expression of extra *bcl-xL* rendered the cells independent of *IL-3* or other trophic factors for survival. Together, these data suggest a role for *bcl-xL* in the development of myelopoeisis and the survival of leukemia cells (Packham *et al.* 1998). *Bcl-xL* has also been demonstrated to be a target of *Epo* regulation; patients with polycythemia vera develop cancer because their erythroid progenitor cells have lost dependence on *Epo*. Investigators studied whether *bcl-xL* levels are also altered in these patients, given the relationship between *Epo* and *bcl-xL*. Patients with polycythemia vera and normal patients were studied using immunocytochemistry and flow cytometry to detect *bcl-x*; the particular isoform of *bcl-x* was determined later by RT-PCR. In patients with polycythemia vera, the loss of *Epo* dependence was directly correlated with an increase in *bcl-xL* expression; very little change was noted in the expression levels of *bcl-xS*. This result suggests that mis-regulation of the proliferation of erythroid progenitors in polycythemia vera is a product of increased *bcl-xL* expression (Silva *et al.* 1998).

The alternative splicing pattern of *bcl-x* provides an attractive target for the treatment of cancer. If the ratio of *bcl-xL/bcl-xS* could be shifted in favor of *bcl-xS* in cancer cells, then perhaps apoptosis could be induced. One group of researchers used an antisense oligonucleotide to alter the splice site of the immature *bcl-x* RNA to promote production of the *bcl-xS* isoform over the *bcl-xL* isoform. This technique sensitized the cells to the induction of apoptosis by chemotherapeutic agents or ultraviolet radiation (Taylor *et al.* 1999). A different group of researchers employed the same approach in

PC-3 prostate cancer cells, using an antisense oligonucleotide to alter the 5' splice site of the *–xL* isoform, shifting the profile in favor of the *–xS* isoform. The treatment induced significant apoptosis in the *PC-3* cells; by comparison, the researchers tried the same technique in *MCF-7* breast cancer cells, and did not find the same degree of induction of apoptosis. *PC-3* cells treated with this oligonucleotide showed a greater degree of inhibition of colony formation *in vitro* than *MCF-7* cells, indicating that the cell type and total genetic expression profile is critical to the response to altering the *bcl-xL/bcl-xS* ratio (Mercatante *et al.* 2001). *Bcl-xL* may also play a significant role in the response of cancer cells to chemotherapy. Most chemotherapeutic agents induce apoptosis by causing DNA damage, but it has been unclear why these agents appear to be specific for tumor cells and not normal healthy cells. DNA damaging agents cause the deamidation of two asparagine residues in the unstructured loop of *bcl-xL*, causing it to lose its ability to bind *bax* and *bad*; ultimately this causes the cells to lose viability and undergo apoptosis. Cancer cells that overexpress *bcl-xL* can be treated by DNA damaging agents because of the deamidation of asparagines in *bcl-xL*. Intriguingly, fibroblasts that are relatively resistant to DNA damage-induced apoptosis demonstrate a suppression of deamidation, likely resulting in the maintenance of the structural integrity of *bcl-xL* (Deverman *et al.* 2002).

Bcl-w

Cellular and Molecular Biology

The initial characterization of *bcl-w* came through a PCR-based screen using sequences from other known *bcl-2* family members. *Bcl-w* was identified and mapped in

both human and murine form; human *bcl-w* lies on chromosome 14q.11 while murine *bcl-w* lies on chromosome 14 as well. Both versions are located in close proximity to the T-cell antigen receptor alpha gene. When expression of *bcl-w* is driven in myeloid and lymphoid cells, the cells became resistant to some, but not all, cytotoxic agents and conditions. Very little *bcl-w* mRNA was detected in B or T lymphoid cell lines but mRNA was found in all myeloid cell lines and a variety of different tissues (Gibson *et al.* 1996). Given that *bcl-w* was initially identified based upon its relationship to the *bcl-2* family, researchers had good reason to suspect it might behave and bind to other family members in a similar fashion. Indeed, *bcl-w* is pulled out of co-immunoprecipitation studies in association with *bax, bak, bad,* and *bik.* Mutations in several glycine residues that are highly conserved across the family of anti-apoptotic *bcl-2* family members did not disrupt the binding of *bcl-w* to pro-apoptotic proteins. Interestingly, overexpression of *bcl-w* protected cells from overexpression of *bax* or *bad* but not *bak* or *bik*, suggesting that *bcl-w* may bind to pro-apoptotic proteins in a manner slightly different from other *bcl-2* anti-apoptotic proteins (Holmgreen *et al.* 1999).

In contrast to other anti-apoptotic *bcl-2* family proteins, *bcl-w* is primarily expressed in mature cells, specifically those of the brain. One group of researchers cloned the cDNA for *bcl-w* to use as a probe for mRNA throughout different stages of mouse development; they used RNA blotting and *in situ* hybridization to detect and localize the mRNA. The highest levels of *bcl-w* expression were found in mature brain regions, specifically the cerebellum, hippocampus, piriform cortex, and locus ceruleus. Double staining for neuronal markers indicated that *bcl-w* was localized to neurons in these regions (Hamner *et al.* 1999). Antibody assays for *bcl-w* expression provided

further insights. Immunohistochemistry using these antibodies indicated that *bcl-w* was not only expressed in the brain, but there was also strong expression in the colon and testes. When these researchers screened several cell lines, they found that *bcl-w* was present, to varying degrees, in epithelial, myeloid, and lymphoid tissues. Subcellular fractionation experiments hinted that *bcl-w* was associated with intracellular membranes, in a fashion similar to its other family members (O'Reilly *et al.* 2001).

Given the demonstrated expression of *bcl-w* in tissues of the gut, researchers sought insight into its potential role in these tissues using massive small bowel resection (SBR). Massive SBR provides a model to study enterocyte proliferation and apoptosis. The pro-apoptotic protein *bax* is also strongly expressed in the gut, so these researchers measured the levels of both proteins, hypothesizing that the ratio of *bcl-w* to *bax* may determine the amount of apoptosis that occurs following SBR. Mice were either subjected to SBR or a sham (control) surgery, and the researchers assessed the mice after 12 hours, 1, 2, 3, or 7 days. The primary measurement was apoptotic index, which is the number of apoptotic bodies per crypt; the levels of mRNA and protein for *bax* and *bcl-w* were also measured. After 12 hours, the apoptotic index in animals subjected to SBR was significantly higher than control animals. The *bax/bcl-w* ratio was elevated at 24 hours, dropped at 3 days, and returned to normal levels at 7 days. When or where an elevated *bax/bcl-w* ratio was found, there was consistently a higher rate of enterocyte apoptosis (Stern *et al.* 2000a). Next, these researchers repeated the experiment, but this time treated the animals with epidermal growth factor (EGF), which is known to increase enterocyte proliferation and inhibit apoptosis. The animals were again subjected either to SBR or sham surgery, and were subsequently treated twice a day with EGF. After three

days, the animals subjected to SBR and treated with EGF showed a decreased apoptotic index and the *bax/bcl-w* ratio was shifted in favor of *bcl-w*. This result suggests that EGF treatment inhibits apoptosis by up-regulating *bcl-w* expression (Stern *et al.* 2000b).

The activity of *bcl-w* is likely mediated through its binding to other proteins, both pro- and anti-apoptotic *bcl-2* family members and unrelated proteins. Co-immunoprecipitation experiments showed that precipitates of *bcl-w* and *bad* contained a phosphatase activity; this activity was identified as *PP1α*. *Bcl-w* contains the R/K X V/I X F consensus *PP1α* targeting motif; if this motif is experimentally disrupted so that *bcl-w* cannot bind to *PP1α*, *bcl-w* also loses its ability to bind to *bad*. This result suggests that formation of a trimolecular complex of *bcl-w*, *PP1α*, and *bad* is a key step in the regulation of apoptosis (Ayllon *et al.* 2002). In a manner similar to that employed for *bcl-2*, the molecular structure of *bcl-w* was solved by triple-resonance NMR spectroscopy and molecular modeling. The cytosolic domain contains eight α-helices with a folding pattern similar to the patterns of *bcl-xL, bcl-2,* and *bax*. The key difference between *bcl-w* and *bcl-2/bcl-xL* is that the C-terminal helix of *bcl-w*, helix α8, folds in a manner similar to the C-terminal transmembrane helix of *bax*. This helix can bind to the BH3 region of *bid*, resulting in local helical unfolding. Deletion of this helix increases the binding affinity of *bcl-w* to *bak-* or *bid*-derived peptides, suggesting that this helix mediates the interaction affinity of *bcl-w* with pro-apoptotic proteins (Denisov *et al.* 2003).

Binding of a ligand to *bcl-w* has a pronounced effect on its structure and activity. In healthy cells, *bcl-w* is only loosely attached to the mitochondrial membrane, and its hydrophobic COOH-terminal domain lies in the hydrophobic groove where BH3 ligands,

such as *bim*, bind. Ligand binding displaces the COOH-terminal residues, allowing the insertion of *bcl-w* into the mitochondrial membrane; that is, *bcl-w* becomes an integral membrane protein upon ligand binding. When researchers experimentally tethered a *bim* BH3 peptide to the amino terminus of *bcl-w*, the chimeric protein bound more strongly to the mitochondrial membrane than wild-type *bcl-w*. This chimeric protein did not cause a change in the rate of apoptosis, suggesting that the binding of BH3-only proteins such as *bim* to *bcl-w* counteracts the survival activity of *bcl-w* (Wilson-Annan *et al.* 2003). In another experiment studing the binding-induced change in *bcl-w*, researchers found that *bcl-w* was active as an anti-apoptotic protein when weakly bound to the mitochondrial membrane. Upon binding by BH3-only proteins at the hydrophobic pocket, the COOH-terminal of *bcl-w* is released and the protein is inserted into the mitochondrial membrane, neutralizing its survival activity (Kaufman *et al.* 2004).

Disease and Models: Murine and Human

Testes

Bcl-w plays a critical role in testes and germ cell development and spermatogenesis. The role for *bcl-w* in germ cell biology was initially identified through a retroviral gene trap screen for genes conferring male infertility. *ROSA41* male mice demonstrate germ cell defects beginning at postnatal day 19, and spermatogenesis is blocked in the later stages, leading to a depletion of all germ cells. After the germ cells die, only Sertoli cells remain; very shortly thereafter, nearly all Sertoli and Leydig cells also die. *ROSA41* mice produce no *bcl-w* polypeptide and are otherwise normal, indicating that it is the *bcl-w* deficiency that leads to the erroneous germ cell development

(Ross *et al.* 1998). The *bcl-w -/-* knockout mouse provides an attractive model for studying the role of *bcl-w in vivo*. The knockout was achieved through homologous recombination and produces no *bcl-w* protein. Adult *bcl-w -/-* mice appear normal, and haematopoiesis is unaffected, likely due to the redundancy with other *bcl-2* family members. Female mice have normal reproductive systems, but the males are infertile due to a failure of spermatogenesis. This failure leads to a gradual depletion of maturing germ cells; initially, spermatogenesis is normal, but in later developmental stages the cells begin to die (Print *et al.* 1998).

In the testis, *bcl-w* dimerizes with pro-apoptotic members of the *bcl-2* family in response to hormonal stimulation. Steady-state levels of *bcl-w* mRNA and protein are highest in Sertoli cells, but both are detected in spermatogonia, spermatocytes, and Leydic cells as well. *In vivo*, *bcl-w* forms complexes with *bax* and *bak* but not *bad*; *bax* and *bak* co-localize with *bcl-w* in immunohistochemical experiments. When adult male mice are treated with follicle-stimulating hormone (FSH), *bcl-w* mRNA levels increase in the seminiferous tubules; testosterone treatment appears to have no effect on *bcl-w* levels. When researchers compared three different mouse models of spermatogonial apoptosis, they found increased ratios of *bax* to *bcl-w* and *bak* to *bcl-w*, indicative of a loss of the *bcl-w*-driven survival signal (Yan *et al.* 2000). Given the functional redundancy of many members of the *bcl-2* family, one group studied the expression pattern of other *bcl-2* family members in the testis. *Bcl-2, bcl-xL,* and *bcl-w* are all present during the early, juvenile stages of spermatogenesis, but only *bcl-w* is expressed in adult mouse spermatogonia. The early expression of several related *bcl-2* family anti-apoptotic proteins may explain why *bcl-w -/-* knockout mice only begin to display spermatogonial

failure after three to four weeks postnatally (Meehan *et al.* 2001). The initiation of apoptosis in *bcl-w -/-* cells is likely mediated by *caspase 3*; *bcl-w* and *casp3* co-localize in Sertoli cells and in pre-pubertal cells in rat testes. *Casp3* may be the effector of apoptosis in cells lacking *bcl-w* because *bcl-w* cannot bind to its targets and prevent *casp3* activity (Giannattasio *et al.* 2002).

Mice that experimentally overexpress *bcl-w* in the testis are, intriguingly, infertile. The developing testis in these mice shows a lower number of spermatogonia and decreased proliferation in germ cells, measured by *in vivo* and *in vitro* 5' BRDU incorporation assays. The adult testis in these mice shows interrupted spermatogenesis and few to no germ cells. When researchers looked at the degenerating germ cells, they found that these cells were TUNEL-negative and did not display characteristic apoptotic features, such as the DNA ladder. *Bcl-w* overexpression may thus inhibit cell cycle entry or progression in germ cells, resulting in the ovserved failure of spermatogenesis (Yan *et al.* 2003).

Brain

Because of the established expression pattern of *bcl-w* in tissues of the brain, researchers hypothesized that *bcl-w* might be expressed in response to brain damage. To experimentally study cell survival after cerebral ischemia, one group of researchers subjected mice to a 20 minute termporary middle cerebral artery occlusion (MCAO), and then performed Western blots and immunocytochemistry at a variety of time points thereafter. There was an increase in *bcl-w* protein in the caudate putamen, parietal cortex, and frontal cortex; the protein was localized in these regions to neurons in the parietal and frontal cortex and to glia in the caudate putamen (Minami *et al.* 2000). The

expression pattern of *bcl-w* following transient MCAO was measured at 6 to 72 hours post-injury; the highest levels were found in ischemic cells at all time points, with a low but steady level of *bcl-w* expression detected in non-ischemic cells. Cells in the penumbral cortex and cells determined to be non-apoptotic by their lack of DNA fragmentation showed the highest levels of *bcl-w* expression. Not surprisingly, expression of *bcl-w* was localized to neurons, specifically to the mitochondria. When researchers studied isolated brain mitochondria, they found that adding recombinant *bax* or increasing the calcium concentration could trigger *cyt c* release. The addition of recombinant *bcl-w* could stop *cyt c* release and maintain the mitochondrial transmembrane potential (Yan *et al.* 2000).

The induction of *bcl-w* in brain tissue has remained somewhat mysterious at the mechanistic level beyond its role as a generalized response to damage. The amino acids L-serine and glycine have a demonstrated protective effect on neurons; researchers built on this observation to search for a relationship to *bcl-w*. When L-serine or glycine are added to cultured rat cerebrocortical neurons, there is a dose-dependent protective effect. Both amino acids acted to up-regulate *bcl-w* but not *bcl-xL*, suggesting that the protection conferred by these amino acids is mediated by *bcl-w* (Yang *et al.* 2000). Because both *bcl-xL* and *bcl-w* have been shown to play neuroprotective roles, the orchestration and communication between these two proteins is of interest. Researchers microinjected sense or antisense plasmids for *bcl-xL* or *bcl-w* into the nuclei of nerve growth factor-dependent trigeminal neurons at a variety of stages covering the beginning and conclusion of naturally occurring cell death (NOCD). Overexpression of sense *bcl-w* showed a protective effect that increased dramatically with the age and developmental

stage; overexpression of antisense *bcl-w* became more and more lethal as the age of induction increased. By contrast, overexpression of sense *bcl-xL* showed a protective effect that attenuated with progressive developmental stages, and antisense *bcl-xL* also became less lethal with age. This result suggests that *bcl-xL* is important as a protective protein during the early stages of NOCD and that *bcl-w* is important primarily in later developmental stages (Middleton *et al.* 2001).

Bcl-w may be expressed as a response to seizures and the subsequent brain damage. When limbic seizures were experimentally induced in rats using kainic acid and then stopped after 40 minutes by treatment with diazepam, there was constitutive expression of *bcl-w* in neurons, as shown by Western blotting. Expression was concentrated primarily in the hippocampus and piriform cortex (Henshall *et al.* 2001). Overexpression of *bcl-w* in the testis did not afford any protective effects, but overexpression in the brain proved to be different. Researchers injected a recombinant adeno-associated virus (rAAV) overexpressing *bcl-w* into the cerebral cortex and striatum of rats and studied the response of these and control animals to temporary focal ischemia induced by middle cerebral artery occlusion. Rats injected with the rAAV expressed more *bcl-w* than control rats in the injected regions, and these rats showed 33-40% increased neurological function as well as a 30% smaller infarct size (Sun *et al.* 2003). Another group of researchers took these observations that *bcl-w* plays a protective effect in the brain and investigated the potential role of *bcl-w* in the pathology of Alzheimer disease (AD). Neurofibrillary neurons are especially long-lived in AD brains; *bcl-w* and *bcl-xL* have both been shown to protect neurons from death. When AD brains are compared to normal age-matched control brains, there is an increased level of *bcl-w* that

correlates with the presence of neurofibrils and intracytoplasmic inclusions in the AD brains. The control age-matched brains showed low and diffuse levels of *bcl-w*. In the AD brains, *bcl-w* was localized to the mitochondria and to neurofibrils, as shown by electron microscopy. *In vitro*, *M17* human neuroblastoma cells express fibrillized amyloid-beta protein, and expression of this protein is correlated with increased levels of *bcl-w* protein. These cells are especially resistant to apoptosis induced by staurosporine, suggesting that the longevity of these neurofibrillary cells in the AD brain is, in part, mediated by elevated expression of *bcl-w* (Zhu *et al.* 2004).

Gut/Colorectal

Bcl-w is expressed at unusually high levels in a variety of gut and colorectal cancers, suggesting that its expression may contribute to the etiology of these diseases. In a screen of various colorectal tumor tissue samples, 69 of 75 colorectal adenocarcinomas tested positive for expression of *bcl-w*; cancers of other tissues did not show such a high level of expression. Amongst these adenocarcinomas, cases with a higher grade of tumor progression and a lower prognostic grade were most likely to show *bcl-w* expression; cases with a higher prognostic grade and lower grade of tumor were less likely to express *bcl-w*. Cancers that involved the lymph nodes were also more likely than node-negative cancers to show *bcl-w* expression. These data suggest that *bcl-w* plays a role in the progression of colorectal epithelial cancers from adenoma to adenocarcinoma (Wilson *et al.* 2000). Two different human adenocarcinoma cell lines, *SNU-620* and *SNU-16*, respond differently to apoptotic conditions, and this difference is due to different expression levels of *bcl-w*. *SNU-620* cells are much more resistant to apoptosis induced by anti-Fas, hydrogen peroxide, or serum withdrawal than *SNU-16*.

SNU-620 cells expressed *bcl-w*, but not other *bcl-2* family members, to a much higher degree than *SNU-16* cells. Stable transfection of *bcl-w* into *SNU-16* cells rescued these cells and stopped activation of the *SAPK/JNK* pathway, indicating that the difference in apoptosis levels between the two cell lines was due to *bcl-w* expression differences. When these researchers looked at 50 different patient samples from cases of gastric adenocarcinoma, they found that *bcl-w* was expressed in cancerous cells but not in the flanking mucosal cells, and that *bcl-w* was expressed in the majority of infiltrative tumors (Lee *et al.* 2003).

Bcl-w serves a protective function in intestinal tissues, mediating the response to damage incurred through cytotoxic treatments and other apoptosis-inducing factors. Using monoclonal antibodies for *bcl-w*, one group detected *bcl-w* in the small intestine, colon, and several epithelial tumor cell lines and colon carcinoma cell lines. When *bcl-w* -/- mice were compared to wild-type mice, there were no differences between the two in their level of apoptosis in intestinal crypts. When these mice were treated with cytotoxic drugs such as 5-fluorouracil or γ-radiation, the *bcl-w* -/- mice showed a significantly higher level of apoptosis than the wild-type mice. The biggest difference between the two genotypes was in the small intestine, where there was a six-fold higher rate of apoptosis in the *bcl-w* -/- than wild-type mice, suggesting that *bcl-w* expression was responsible for the difference in response to damage (Pritchard *et al.* 2000).

Bcl-b

Cellular and Molecular Biology

One of the newest additions to the *bcl-2* family is *bcl-b*, which was identified through a screen searching for novel family members showing homology to *bcl-2*. Not much is known about the activity of *bcl-b*, although studies are ongoing. The closest sequence homology of *bcl-b* is to *boo/diva*, and it contains BH1, BH2, BH3, and BH4 domains. The mRNA of *bcl-b* is found in a large variety of human tissues, as shown by extensive Northern blotting. *Bcl-b* likely acts by suppressing *bax*-induced apoptosis, but not *bak*-induced apoptosis. In addition, *bcl-b* appears to be an inner mitochondrial membrane protein; deletion of its COOH-terminal transmembrane domain disrupts its association with intracellular organelles and also disrupts its anti-apoptotic activity (Ke *et al.* 2001). Because *bcl-b* interacts with *bax* and not *bak*, researchers studied the domains of *bcl-b* necessary for this interaction. *Bcl-b* binds to *bax* at the BH3 domain of *bax*; to investigate this binding, they constructed mutant *bax* containing the *bak* BH3 domain and mutant *bak* containing the *bax* BH3 domain. *Bcl-b* was able to bind to wild-type *bax* and *bak* with the *bax* BH3 domain, and it could not bind to wild-type *bak* or *bax* with the *bak* BH3 domain, suggesting that it is indeed the *bax* BH3 domain that is critical for *bcl-b* binding. *Bcl-xL*, by contrast, associated with both wild-type and chimeric proteins, showing that the specificity of the interaction is exclusive to *bcl-b*. Alanine substitution mapping showed that there are several defined residues in the *bax* BH3 domain that are required for *bcl-b* binding. This technique also identified several residues in *bcl-b* that are necessary for *bax* binding; leucine 86 and arginine 96 are each required for *bcl-b* to bind to *bax*. Mutant versions of *bcl-b* bearing alanine in place of either of these residues are unable to bind to *bax* and cannot suppress apoptosis driven by *bax* overexpression. *Bcl-b* mutants that retained the ability to bind to *bax* were still able to suppress apoptosis,

confirming that it is the binding of *bcl-b* to *bax* that is necessary to inhibit apoptosis (Zhai *et al.* 2003).

Mcl-1

Cellular and Molecular Biology

The *mcl-1* gene was isolated from a screen of a human myeloid leukemia cell line, *ML-1*, studying the response of the cells to cues driving differentiation. The cells were driven to differentiate by treatment with phorbol ester. At approximately one to three hours, *mcl-1* expression was first detected; this time frame marks the programming phase, when the induction of differentiation occurs, and before mature markers and signs of differentiation appear. There is a strong sequence similarity between *mcl-1* and *bcl-2*; both genes show cell protective effects but do not appear to affect rates of proliferation (Kozopas *et al.* 1993). *Mcl-1* has been mapped in both the human and murine forms. Researchers used a combination of somatic cell hybrid analysis and *in situ* hybridization to map human *mcl-1* to chromosome 1q21, a site on the long arm of chromosome 1. The murine version of *mcl-1* is more enigmatic, mapping to two locations in the genome. One copy resides on chromosome 3, and this copy is homologous to human *mcl-1*. Another copy resides on chromosome 5, and this copy is likely to be a pseudogene, as expression is not detectable. Initial screens showed that rearranged versions of *mcl-1* are frequently expressed in some neoplastic diseases, suggesting that aberrant expression may be linked to disease development (Craig *et al.* 1994).

Because *mcl-1* expression was known to be associated with the induction of differentiation, researchers studied the expression pattern of *mcl-1* using agents that can

induce differentiation in cell culture. *Mcl-1* mRNA is induced by agents that drive monocyte and macrophage differentiation, such as phorbol esters and lymphocyte conditioned medium, as well as by treatment with cytotoxic agents, such as colchicines or vinblastine. Expression is not induced by agents that drive granulocyte differentiation, such as retinoic acid, suggesting that the expression of *mcl-1* is not a general feature of all types of differentiation. The turnover between *mcl-1* mRNA and protein is quite rapid, implying that *mcl-1* is expressed quickly in response to the initiation of monocyte or macrophage differentiation or to signals of cell death (Yang *et al.* 1996). Researchers looking at the *NCR-G3* testicular embryonal cell line found that *mcl-1* was a hallmark of differentiation in these cells. The cells were experimentally driven to differentiate, and 50 thousand clones were then screened for expression of a variety of genes. *Mcl-1* was consistently up-regulated at very early stages of differentiation, before the expression of genes specific to later stages of differentiation. *Mcl-1* was also induced in response to heat shock, which has been suggested to induce differentiation (Umezawa *et al.* 1996). The structure of *mcl-1* is also consistent with its rapid induction behavior. *Mcl-1* contains three BH regions homologous to *bcl-2*, and its sequence encodes one immediate response box (IRB). The presence of the IRB suggests that *mcl-1* is an immediate-early gene; that is, a gene that is expressed rapidly in response to signals of differentiation (Okita *et al.* 1998).

Given the relationship between *mcl-1* and *bcl-2*, *mcl-1* is a strong candidate to have anti-apoptotic activity. Treatment of cells with vascular endothelial growth factor (VEGF) is known to inhibit cell death driven by a variety of cytotoxic agents, including ionizing radiation and the chemotherapeutic drugs etoposide and doxorubicin.

Researchers treated cells with VEGF and cytotoxic agents, and then performed Northern blotting and immunoblotting to see if any members of the *bcl-2* family were expressed. The only family member expressed in response to VEGF was *mcl-1*. To confirm that *mcl-1* was indeed responsible for the rescue of the cells from apoptosis, the researchers transfected the *U937* clonal myeloid leukemia cell line with *mcl-1*. These cells showed decreased *caspase 3* activity as well as increased viability after etoposide treatment when transfected with *mcl-1*, substantiating the anti-apoptotic activity of *mcl-1* (Katoh *et al.* 1998).

Expression of *mcl-1* occurs in response to a highly coordinated upstream signaling cascade. *Mcl-1* is a target of the mitogen activated protein (*MAP*) kinase cascade. Both the *MAP* kinase cascade and *bcl-2* family are known to be involved in promoting viability, but the relationship between the two was previously unclear. To elucidate this relationship, researchers treated *ML-1* cells with colchicine or vinblastine, both of which are microtubule disrupting agents, or 12-O-tetradecanoylphorbol 13-acetate (*TPA*). Treatment with these agents drove an increase in phosphorylation of the extracellular signal-regulated kinase (*ERK*) and to an increase in *mcl-1* expression. Phosphorylation of *ERK* is necessary for *mcl-1* expression to occur; if *ERK* phosphorylation is inhibited with *PD 98059*, *mcl-1* expression is prevented. If *ERK* phosphorylation is increased by treatment with lipopolysaccharide or okadaic acid, *mcl-1* is coordinately increased as well. Upstream of *ERK*, the pathway is stimulated by microtubule damage, which triggers the activity of protein kinase C (Townsend *et al.* 1998). At the nuclear level, several different factors act to drive *mcl-1* transcription.

Serum response factor (*SRF*), *Elk-1*, and *Sp1* bind to the 162 base pair 5' flank of *mcl-1* to direct both the basal and *TPA*-inducible expression of *mcl-1* (Townsend *et al.* 1999).

The activity of *mcl-1* is regulated through its phosphorylation, and this modification can be achieved through several pathways. Researchers initially noted that phosphorylation of *mcl-1* in a Burkitt lymphoma cell line through *TPA* treatment yielded a protein with a different electrophoretic mobility than *mcl-1* phosphorylated by microtubule-damaging agents such as taxol. The *TPA/ERK* pathway to *mcl-1* phosphorylation happens quite rapidly, and the cells remain viable after *TPA* treatment. By contrast, taxol-driven *mcl-1* phosphorylation occurs more slowly, and the cells tend to accumulate at the *G2/M* transition and ultimately die. These results suggest that there are two distinct pathways leading to *mcl-1* phosphorylation, one that is *ERK*-dependent and another that is *ERK*-independent (Domina *et al.* 2000). Additional support for this model comes from the finding that the two pathways lead to phosphorylation of *mcl-1* at different sites on the protein. *TPA* treatment and *ERK* activation leads to phosphorylation at a conserved *ERK*-binding site in the *PEST* region at threonine 163. Phosphorylation at this residue slows down the turnover of *mcl-1* protein, which is otherwise rapidly degraded. Okadaic acid and taxol treatment lead to phosphorylation of *mcl-1* at different but discrete sites on the protein (Domina *et al.* 2004).

Once *mcl-1* is phosphorylated, its target within the cell is the proliferating cell nuclear antigen (*PCNA*). Researchers used *in vitro* and *in vivo Brdu* uptake assays to show that overexpression of *mcl-1* inhibits the cell cycle by arresting the cells at *S* phase, a known target of *PCNA*. A mutant version of *mcl-1*, *mcl-1-δ4*, cannot bind to *PCNA*. *Mcl-1-δ4* localizes to the same intracellular locations as wild-type *mcl-1* and it has the

same anti-apoptotic function in cells treated with etoposide, but it does not inhibit the cell cycle. *In vitro* pull-down assays show that *mcl-1* is the only member of the *bcl-2* family that binds to *PCNA*; *mcl-1* is also the only member of the *bcl-2* family that contains a *PCNA* binding motif. These results suggest that *mcl-1* regulates the progression of the cell cycle through its specific *PCNA* binding activity (Fujise *et al.* 2000). In addition to *PCNA*, *mcl-1* interacts with *fortilin*, a 172 amino acid anti-apoptotic polypeptide. The activity and function of *fortilin* remain poorly defined, but it does have demonstrated anti-apoptotic activity. The interaction between *mcl-1* and *fortilin* increases the half-life and stability of *fortilin*, but it does not appear to have the same effect on *mcl-1*. Researchers used *si*RNA to silence either one or both of these genes in the *U20S* cell line, and treated the cells with the cytotoxic agent 5-fluorouracil. When either *fortilin* or *mcl-1* were silenced, the other protein showed a protective effect in a strictly dose-dependent manner, and both proteins localized normally on their own to the same intracellular site. These data suggest that *mcl-1* interacts with *fortilin* so that both can act as inhibits of apoptosis for the cell (Zhang *et al.* 2002 and Graidist *et al.* 2004).

Disease and Models: Murine and Human

As is the case for most members of the *bcl-2* anti-apoptotic family, *mcl-1* is expressed in response to damaging drugs or conditions. *ML-1* cells treated with DNA-damaging agents show decreased levels of *bcl-2*, increased levels of pro-apoptotic *bax*, and increased levels of *mcl-1* protein in response to ionizing and ultraviolet radiation as well as alkylating drugs. The increase in *mcl-1* protein level is brief, and the levels of both mRNA and protein return to baseline in approximately 24 hours. Researchers

screening a variety of human cell lines found that *mcl-1* levels were increased in response to DNA damage, and that this increase was independent of the presence or absence of the tumor suppressor protein *p53*. The increase in *bax* expression, by comparison, depended upon the presence of *p53* in the cell. *Mcl-1* expression in these cell lines was also contingent upon the sensitivity to the cell line to apoptosis induced by DNA-damaging agents (Zhan *et al.* 1997). Expression of *mcl-1* is likely to play a role in the tumorigenicity of certain cancer cell lines. Another group of researchers looked at the Burkitt lymphoma cell line *BL41* and its derivative, *BL41-3*. The key difference between the two cell lines is that *BL41-3* expresses three-fold elevated levels of *mcl-1*, and five-fold more *mcl-1* in response to *TPA* treatment when compared to *BL41* or wild-type cells. To investigate whether *mcl-1* expression performs a protective function in these *BL41-3* cells, the researchers exposed both *BL41* and *BL41-3* cells to a variety of cytotoxic conditions, including growth factor withdrawal, staurosporine, etoposide, and camptothecin. The *BL41-3* cells remained viable for significantly longer than the *BL41* cells after treatment with any of the above agents. Intriguingly, *BL41-3* cells were unusually sensitive to *1-beta-D-arabinofuranosylcytosine* treatment, suggesting that the overexpression of *mcl-1* did not render the *BL41-3* cells resistant to all chemotherapeutic agents (Vrana *et al.* 2002).

A transgenic mouse model for *mcl-1* was recently developed, and it provides valuable clues to the relationship between *mcl-1* expression and disease pathogenesis. Mice bearing a transgene in hematolymphoid tissues overexpressed *mcl-1* and ultimately developed a variety of lymphomas. Most of the lymphomas had a long latency, but 85% of the transgenic mice studied did develop cancer within two years. Most of the

lymphomas were of clonal B-cell origin. At the histological level, most of these were follicular lymphomas or diffuse large-cell lymphomas, but a variety of different subtypes also developed (Zhou *et al.* 2001). Beyond generalized transgenic mouse lymphoma models, *mcl-1* has been implicated as a neuroprotective agent. *Bcl-2* expression was demonstrated in the neurons of the central nervous system by multiple research groups, but there was not much evidence for *mcl-1* expression in CNS neurons. Researchers studying prolonged seizures in mice found that *mcl-1* expression was a key facet of resistance to apoptosis following seizure. *C57 Bl/6J* mice are relatively resistant to hippocampal cell death, while *C3H/HeJ* mice are comparatively susceptible. The difference in the susceptibility of these two strains to hippocampal cell death is mediated by their differential expression of *mcl-1*. *C57 Bl/6J* mice express *mcl-1* protein at elevated levels in hippocampal pyramidal neurons even after the seizure is done, while *C3H/HeJ* mice show a drop in *mcl-1* protein levels. *C3H/HeJ* mice subsequently undergo a high degree of neuronal apoptosis, suggesting that *mcl-1* expression in *C57 Bl/6J* mice was key to the survival of the hippocampal pyramidal neurons. In contrast to overexpressing transgenic models, *mcl-1 -/-* mice that do not express any *mcl-1* protein show elevated levels of DNA damage. These mice also undergo caspase-mediated cell death after seizures when compared to wild-type *mcl-1* expressing control littermates, further confirming the neuroprotective role of *mcl-1* (Mori *et al.* 2004).

Conclusion

The control of cell proliferation and death in the immune system is a function of the coordination of multiple signaling pathways, including ligation of the death receptor

and the regulation of *bcl-2*-family members. This review has focused on our current knowledge and understanding of the anti-apoptotic members of the *bcl-2* protein family. Very small changes and adjustments in a variety of the steps along these pathways are critical to the fine-tuning and regulation of cell death in an organism. When mis-regulated, a variety of mishaps may occur, including the development of cancer, autoimmunity, and infection. Future research is required to further elucidate the many and varied features of these genetic pathways.

Figure 1: An Overview of the Mitochondrial Cell Death Pathway

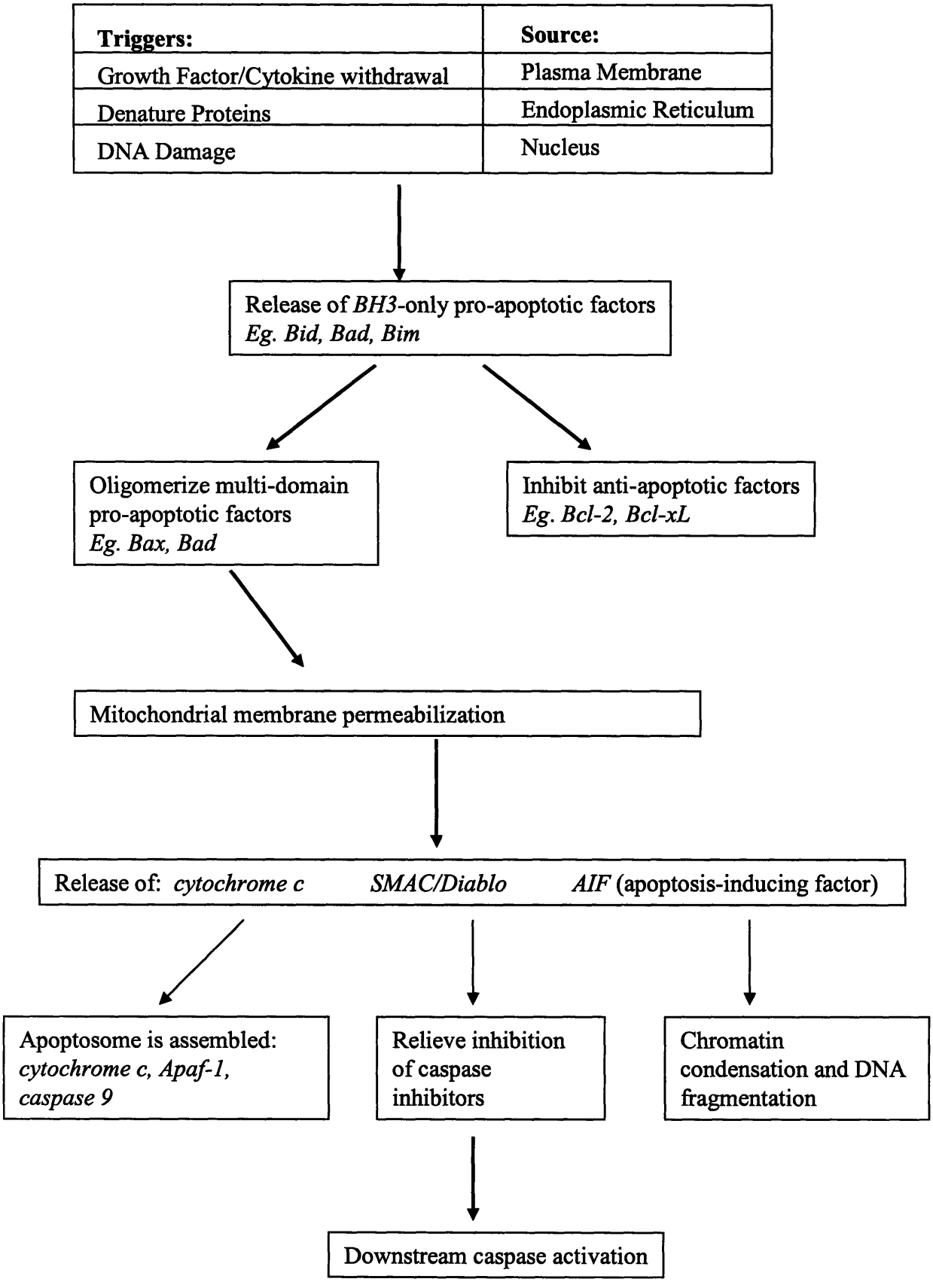


Figure 2:

A Comparison of Anti-Apoptotic *Bcl-2* Family Members

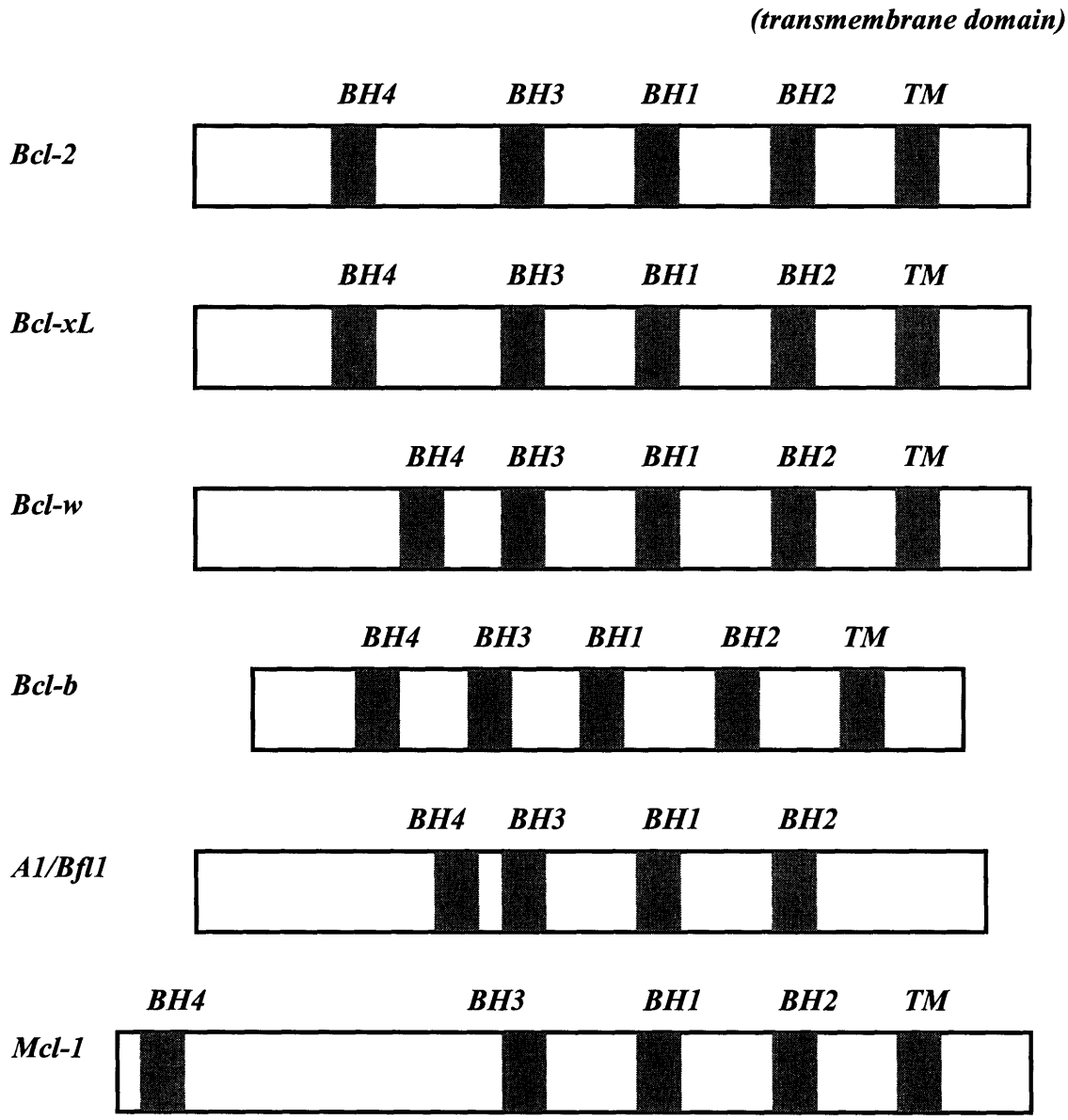
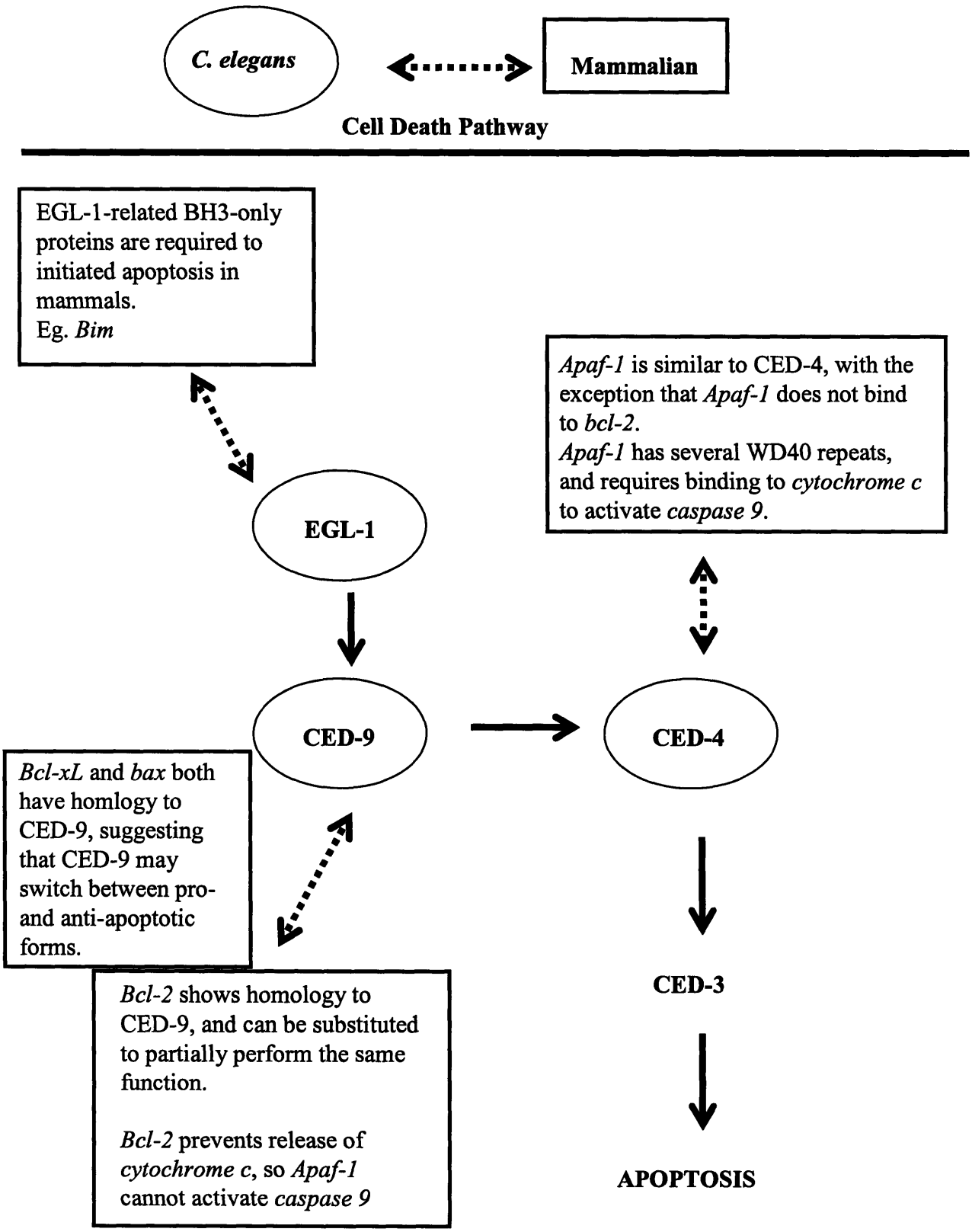


Figure 3:

A Comparison of Apoptosis in Metazoans and Mammals



References

Adams, J.M. and Cory, S. The Bcl-2 protein family: arbiters of cell survival. Science 1998, 281(5381):1322-1326.

Akatsuka, Y., Nishida, T., Kondo, E., Miyazaki, M., Taji, H., Iida, H., Tsujimura, K., Yazaki, M., Naoe, T., Morishima, Y., Kodera, Y., Kuzushima, K., and Takahashi, T. Identification of a Polymorphic Gene, BCL2A1, Encoding Two Novel Haematopoietic Lineage-specific Minor Histocompatibility Antigens. The Journal of Experimental Medicine 2003, 197(11):1489-1500.

Aisenberg, A.C., Wilkes, B.M., and Jacobson, J.O. The bcl-2 gene is rearranged in many diffuse B-cell lymphomas. Blood 1988, 71(4):969-972.

Ayllon, V., Cayla, X., Garcia, A., Fleischer, A., and Rebollo, A. The anti-apoptotic molecules Bcl-xL and Bcl-w target protein phosphatase 1alpha to Bad. European Journal of Immunology 2002, 32(7):1847-1855.

Bakhshi, A., Jensen, J.P., Goldman, P., Wright, J.J., McBride, O.W., Epstein, A.L., and Korsmeyer, S.J. Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. Cell 1985, 41(3):899-906.

Ban, J., Eckhart, L., Weninger, W., Mildner, M., and Tschachler, E. Identification of a human cDNA encoding a novel Bcl-x isoform. Biochemistry and Biophysics Research Communication 1998, 248(1):147-152.

Boise, L.H., Gonzalez-Garcia, M., Postema, C.E., Ding, L., Lindsten, T., Turka, L.A., Mao, X., Nunez, G., and Thompson, C.B. Bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell 1993, 74(4):597-608.

Boise, L.H., Minn, A.J., Noel, P.J., June, C.H., Accavitti, M.A., Lindsten, T., and Thompson, C.B. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-xL. Immunity 1995, 3(1):87-98.

Bojes, H.K., Datta, K., Xu, J., Chin, A., Simonian, P, Nunez, G., and Kehrer, J.P. Bcl-xL overexpression attenuates glutathione depletion in FL5.12 cells following interleukin-3 withdrawal. Biochemistry Journal 1997, 325(2):315-319.

Bouillet, P., Purton, J.F., Godfrey, D.I., Zhang, L.-C., Coultas, L., Puthalakath, H., Pellegrini, M., Cory, S., Adams, J.M., and Strasser, A. BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. Nature 415(6874):922-926.

Broome, H.E., Dargan, C.M., Krajewski, S., and Reed, J.C. Expression of Bcl-2, Bcl-x, and Bax after T cell activation and IL-2 withdrawal. Journal of Immunology 1995, 155(5):2311-2317.

Chen, J., Flannery, J.G., LaVail, M.M., Steinberg, R.H., Xu, J., and Simon, M.I. Bcl-2 overexpression reduces apoptotic photoreceptor cell death in three different retinal degenerations. Proceedings of the National Academy of Sciences USA 1996, 93(14):7042-7027.

Chen, Q., Turner, J., Watson, A.J., and Dive, C. V-Abl protein tyrosine kinase (PTK) mediated suppression of apoptosis is associated with the up-regulation of Bcl-XL. Oncogene 1997, 15(18):2249-2254.

Cheng, E.H., Levine, B., Boise, L.H., Thompson, C.B., and Hardwick, J.M. Bax-independent inhibition of apoptosis by Bcl-XL. Nature 1996, 379(6565):554-556.

Chinnaiyan, A.M., O'Rourke, K., Tewari, M., and Dixit, V.M. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell 1995, 81(4):505-512.

Choi, S.S., Park, I.C., Yun, J.W., Sung, Y.C., Hong, S.I., and Shin, H.S. A novel Bcl-2 related gene, Bfl-1, is overexpressed in stomach cancer and preferentially expressed in bone marrow. Oncogene 1995, 11(9):1693-1698.

Chuang, P.I., Morefield, S., Liu, C., Chen, S., Harlan, J.M., and Willerford, D.M. Perturbation of B-cell development in mice overexpressing the Bcl-2 homolog A1. Blood 2002, 99(9):3350-3359.

Cleary, M.L. and Sklar, J. Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18. Proceedings of the National Academy of Sciences USA 1985, 82(21):7439-7443.

Craig, R.W. The BCL-2 gene family. Seminars in Cancer Biology 1995, 6:35-43.

Craig, R.W., Jabs, E.W., Zhou, P., Kozopas, K.M., Hawkins, A.L., Rochelle, J.M., Seldin, M.F., and Griffin, C.A. Human and mouse chromosomal mapping of the myeloid cell leukemia-1 gene: MCL1 maps to human chromosome 1q21, a region that is frequently altered in preneoplastic and neoplastic disease. Genomics 1994, 23(2):457-463.

Deng, G. and Podack, E.R. Suppression of apoptosis in a cytotoxic T-cell line by interleukin 2-mediated gene transcription and deregulated expression of the protooncogene bcl-2. Proceedings of the National Academy of Sciences USA 1993, 90(6):2189-2193.

Denisov, A.Y., Madiraju, M.S., Chen, G., Khadir, A., Beauparlant, P., Attardo, G., Shore, G.C., and Gehring, K. Solution structure of human BCL-w: modulation of ligand binding by the C-terminal helix. Journal of Biological Chemistry 2003, 278(23):21124-21128.

Deverman, B.E., Cook, B.L., Manson, S.R., Niederhoff, R.A., Langer, E.M., Rosova, I., Kulans, L.A., Fu, X., Weinberg, J.S., Weinecke, J.W., Roth, K.A., Weintraub, S.J. Bcl-xL deamidation is a critical switch in the regulation of the response to DNA damage. Cell 2002, 111(1):51-62.

Domina, A.M., Smith, J.H., and Craig, R.W. Myeloid cell leukemia 1 is phosphorylated through two distinct pathways, one associated with extracellular signal-regulated kinase activation and the other with G2/M accumulation or protein phosphatase 1/2A inhibition. Journal of Biological Chemistry 2000, 275(28):21688-21694.

Domina, A.M., Vrana, J.A., Gregory, M.A., Hann, S.R., and Craig, R.W. MCL1 is phosphorylated in the PEST region and stabilized upon ERK activation in viable cells, and at additional sites with cytotoxic okadaic acid or taxol. Oncogene 2004, 23(31):5301-5315.

D'Sa-Eipper, C., Subramanian, T., and Chinnadurai, G. Bfl-1, a bcl-2 homologue, suppresses p53-induced apoptosis and exhibits potent cooperative transforming activity. Cancer Research 1996, 56(17):3879-3882.

D'Souza, B., Rowe, M., and Walls, D. The bfl-1 Gene is Transcriptionally Upregulated by the Epstein-Barr Virus LMP1, and Its Expression Promotes the Survival of a Burkitt's Lymphoma Cell Line. Journal of Virology 2000, 74(14):6652-6658.

Edelstein, L.C., Lagos, L., Simmons, M., Tirumalai, H., and Gelinas, C. NF-kB-Dependent Assembly of an Enhanceosome-Like Complex on the Promoter Region of Apoptosis Inhibitor Bfl-1/A1. Molecular and Cellular Biology 2003, 23(8):2749-2761.

Ellis, R.E., Yuan, J., and Horvitz, H.R. Mechanisms and functions of cell death. Annual Review of Cell Biology 1991, 7:663-698.

Fang, W., Rivard, J.J., Mueller, D.L., and Behrens, T.W. Cloning and molecular characterization of mouse bcl-x in B and T lymphocytes. Journal of Immunology 1994, 153(10):4388-4398.

Farlie, P.G., Dringen, R., Rees S.M., Kannourakis, G., and Bernard, O. Bcl-2 transgene expression can protect neurons against developmental and induced cell death. Proceedings of the National Academy of Sciences USA1995, 92(10):4397-4401.

Fujise, K., Zhang, D., Liu, J., and Yeh, E.T. Regulation of apoptosis and cell cycle progression by MCL1. Differential role of proliferating cell nuclear antigen. Journal of Biological Chemistry 2000, 275(50):39458-39465.

Gazzaniga, P., Gradilone, A., Silvestri, I., Gandini, O,, Guiliani, L., Vincenzoni, A., Gallucci, M., Fratti, L., and Agliano, A.M. Variable levels of bcl-2, bcl-x and bax mRNA in bladder cancer progression. Oncology Report 1998, 5(4):901-904.

Giannattasio, A., Angeletti, G., De Rosa, M., Zarrilli, S., Ambrosino, M., Cimmino, A., Coppola, C., Panza, G., Calafiore, R., Colao, A., Abete, O., and Lombardi, G. RNA expression bcl-w, a new related protein Bcl-2 family, and caspase-3 in isolated sertoli cells from pre-pubertal rat testes. Journal of Endocrinology Investigation 2002, 25(7):RC23-25.

Gibson, L, Holmgreen, S.P., Huang, D.C., Bernard, O., Copeland, N.G., Jenkins, N.A., Sutherland, G.R., Baker, E., Adams, J.M., and Cory, S. Bcl-w, a novel member of the bcl-2 family, promotes cell survival. Oncogene 1996, 13(4):665-675.

Gonzalez-Garcia, M., Perez-Ballestero, R., Ding, L., Duan, L., Boise, L.H., Thompson, C.B., and Nunez, G. Bcl-XL is the major bcl-x mRNA form expressed during murine development and its product localizes to mitochondria. Development 1994, 120(10):3033-3042.

Graidist, P., Phongdara, A., and Fujise, K. Antiapoptotic protein partners fortilin and MCL1 independently protect cells from 5-FU-induced cytotoxicity. Journal of Biological Chemistry 2004, E-publication ahead of print.

Graninger, W.B., Seto, M., Boutain, B., Goldman, P., and Korsmeyer, S.J. Expression of Bcl-2 and Bcl-2-Ig fusion transcripts in normal and neoplastic cells. Journal of Clinical Investigation 1987, 80(5):1512-1515.

Green, D.R. Overview: apoptotic signaling pathways in the immune system. Immunological reviews 2003, 193:5-9.

Gonzalez, J., Orlofsky, A., and Prystowsky, M.B. A1 is a growth-permissive antiapoptotic factor mediating postactivation survival in T cells. Blood 2003, 101(7):2679-2685.

Grumont, R.J., Rourke, I.J., and Gerondakis, S. Rel-dependent induction of A1 transcription is required to protect B cells from antigen receptor-ligation induced apoptosis. Genes and Development 1999, 13(4):400-411.

Hamasaki, A., Sendo, F., Nakayama, K., Ishida, N., Negishi, I., Nakayama, K., and Hatakeyama, S. Accelerated Neutrophil Apoptosis in Mice Lacking A1-a, a Subtype of the bcl-2-related A1 Gene. Journal of Experimental Medicine 1998, 188(11):1985-1992.

Hamner, S., Skoglosa, Y., and Lindholm, D. Differential expression of bcl-w and bcl-x messenger RNA in the developing and adult rat nervous system. Neuroscience 1999, 91(2):673-684.

Hausmann, G., O'Reilly, L.A., van Driel, R., Beaumont, J.G., Strasser, A., Adams, J.M., and Huang, D.C.S. Pro-apoptotic apoptosis protease-activating Factor 1 (Apaf-1) has a cytoplasmic localization distinct from Bcl-2 of Bcl-xL. Journal of Cell Biology 2000, 149(3):623-634.

Hengartner, M.O. The biochemistry of apopsosis. Nature 2000, 407(6805):770-776.

Hengartner, M.O. and Horvitz, H.R. Activation of C. elegans cell death protein CED-9 by an amino-acid substitution in a domain conserved in Bcl-2. Nature 369(6478):318-320.

Hengartner, M.O. and Horvitz, H.R. C. elegans cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. Cell 1994, 76(4):665-676.

Hendhall, D.C., Skradski, S.L., Lan, J.Q., Ren, T., and Simon, R.P. Increased Bcl-w expression following focally evoked limbic seizures in the rat. Neuroscience Letters 2001, 305(3):153-156.

Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R.D., and Korsmeyer, S.J. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 1990, 348(6299):334-336.

Holmgreen, S.P., Huang, D.C., Adams, J.M., and Cory, S. Survival activity of Bcl-2 homologs Bcl-w and A1 only partially correlates with their ability to bind pro-apoptotic family members. Cell Death and Differentiation 1999, 6(6):525-532.

Jacobson, M.D., Burne, J.F., King, M.P., Miyashita, T., Reed, J.C., and Raff, M.C. Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA. Nature 1993, 361(6410):365-369.

Ji, L., Mochon, E., Arcinas, M., and Boxer, L.M. CREB proteins function as positive regulators of the translocated bcl-2 allele in t(14;18) lymphomas. Journal of Biological Chemistry 1996, 271(37):22687-22691.

Katoh, O., Takahashi, T., Oguri, T., Kuramoto, K., Mihara, K., Kobayashi, M., Hirata, S., and Watanabe, H. Vascular endothelial growth factor inhibits apoptotic death in hematopoietic cells after exposure to chemotherapeutic drugs by inducing MCL1 acting as an antiapoptotic factor. Cancer Research 1998, 58(23):5565-5569.

Kaufman, T., Schnizel, A., and Borner, C. Bcl-w(edding) with mitochondria. Trends in Cell Biology 2004, 14(1):8-12.

Ke, N., Godzik, A., and Reed, J.C. Bcl-B, a novel Bcl-2 family member that differentially binds and regulates Bax and Bak. Journal of Biological Chemistry 2001, 276(16):12481-12484.

Kelekar, A., Chang, B.S., Harlan, J.E., Fesik, S.W., and Thompson, C.B. Bad is a BH3 domain-containing protein that forms an inactivating dimmer with Bcl-XL. Molecular and Cellular Biology 1997, 17(12):7040-7046.

Kerr, J.F.R., Wyllie, A.H., and Currie, A.R. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. British Journal of Cancer 1972, 26(4):239-257.

Kischkel, F.C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P.H., and Peter, M.E. Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex with the receptor. EMBO Journal 1995, 14(22):5579-5588.

Kozopas, K.M., Yang, T., Buchan, H.L., Zhou, R., and Craig, R.W. MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. Proceedings of the National Academy of Sciences USA 1993, 90(8):3516-3520.

Lee, H.W., Lee, S.S., Lee, S.J., and Um, H.D. Bcl-w is expressed in a majority of infiltrative gastric adenocarcinomas and suppresses the cancer cell death by blocking stress-activated protein kinase/c-Jun NH2-terminal kinase activation. Cancer Research 2003, 63(5):1093-1100.

Leverrier, Y., Thomas, J., Mathieu, A.L., Low, W., Blanquier, B., and Marvel, J. Role of PI3-kinase in Bcl-X induction and apoptosis inhibition mediated by IL-3 or IGF-1 in Baf-3 cells. Cell Death and Differentiation 1999, 6(3):290-296.

Limana, F., Urbanek, K., Chimenti, S., Quaini, F., Leri, A., Kajstura, J., Nadal-Ginard, B., Izumo, S., and Anversa, P. Bcl-2 overexpression promotes myocyte proliferation. Proceedings of the National Academy of Sciences USA 2002, 99(9):6257-6262.

Lin, E.Y., Orlofsky, A., Berger, M.S., and Prystowsky, M.B. Characterization of A1, a novel hemopoietic-specific early-response gene with sequence similarity to bcl-2. Journal of Immunology 1993, 151(4):1979-1988.

Lin, E.Y., Orlofsky, A., Wang, H.G., Reed, J.C., and Prystowsky, M.B. A1, a Bcl-2 family member, prolongs cell survival and permits myeloid differentiation. Blood 1996, 87(3):983-992.

Liu, Y., Hernandez, A.M., Shibata, D., and Cortopassi, G.A. BCL2 translocation frequency rises with age in humans. Proceedings of the National Academy of Sciences USA 1994, 91(19):8910-8914.

Marsden, V.S., O'Connor, L., O'Reilly, L.A., Silke, J., Metcalf, D., Ekert, D., Huang, D.C., Cecconi, F., Kuida, K., Tomaselli, K.J., Roy, S., Nicholson, D.W., Vaux, D.L., Bouillet, P., Adams, J.M., and Strasser, A. Apoptosis initiated by Bcl-2-regulated caspase activation independently of the cytochrome c/Apaf-1/caspase-9 apoptosome. Nature 2002, 419(6907):634-637.

Marsden, V.S. and Strasser, A. Control of Apoptosis in the Immune System: Bcl-2, BH3-Only Proteins and More. Annual Review of Immunology 2003, 21:71-105.

Martinou, J.C., Dubois-Dauphin, M., Staple, J.K., Rodriguez, I., Frankowski, H., Missotten, M., Albertini, P., Talabot, D., Catsicas, S., Pietra, C., *et al.* Overexpression of BCL-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. Nature 1994, 13(4):1017-1030.

McDonnell, T.J., Deane, N., Platt, F.M., Nunez, G., Jaeger, U., McKearn, J.P., and Korsmeyer, S.J. Bcl-2-immunoglobin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. Cell 1989, 57(1):79-88.

Meehan, T., Loveland, K.L., de Kretser, D., Cory, S., and Print, C.G. Developmental regulation of the bcl-2 family during spermatogenesis: Insights into the sterility of bcl-w -/- male mice. Cell Death and Differentiation 2001, 8(3):225-233.

Mercatante, D.R., Bortner, C.D., Cidlowski, J.A., and Kole, R. Modification of alternative splicing of Bcl-x pre-mRNA in prostate and breast cancer cells; analysis of apoptosis and cell death. Journal of Biological Chemistry 2001, 276(19):16411-16417.

Middleton, G., Wyatt, S., Ninkina, N., and Davies, A.M. Reciprocal developmental changes in the roles of Bcl-w and Bcl-x(L) in regulating sensory neuron survival. Development 2001, 128(3):447-457.

Migheli, A., Cavalla, P., Piva, R., Giordana, M.T., and Schiffer, D. Bcl-2 protein expression in aged brain and neurodegenerative diseases. Neuroreport 1994, 5(15):1906-1908.

Minami, M., Jin, K.L., Li, W., Nagayama, T., Henshall, D.C., and Simon, R.P. Bcl-w expression is increased in brain regions affected by focal cerebral ischemia in the rat. Neuroscience Letters 2000, 279(3):193-195.

Mori, M., Burgess, D.L., Gefrides, L.A., Foreman, P.J., Opferman, J.T., Korsmeyer, S.J., Cavalheiro, E.A., Naffah-Mazzacoratti, M.G., and Noebels, J.L. Cell Death and Differentiation 2004, E-publication ahead of print.

Nakayama, K., Nakayama, K., Negishi, I., Kuida, K., Sawa, H., and Loh, D.Y. Targeted disruption of Bcl-2 alpha beta in mice: occurrence of gray hair, polycystic kidney disease, and lymphocytopenia. Proceedings of the National Academy of Sciences USA 1994, 91(9):3700-3704.

Nunez, G., Hockenbery, D., McDonnell, T.J., Sorensen, C.M., and Korsmeyer, S.J. Bcl-2 maintains B cell memory. Nature 1991. 353(6339):71-73.

Nunez, G., Seto, M., Seremetis, S., Ferrero, D., Grignani, F., Korsmeyer, S.J., and Dalla-Favera, R. Growth- and tumor-promoting effects of deregulated BCL2 in human B-lymphoblastoid cells. Proceedings of the National Academy of Sciences USA 1989, 86(12):4589-4593.

Okita, H., Umezawa, A., Suzuki, A., and Hata, J. Up-regulated expression of murine Mcl1/EAT, a bcl-2 related gene, in the early stage of differentiation of murine embryonal carcinoma cells and embryonic stem cells. Biochemistry and Biophysics Acta 1998, 1398(3):335-341.

O'Reilly, L.A., Print, C., Hausmann, G., Moriishi, K., Cory, S., Huang, D.C., and Strasser, A. Tissue expression and subcellular localization of the pro-survival molecule Bcl-w. Cell Death and Differentiation 2001, 8(5):486-494.

Orlofsky, A., Somogyi, R.D., Weiss, L.M., and Prystowsky, M.B. The Murine Antiapoptotic Protein A1 Is Induced in Inflammatory Macrophages and Constitutively Expressed in Neutrophils. The Journal of Immunology 1999, 163:412-419.

Orlofsky, A., Weiss, L.M., Kawachi, N., and Prystowsky, M.B. Deficiency in the Anti-Apoptotic Protein A1-a Results in a Diminished Acute Inflammatory Response. The Journal of Immunology 2002, 168:1840-1846.

Packham, G., White, E.L., Eischen, C.M., Yang, H., Parganas, E., Ihle, J.N., Grillot, D.A., Zambetti, G.P., Nunez, G., and Cleveland, J.L. Selective regulation of Bcl-XL by a Jak kinase-dependent pathway is bypassed in murine haematopoietic malignancies. Genes and Development 1998, 12(16):2475-2487.

Parrizas, M. and LeRoith, D. Insulin-like growth factor-1 inhibition of apoptosis is associated with increased expression of the bcl-xL gene product. Endocrinology 1997, 138(3):1355-1358.

Print, C.G., Loveland, K.L., Gibson, L., Meehan, T., Stylianou, A., Wreford, N., de Kretser, D., Metcalf, D., Kontgen, F., Adams, J.M., and Cory, S. Apoptosis

regulator bcl-w is essential for spermatogenesis but appears otherwise redundant. Proceedings of the National Academy of Sciences 1998, 95(21):12424-12431.

Pritchard, D.M., Print, C., O'Reilly, L., Adams, J.M., Potten, C.S., and Hickman, J.A. Bcl-w is an important determinant of damage-induced apoptosis in epithelia of small and large intestine. Oncogene 2000, 19(34):3955-3959.

Raghavan, S.C., Swanson, P.C., Wu, X., Hsieh, C.L., and Lieber, M.R. A non-B-DNA structure at the Bcl-2 major breakpoint region is cleaved by the RAG complex. Nature 2004, 428(6978):88-93.

Reed, J.C., Cuddy, M., Slabiak, T., Croce, C.M., and Nowell, P.C. Oncogenic potential of bcl-2 demonstrated by gene transfer. Nature 1988, 336(6196):259-261.

Ross, A.J., Waymire, K.G., Moss, J.E., Parlow, A.F., Skinner, M.F., Russell, L.D., and MacGregor, G.R. Testicular degeneration in Bclw-deficient mice. Nature Genetics 18(3):251-256.

Sagot, Y., Dubois-Dauphin, M., Tan, S.A., de Bilbao, F., Aebischer, P., Martinou, J.C., and Kato, A.C. Bcl-2 overexpression prevents motoneurons cell body loss but not axonal degeneration in a mouse model of a neurodegenerative disease. Journal of Neuroscience 1995, 15(11):7727-7733.

Sentman, C.L., Shutter, J.R., Hockenbery, D., Kanagawa, O., and Korsmeyer, S.J. Bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. Cell 1991, 67(5):879-888.

Shimizu, S., Narita, M., and Tsujimoto, Y. Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature 1999, 399(6735):483-487.

Shiraiwa, N., Inohara, N., Okada, S., Yuzaki, M., Shoji, S., and Ohta, S. An additional form of rat Bcl-x, Bcl-xbeta, generated by an unspliced RNA, promotes apoptosis in promyeloid cells. Journal of Biological chemistry 1996, 271(22):13258-13265.

Silva, M., Grillot, D., Benito, A., Richard, C., Nunez, G., and Fernandez-Luna, J.L. Erythropoietin can promote erythroid progenitor survival by repressing apoptosis through Bcl-XL and Bcl-2. Blood 1996, 88(5):1576-1582.

Silva, M., Richard, C., Benito, A., Sanz, C., Olalla, I., and Fernandez-Luna, J.L. Expression of Bcl-x in erythroid precursors from patients with polycythemia vera. New England Journal of Medicine 1998, 338(9):564-571.

Sorenson, C.M. Bcl-2 family members and disease. Biochimica et Biophysica Acta (BBA) – Molecular Cell Research 2004, 1644(2-3):169-177.

Stern, L.E., Falcone, R.A. Jr., Huang, F., Kemp, C.J., Erwin, C.R., and Warner, B.W. Epidermal growth factor alters the bax:bcl-w ratio following massive small bowel resection. Journal of Surgery Research 2000, 91(1):38-42.

Stern, L.E., Falcone, R.A., Kemp, C.J., Stuart, L.A., Erwin, C.R., and Warner, B.W. Effect of massive small bowel resection on the Bax/Bcl-w ratio and enterocyte apopsosis. Journal of Gastrointestinal Surgery 2000, 4(1):93-100.

Strasser, A., Harris, A.W., and Cory, S. Bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship. Cell 1991, 67(5):889-899.

Stroka, D.M., Badrichani, A.Z., Bach, F.H., and Ferran, C. Overexpression of A1, an NF-kB-Inducible Anti-Apoptotic Bcl Gene, Inhibits Endothelial Cell Activation. Blood 1999, 93(11):3803-3810.

Sun, Y., Jin, K., Clark, K.R., Peel, A., Mao, X.O., Chang, Q., Simon, R.P., and Greenberg, D.A. Adeno-associated virus-mediated delivery of BCL-w gene improves outcome after transient focal ischemia. Gene Therapy 2003, 10(2):115-122.

Taylor, J.K., Zhang, Q.Q., Wyatt, J.R., and Dean, N.M. Induction of endogenous Bcl-xS through the control of Bcl-x pre-mRNA splicing by antisense oligonucleotides. Nature Biotechnology 1999, 17(11):1097-1100.

Thornberry, N.A. and Lazebnik, Y. Caspases: enemies within. Science 1998, 281(5381):1312-1316.

Tomayko, M.M. and Cancro, M.P. Long-Lived B Cells Are Distinguished by Elevated Expression of A1. The Journal of Immunology 1998, 160:107-111.

Tomayko, M.M., Punt, J.A., Bolcavage, J.M., Levy, S.L., Allman, D.M., and Cancro, M.P. Expression of the Bcl-2 family member A1 is developmentally regulated in T cells. International Immunology 1999, 11(11):1753-1761.

Townsend, K.J., Trusty, J.L., Traupman, M.A., Eastman, A., and Craig, R.W. Expression of the antiapoptotic MCL1 gene product is regulated by a mitogen activated protein kinase-mediated pathway triggered through microtubule disruption and protein kinase C. Oncogene 1998, 17(10):1223-1234.

Townsend, K.J., Zhou, P., Qian, L., Bieszczad, C.K., Lowrey, C.H., Yen, A., and Craig, R.W. Regulation of MCL1 through a serum response factor/Elk-1-mediated mechanism links expression of a viability-promoting member of the BCL2 family to the induction of hematopoietic cell differentiation. Journal of Biological Chemistry 1999, 274(3):1801-1813.

Tsujimoto, Y. Overexpression of the human BCL-2 gene product results in growth enhancement of Epstein-Barr virus-immortalized B cells. Proceedings of the National Academy of Sciences USA 1989, 86(6):1958-1962.

Tsujimoto, Y., Cossman, J., Jaffe, E., and Croce, C.M. Involvement of the bcl-2 gene in human follicular lymphoma. Science 1985, 228(4706):1440-1403.

Tsujimoto, Y. and Croce, C.M. Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. Proceedings of the National Academy of Sciences USA 1986, 83(14):5214-5218.

Tsujimoto, Y., Gorham, J., Cossman, J., Jaffe, E., and Croce, C.M. The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. Science 1985, 229(4720):1390-1393.

Umezawa, A., Maruyama, T., Inazawa, J., Imai, S., Takano, T., and Hata, J. Cell Structure and Function 1996, 21(2):143-150.

Vander Heiden, M.G., Chandel, N.S., Williamson, E.K., Schumacker, P.T., and Thompson, C.B. Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. Cell 1997, 91(5):627-637.

Vaux, D.L., Cory, S., and Adams, J.M. Bcl-2 gene promotes haematopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature 1988, 335(6189):440-442.

Vaux, D.L. and Strasser, A. The molecular biology of apoptosis. Proceedings of the National Academy of Sciences USA 1996, 93(6):2239-2244.

Vrana, J.A., Bieszczad, C.K., Cleaveland, E.S., Ma, Y., Park, J.P., Mohandas, T.K., and Craig, R.W. An MCL1-overexpressing Burkitt lymphoma subline exhibits enhanced survival on exposure to serum deprivation, topoisomerase inhibitors, or staurosporine but remains sensitive to 1-beta-D-arabinofuranosylcytosine. Cancer Research 2002, 62(3):892-900.

Wang, H.G., Rapp, U.R., and Reed, J.C. Bcl-2 targets the protein kinase Raf-1 to mitochondria. Cell 1996, 87(4):629-638.

Wen, R., Chen, Y., Xue, L., Schuman, J., Yang, S., Morris, S.W., and Wang, D. Phospholipase Cg2 Provides Survival Signals via Bcl2 and A1 in Different Subpopulations of B Cells. Journal of Biological Chemistry 2003, 278(44):43654-43662.

Wilson-Annan, J., O'Reilly, L.A., Crawford, S.A., Hausmann, G., Beaumont, J.G., Parma, L.P., Chen, L., Lackmann, M., Lithgow, T., Hinds, M.G., Day, C.L., Adams, J.M., and Huang, D.C. Proapoptotic BH3-only proteins trigger

membrane integration of prosurvival Bcl-w and neutralize its activity. Journal of Cell Biology 2003, 162(5):877-887.

Wilson, J.W., Nostro, M.C., Balzi, M., Faraoni, P., Cianchi, F., Becciolini, A., and Potten, C.S. British Journal of Cancer 2000, 82(1):178-185.

Wyllie, A.H., Kerr, J.F.R., and Currie, A.R. Cell death: the significance of apoptosis. International Reviews in Cytology 1980, 68:251-306.

Xerri, L., Parc, P., Brousset, P., Schlaifer, D., Hassoun, J., Reed, J.C., Krajewski, S., Birnbaum, D. Predominant expression of the long isoforms of Bcl-x (Bcl-xL) in human lymphomas. British Journal of Haematology 1996, 92(4):900-906.

Xiang, Z., Ahmed, A.A., Moller, C., Nakayama, K., Hatakeyama, S., and Nilsson, G. Essential Role of the Prosurvival bcl-2 Homologue A1 in Mast Cell Survival After Allergic Activation. The Journal of Experimental Medicine 2001, 194(11):1561-1570.

Yan, C., Chen, J., Chen, D., Minami, M., Pei, W., Yin, X.M., and Simon, R.P. Overexpression of the cell death suppressor Bcl-w in ischemic brain: implications for a neuroprotective role via the mitochondrial pathway. Journal of Cerebral Blood Flow Metabolism 2000, 20(3):620-630.

Yan, W., Huang, J.X., Lax, A.S., Pelliniemi, L., Salminen, E., Poutanen, M., and Toppari, J. Overexpression of Bcl-W in the testis disrupts spermatogenesis: revelation of a role of BCL-W in male germ cell cycle control. Molecular Endocrinology 2003, 17(9):1868-1879.

Yan, W., Samson, M., Jegou, B., and Toppari, J. Bcl-w forms complexes with Bax and Bak, and elevated ratios of Bax/Bcl-w and Bak/Bcl-w correspond to spermatogonial and spermatocyte apoptosis in the testis. Molecular Endocrinology 2000, 14(5):682-699.

Yang, L., Zhang, B., Toku, K., Maeda, N., Sakanaka, M., and Tanaka, J. Improvement of the viability of cultured rat neurons by the non-essential amino acids L-serine and glycine that upregulates expression of the anti-apoptotic gene product Bcl-w. Neuroscience Letters 2000, 295(3):97-100.

Yang, T., Buchan, H.L., Townsend, K.J., and Craig, R.W. MCL-1, a member of the BCL-2 family, is induced rapidly in response to signals for cell differentiation or death, but not to signals for cell proliferation. Journal of Cellular Physiology 1996, 166(3):523-536.

Yang, X.F., Weber, G.F., and Cantor, H. A novel Bcl-x isoform connected to the T cell receptor regulates apoptosis in T cells. Immunity 1997, 7(5)629-639.

Yunis, J.J., Mayer, M.C., Arnesen, M.A., Aeppli, D.P., Oken, M.M., and Frizzera, G. Bcl-2 and other genomic alterations in the prognosis of large-cell lymphoma. New England Journal of Medicine 1989, 320(16):1047-1054.

Zhai, D., Ke, N., Zhang, H., Ladror, U., Joseph, M., Eichinger, A., Godzik, A., Ng, S.C., and Reed, J.C. Characterization of the anti-apoptotic mechanism of Bcl-B. Biochemistry Journal 2003, 376(1):229-236.

Zhan, Q., Bieszczad, C.K., Bae, I., Fornace, A.J. Jr., and Craig, R.W. Induction of BCL2 family member MCL1 as an early response to DNA damage. Oncogene 1997, 14(9):1031-1039.

Zhang, D., Li, F., Weidner, D., Mnjoyan, Z.H., and Fujise, K. Physical and functional interaction between myeloid cell leukemia 1 protein (MCL1) and Fortilin. The potential role of MCL1 as a fortilin chaperone. Journal of Biological Chemistry 2002, 277(40):37430-37438.

Zhang, L., Nair, A., Krady, K., Corpe, C., Bonneau, R.H., Simpson, I.A., and Vannucci, S.J. Estrogen stimulates microglia and brain recovery from hypoxia-ischemia in normoglycemic but not diabetic female mice. Journal of Clinical Investigation 2004, 113:85-95.

Zhou, P., Levy, N.B., Xie, H., Qian, L., Lee, C.Y., Gascoyne, R.D., and Craig, R.W. MCL1 transgenic mice exhibit a high incidence of B-cell lymphoma manifested as a spectrum of histologic subtypes. Blood 2001, 97(12):3902-3909.

Zhu, X., Wang, Y., Ogawa, O., Lee, H.G., Raina, A.K., Siedlak, S.L., Harris, P.L., Fujioka, H., Shimohama, S., Tabaton, M., Atwood, C.S., Petersen, R.B., Perry, G., and Smith, M.A. Neuroprotective properties of Bcl-w in Alzheimer disease. Journal of Neurochemistry 2004, 89(5):1233-1240.