Colloquium series on Building Blocks of the Cell: Cell Structure and Function

Series Editor: Ivan Robert Nabi

Apoptosis

Ning Yang
Ing Swie Goping

MORGAN & CLAYPOOL LIFE SCIENCES

Apoptosis

Colloquium
Digital Library of Life Sciences

This e-book is a copyrighted work in the Colloquium Digital Library—an innovative collection of time saving references and tools for researchers and students who want to quickly get up to speed in a new area or fundamental biomedical/life sciences topic. Each PDF e-book in the collection is an in-depth overview of a fast-moving or fundamental area of research, authored by a prominent contributor to the field. We call these e-books *Lectures* because they are intended for a broad, diverse audience of life scientists, in the spirit of a plenary lecture delivered by a keynote speaker or visiting professor. Individual e-books are published as contributions to a particular thematic **series**, each covering a different subject area and managed by its own prestigious editor, who oversees topic and author selection as well as scientific review. Readers are invited to see highlights of fields other than their own, keep up with advances in various disciplines, and refresh their understanding of core concepts in cell & molecular biology.

For the full list of published and forthcoming Lectures, please visit the Colloquium homepage:
www.morganclaypool.com/page/lifesci

Access to the Colloquium Digital Library is available by institutional license. Please e-mail info@morganclaypool.com for more information.

Colloquium Series on Building Blocks of the Cell: Cell Structure and Function

Editor

Ivan Robert Nabi, *Professor, University of British Columbia, Department of Cellular and Physiological Sciences*

This Series is a comprehensive, in-depth review of the key elements of cell biology including 14 different categories, such as Organelles, Signaling, and Adhesion. All important elements and interactions of the cell will be covered, giving the reader a comprehensive, accessible, authoritative overview of cell biology. All authors are internationally renowned experts in their area.

Published titles

(for future titles please see the website, www.morganclaypool.com/page/lifesci)

Apoptosis
Ning Yang and Ing Swie Goping
www.morganclaypool.com

ISBN: 9781615045389 paperback

ISBN: 9781615045396 ebook

DOI: 10.4199/C00080ED1V01Y201303BBC003

A Publication in the

COLLOQUIUM SERIES ON BUILDING BLOCKS OF THE CELL

Lecture #3

Series Editor: Ivan Robert Nabi, University of British Columbia, Department of Cellular and Physiological Sciences

Series ISSN:

ISSN 2328-3041 print

ISSN 2328-305X electronic

Apoptosis

Ning Yang and Ing Swie Goping
Department of Biochemistry,
University of Alberta

COLLOQUIUM SERIES ON BUILDING BLOCKS OF THE CELL #3

MORGAN & CLAYPOOL LIFE SCIENCES

ABSTRACT

Multi-cellular organisms eliminate individual cells through a self-destruct process known as apoptosis. Apoptosis is critical for proper development and maintenance of tissue homeostasis. The importance of this process is highlighted by the fact that too much or too little apoptosis is the underlying cause of pathologies such as cancer, autoimmune diseases (e.g., lupus, arthritis), and neurodegenerative disorders (e.g., Parkinson's, Alzheimer's). In the early days, apoptotic cells were identified strictly by cell morphology. Now we know that biochemical signatures define a number of death programs, of which apoptosis is the most widely understood. In this review, we discuss genetic insights gained from *C. elegans*, the importance of caspases, engulfment of apoptotic cells, apoptotic signals, the role of mitochondria, the Bcl-2 family, and the link between dysfunctional apoptosis and disease. Within each topic, we highlight landmark studies that contributed to our current understanding of apoptosis. All together, this research exemplifies tremendous scientific synergy between the disciplines of genetics, biochemistry, developmental cell biology, and structural biology. Continued exploration into mechanisms that regulate apoptosis will undoubtedly lead to insights into disease processes with potential therapeutic strategies.

KEYWORDS

apoptosis, cell death, caspases, engulfment, apoptotic signals, Bcl-2, dysfunctional apoptosis

Contents

CHAPTER 1

Historical Perspective of Apoptosis

1.1 NATURAL CELL DEATH IN THE 19TH CENTURY

Research into cell death exploded near the end of the 20th century, leading to a general misconception that the study of cellular suicide is a relatively new field. In reality, naturally occurring cell death was described over 150 years earlier and appears to have been forgotten and then re-discovered multiple times. The early history of cell death is discussed in excellent reviews by Clarke and Clarke (1996) and Majno and Joris (1995). According to Clarke and Clarke (1996), cell death studies progressed slowly in part because authors did not routinely recognize early seminal works of others and that cell death in itself was not considered to be an interesting scientific process. Around that time, scientists were more intrigued with how cells contributed to growth as opposed to cell death. Notwithstanding, a number of key reports outline morphological descriptions of dying cells and some postulate on how this process is regulated. With modern day molecular biology and genetics, many of these centuries-old predictions have turned out to be true.

As described by Clarke and Clarke (1996), by the 19th century, it was already understood that in some instances, specific structures would form during development but would not be found in the mature organism. For example, it was known in the 1600s that in the fetus, a temporary vessel (ductus arteriosus) allowed most of the blood from the right ventricle to bypass the non-functioning lung. This fetal blood vessel regressed after birth (Harvey, 1628). In 1835, more obvious examples of structural regression were reported in organisms that underwent metamorphosis, such as the transition from tadpole to adult toads with the accompanying loss of tadpole gills and tails (Dugès, 1835). It was not until the breakthrough proposition of the cell theory in 1839 (Schleiden, 1842; Schwann, 1839), when it was suggested that these structural regressions were a result of disappearance of individual cells. In 1842, Carl Vogt published a 130-page report that described cellular changes during cartilage development of the toad (Vogt, 1842). Specifically, Vogt detailed the disappearance of the cells of the notochord and their replacement by cells of the vertebrae. His pioneering scientific contribution was that he equated the well-known loss of specific structures in development to the "destruction," "disappearance," or "resorption" of cells. However, Vogt does not focus or elaborate on the disappearance of cells, and this novel observation was not acknowledged by his peers, which set the stage for "cell death" to be re-discovered.

Twenty years later, August Weismann (Weismann, 1864; Weissman, 1863) outlined regression during the metamorphosis of the pupating fly. He coined the term "histolyse" to describe the morphology of the dying cells and perhaps due to the proposal of new terminology, unlike Vogt's study, Weismann's work was well acknowledged (Clarke and Clarke, 1996). Subsequent scientists adapted his terminology to describe "histolysis" in insects (Viallanes, 1882) and amphibians (Mayer, 1886). An example of cellular changes documented in 1887 by Barfurth (1887) on the regressing tadpole tail is shown in Figure 1, which is taken from Clarke and Clarke (1996). Here the authors illustrated the marked different appearance of dying cells from healthy muscle tissue. The dying cells are labeled as "sp" for "Sarcoplastin"—"a product of degeneration," and "fk" for fatty granules—named because the numerous vacuoles seen in dying cells were assumed to be filled with lipids. Further morphological descriptions included the observation that dying cells were associated with

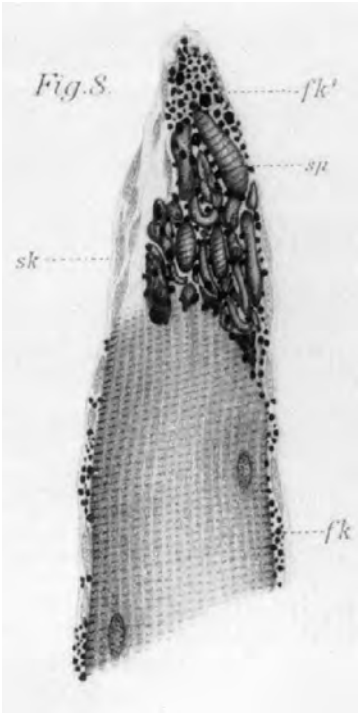


FIGURE 1: Early example of apoptotic cell morphology. Muscle fiber in longitudinal section from the tail of *Rana fusca* on the third day of regression. *sp*, "sarcoplastin" a product of degeneration, *fk*, fatty granules labeled because numerous vacuoles seen in dying cells were assumed to be filled with lipids. *sk*, nuclear proliferation in the perimysium internum, Originally from Barfurth (1887). Taken with permission from Clarke and Clarke (1996).

ameboid phagocytic cells (Kowalevsky, 1887), leading scientists to wonder whether the phagocytes caused histolysis or only acted later to clean up the debris. As fixation, staining, and visualization techniques improved, more detailed descriptions of cell death were reported. In particular in 1885, Flemming (1885) observed that nuclear staining was diminished in dying cells, and he labeled this form of cell death "chromatolytic." It appears now that "chromatolysis" and what later came to be called "apoptosis" are synonymous.

1.2 EXPLOSION OF INTEREST IN CELL DEATH IN THE 20TH CENTURY

By the mid-20th century, developmental biologists had well accepted that cell death was an important process shaping insect metamorphosis (Lockshin, 1997) and had labeled this process as "programmed cell death" (PCD) (Lockshin and Williams, 1965). On the other hand, although similar morphological structures were reported in tissues that were not undergoing metamorphosis (the first example being Vogt's article in 1842), it was difficult for the scientific community to accept that cell death had a role in rapidly growing tissues. In other words, what would be the point of cell death without the regression of some specific structure? Thus, dying cells in normally developing tissues were instead postulated to be metabolic deposits of mitotic cells. In 1951, the developmental biologist, Glücksmann (1951) published a review entitled "Cell deaths in normal vertebrate ontogeny," that systematically summarized the literature of cell death during embryonic development and presented the hypothesis that cell death actively contributed to tissue and organ formation. Years later, influential observations by embryologist Saunders (1966) demonstrated the importance of programmed cell death in normal development. As an example, Saunders proposed that death of tissue sculpted the formation of mature digits, as he contrasted the increased cell death of tissues between the developing digits of the chick with the reduced cell death seen in web-footed ducks (Saunders and Fallon, 1966) (Figure 2). The idea that cell death between insect and vertebrate systems was similar and potentially represented a conserved program was formally discussed by Whitten in an article published in 1969, entitled "Cell death during early morphogenesis: parallels between insect limb and vertebrate limb development" (Whitten, 1969). Clearly, developmental biologists had firmly recognized the importance of programmed cell death in morphogenesis.

Meanwhile, in the related yet separate field of medicine, pathologist John Kerr and colleagues were cataloguing the morphology of a class of cell death that they labeled "shrinkage necrosis." Based on ultrastructural features, this form of cell death was reported in both normal tissue and pathological specimens. Kerr and colleagues observed shrinkage necrosis in normal embryonic and adult tissue (Kerr, 1965; Saunders, 1966), in neoplastic tissues (Kerr and Searle, 1972a; Kerr and Searle, 1972b), and in organs after injury or withdrawal of hormone (Kerr et al., 1972). In

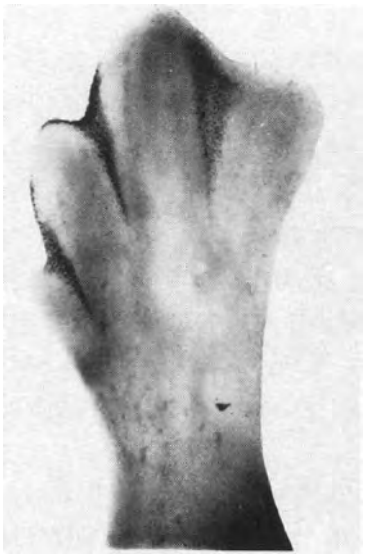
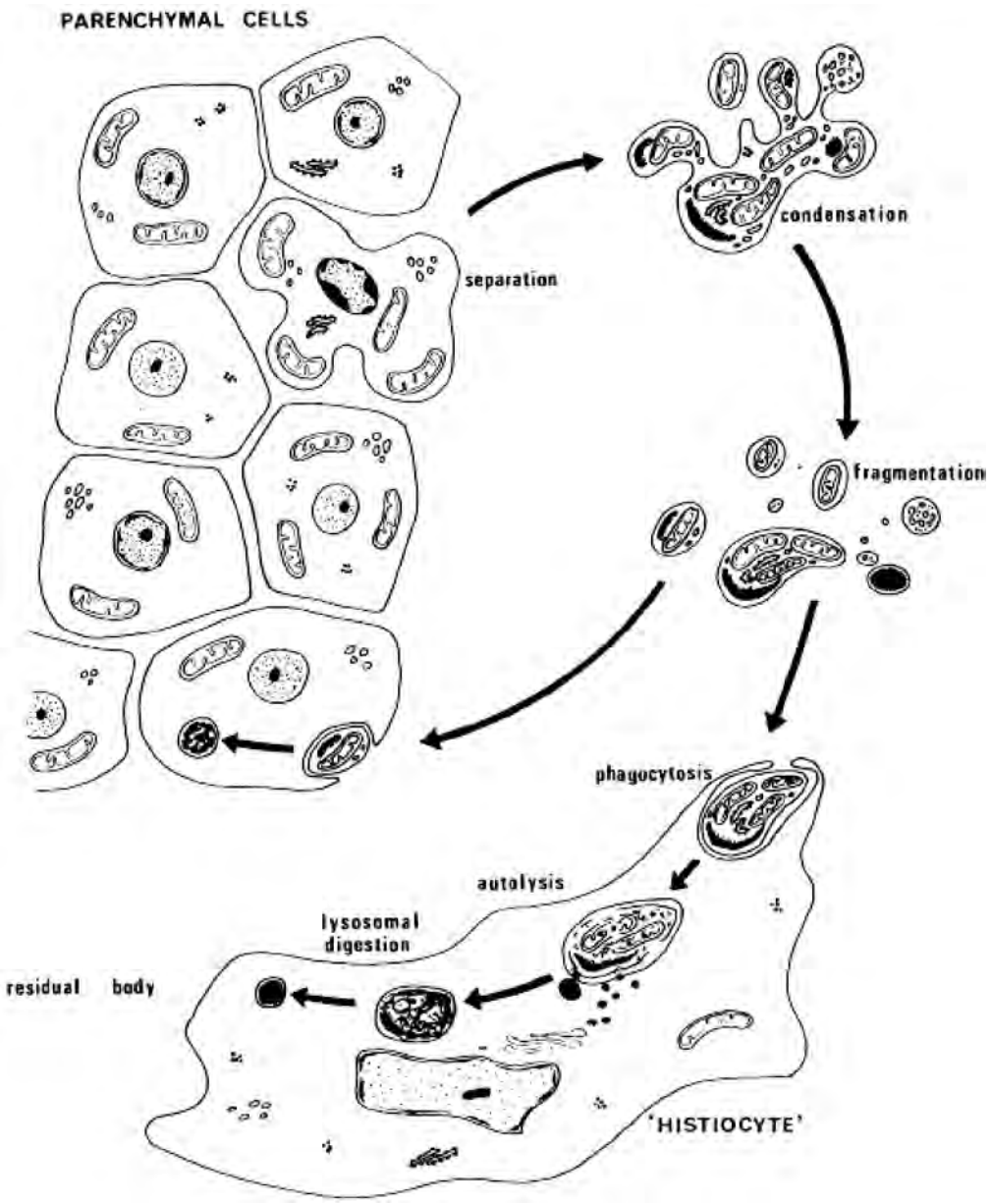


FIGURE 2: Early example of programmed cell death in the leg bud of a chick embryo. A classical picture of programmed cell death in the leg bud of a chick embryo, stained in vivo with Nile red (which becomes concentrated in phagosomes). The dark interdigital zones represent myriads of red-stained macrophages scavenging the debris of cells that died on schedule. Originally from Saunders and Fallon (1966). Legend and figure taken with permission from Majno and Joris (1995).

1972, John Kerr, Andrew Wyllie, and Alistair Currie published a milestone article whereby they concluded that this type of cell death was the same as the embryological cell death summarized in Alfred Glucksmann's extensive review article (Kerr et al., 1972). Kerr et al. described that apoptosis occurred in two stages comprising nuclear and cytoplasmic condensation and breaking up of cell components into membrane-bound apoptotic bodies, followed by phagocytosis of apoptotic bodies by neighboring cells (Figure 3). These morphological descriptions of cell death were a synopsis of over one hundred years of observations first suggested by Vogt in 1842, with cell death morphol-

FIGURE 3: Two-step model of apoptosis. Apoptosis occurred in two stages comprising nuclear and cytoplasmic condensation and breaking up of cell components into membrane-bound apoptotic bodies, followed by phagocytosis of apoptotic bodies by neighboring cells or circulating histocytes. Of interest, the authors recommended that for the pronunciation of apoptosis, "the stress should be on the penultimate syllable, the second half of the word being pronounced like "ptosis (with the "p" silent)," although this intonation was not accepted by all (Funder, 1994). Taken with permission from Kerr et al. (1972).


PARENCHYMAL CELLS
separation
condensation
fragmentation
phagocytosis
autolysis
lysosomal
digestion
residual body
'HISTIOCYTE'

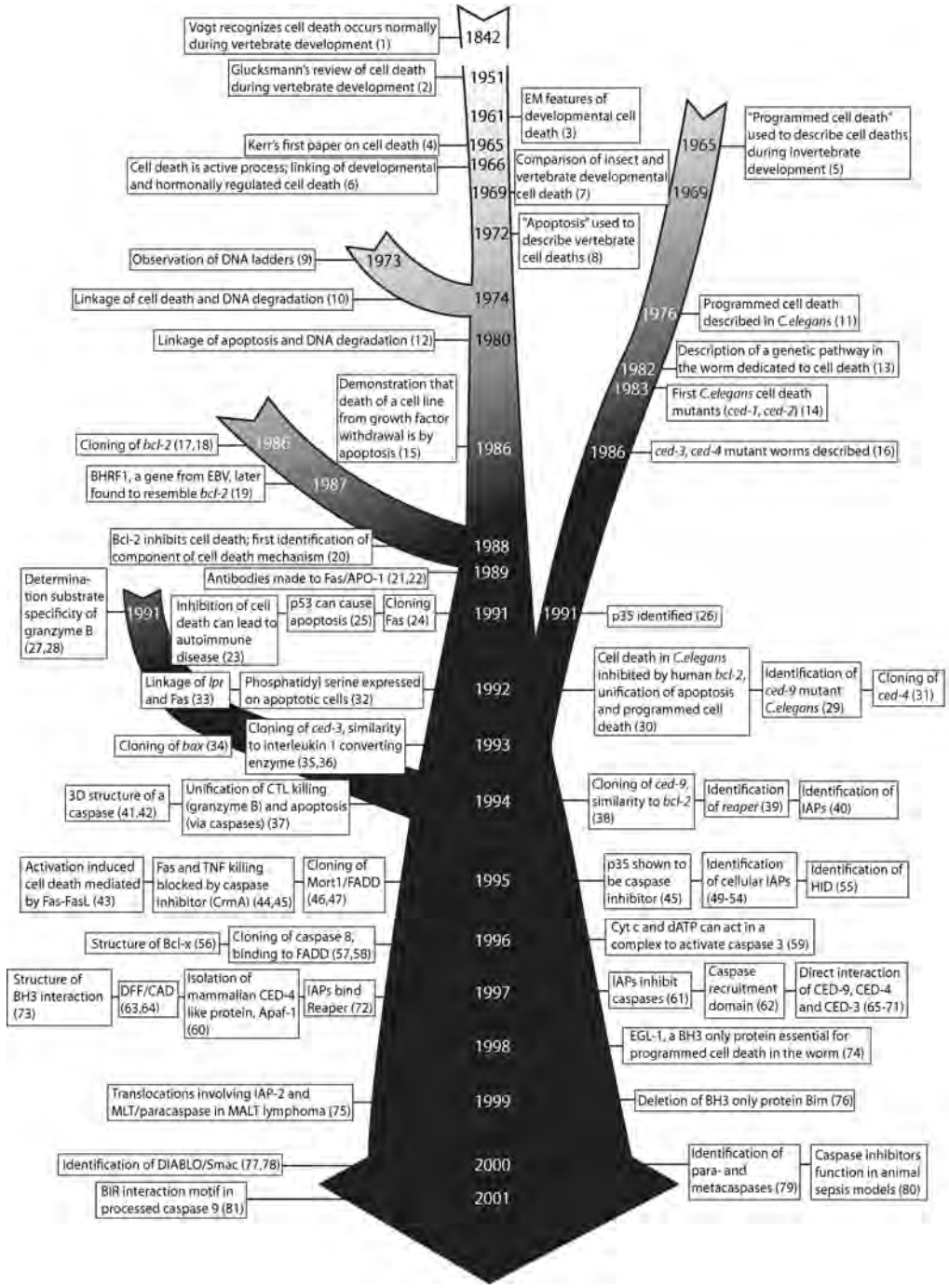

Vogt recognizes cell death occurs normally during vertebrate development (1)
1842
Glucksmann's review of cell death during vertebrate development (2)
1951
EM features of developmental cell death (3)
1961
Kerr's first paper on cell death (4)
1965
Cell death is active process; linking of developmental and hormonally regulated cell death (6)
1966
Comparison of insect and vertebrate developmental cell death (7)
1969
"Programmed cell death" used to describe cell deaths during invertebrate development (5)
1965
1969
"Apoptosis" used to describe vertebrate cell deaths (8)
1972
Observation of DNA ladders (9)
1973
Linkage of cell death and DNA degradation (10)
1974
Programmed cell death described in C.elegans (11)
1976
Linkage of apoptosis and DNA degradation (12)
1980
Description of a genetic pathway in the worm dedicated to cell death (13)
1982
First C.elegans cell death mutants (ced-1, ced-2) (14)
1983
Demonstration that death of a cell line from growth factor withdrawal is by apoptosis (15)
Cloning of bcl-2 (17,18)
1986
1986
ced-3, ced-4 mutant worms described (16)
1986
BHRF1, a gene from EBV, later found to resemble bcl-2 (19)
1987
Bcl-2 inhibits cell death; first identification of component of cell death mechanism (20)
1988
Antibodies made to Fas/APO-1 (21,22)
1989
Determination substrate specificity of granzyme B (27,28)
1991
Inhibition of cell death can lead to autoimmune disease (23)
p53 can cause apoptosis (25)
Cloning Fas (24)
1991
1991
p35 identified (26)
Linkage of lpr and Fas (33)
Phosphatidyl serine expressed on apoptotic cells (32)
1992
Cell death in C.elegans inhibited by human bcl-2, unification of apoptosis and programmed cell death (30)
Identification of ced-9 mutant C.elegans (29)
Cloning of ced-4 (31)
Cloning of bax (34)
Cloning of ced-3, similarity to interleukin 1 converting enzyme (35,36)
1993
3D structure of a caspase (41,42)
Unification of CTL killing (granzyme B) and apoptosis (via caspases) (37)
1994
Cloning of ced-9, similarity to bcl-2 (38)
Identification of reaper (39)
Identification of IAPs (40)
Activation induced cell death mediated by Fas-FasL (43)
Fas and TNF killing blocked by caspase inhibitor (CrmA) (44,45)
Cloning of Mort1/FADD (46,47)
1995
p35 shown to be caspase inhibitor (45)
Identification of cellular IAPs (49-54)
Identification of HID (55)
Structure of Bcl-x (56)
Cloning of caspase 8, binding to FADD (57,58)
1996
Cyt c and dATP can act in a complex to activate caspase 3 (59)
Structure of BH3 interaction (73)
DFF/CAD (63,64)
Isolation of mammalian CED-4 like protein, Apaf-1 (60)
IAPs bind Reaper (72)
1997
IAPs inhibit caspases (61)
Caspase recruitment domain (62)
Direct interaction of CED-9, CED-4 and CED-3 (65-71)
1998
EGL-1, a BH3 only protein essential for programmed cell death in the worm (74)
Translocations involving IAP-2 and MLT/paracaspase in MALT lymphoma (75)
1999
Deletion of BH3 only protein Bim (76)
Identification of DIABLO/Smac (77,78)
2000
Identification of para- and metacaspases (79)
Caspase inhibitors function in animal sepsis models (80)
BIR interaction motif in processed caspase 9 (81)
2001

FIGURE 4: Apoptotic timeline. Publication timeline of seminal discoveries in the field of apoptosis. At the time when this figure was originally published (Vaux, 2002), David Vaux indicated that over 50,000 papers had been published on apoptosis, and it was only possible to give an indication of some of them in the figure. Many, many important papers had therefore been omitted. In most cases, only the first member of a protein family was mentioned. Numbers in parantheses refer to references cited in original publication (Vaux 2002). Modified figure and modified legend are taken with permission from Vaux (2002).

ogy extensively described by Flemming (1885) and phagocytosis described by Kowalevsky (1887). Importantly, Kerr et al. (1972) discussed that most physiological cell deaths were not accidental, but followed a similar morphological pattern as PCD in development. It should be noted that while morphologically similar, there was still no definitive link between programmed cell death (as first defined in invertebrates) and apoptosis. It would be 20 years until the identification of death genes in the nematode provided the molecular framework with which scientists could functionally define apoptosis.

While many use the terms "apoptosis" and "programmed cell death" interchangeably, this is not entirely correct. PCD refers to all physiological cell deaths that occur in a time- and position-dependent manner during the normal development of an organism. Most cell deaths during PCD have apoptotic features (Majno and Joris, 1995); however, some exceptions exist. For example, programmed cell death in the salivary gland and midgut during *D. melanogaster* development shows morphologies of cytoplasmic vacuolization associated with autophagic and not apoptotic cell death (Berry and Baehrecke, 2007; Denton et al., 2009). As more non-apoptotic forms of cell death are uncovered (Galluzzi et al., 2012), it is perhaps not surprising that developmental programmed cell death is accomplished through distinct cell death mechanisms. Nevertheless, at the time of the Kerr et al.'s publication (Kerr et al., 1972), the most widely reported cell death had shared features of "apoptosis," and the new terminology caught the interest of the scientific community. In retrospect, it is remarkable that the authors' final comments speculating on the existence of apoptotic regulators and the mechanisms of cellular condensation, foreshadow the explosion of discoveries in the field that would occur in the ensuing few decades.

At this point, the morphological descriptions of cell death had led the field as far as it would go. That changed with the identification of the first non-morphological marker of apoptosis. Williams et al. (1974) discovered that mammalian cell death was associated with cleavage of DNA. Endonuclease activity could be detected by electrophoretic separation of DNA into "ladders" characteristic of DNA digestion at internucleosomal sites (Hewish and Burgoyne, 1973). Wyllie identified that these DNA ladders were associated with apoptotic cells (Wyllie, 1980). Importantly, this simple biochemical characterization opened the door for the observation of apoptosis to a larger

scientific community. Apoptosis could now be identified without the need for electron or even light microscopes but by agarose gel electrophoresis—a common tool in many scientific laboratories.

Moreover, in addition to technical advances, increased interest in the field came about with the discovery that apoptosis was an active process, directed by the dying cell. In 1966, Tata analyzed RNA and protein synthesis in regressing tissue. Using organ culture of isolated tadpole tails that regressed in response to triiodothyronine, Tata observed that actinomycin D (RNA transcription inhibitor) and cycloheximide (protein synthesis inhibitor) inhibited tissue regression (Tata, 1966). Years later, these observations were confirmed in rodent thymocytes that underwent apoptosis in response to glucocorticoid treatment and using the same inhibitors, demonstrated that DNA fragmentation was dependent on RNA and protein synthesis (Cohen and Duke, 1984; Wyllie et al., 1984). That both apoptosis and PCD required RNA and protein synthesis (Cohen and Duke, 1984; Tata, 1966; Wyllie et al., 1984) led to the conceptualization of apoptosis as "cellular suicide."

Altogether, these discoveries sparked increased interest in apoptosis. In 2002, David Vaux published a review, which summarizes many of the transformative reports that shaped this field (Vaux, 2002) (Figure 4). From his apoptotic timeline, we can see that multiple factors influenced the progression of apoptotic research. As we have already discussed, initial early observations cemented by morphological definition and adoption of terminology (Kerr et al., 1972) were followed by identification of biochemical features of apoptosis that opened the door for more laboratories to study apoptosis (Wyllie, 1980). Importantly, realization of the conservation of the apoptotic program between humans and tractable model organisms led to the merging of research disciplines of pathology, invertebrate PCD and eventually cancer biology. This melding of fields allowed rapid progress in understanding of the molecular mechanisms of the apoptotic pathway, and revealed how dysfunction led to human diseases.

1.3 DEFINITIONS OF CELL DEATH

Morphological features determined the first accounts of cell death. In general, cell death was first largely classified into either "cell death by suicide" (apoptosis) or "cell death by murder" (accidental cell death—eventually labeled necrotic cell death) (Majno and Joris, 1995). Apoptosis causes cells to shrink and lose contact with neighboring cells. The chromatin condenses and is fragmented between nucleosomes by endonucleases. The nucleus buds off forming multiple membrane-bound fragments. Cellular budding leads to breaking up of the cell into membrane-encapsulated spheres identified as apoptotic bodies (Kerr et al., 1972). Specific membrane signals target apoptotic bodies for engulfment by professional phagocytes or adjacent cells. Figure 5 shows apoptotic bodies engulfed by phagocytes (Jacobson et al., 1997). Due to the encapsulation of cellular components

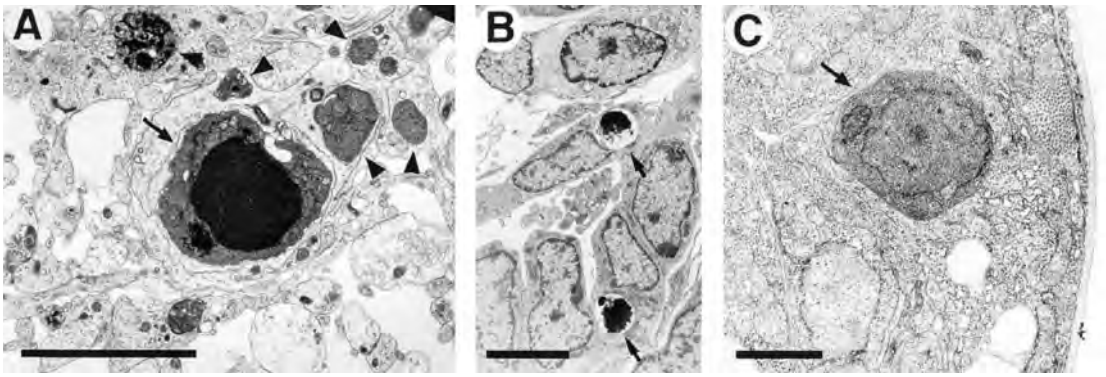


FIGURE 5: Examples of phagocytosed apoptotic cells. Arrows point to dead cells. (A) An early apoptotic cell in the cerebellum of a developing rat (courtesy of J. Burne). Arrowheads point to phagocytosed fragments of apoptotic cells. (B) Two phagocytosed apoptotic cells in a developing rat kidney (Coles et al., 1993). (C) A dead cell in the ventral nerve cord of a developing *C. elegans* embryo (courtesy of E. Hartwieg, G. Stanfield, and H. R. Horvitz). Figure and legend taken with permission from Jacobson et al. (1997).

within membrane-bound vesicles and subsequent engulfment by phagocytic cells, apoptosis occurs without leakage of intracellular components. Moreover, the engulfment of apoptotic cells by phagocytes induces the phagocyte to express and secret anti-inflammatory cytokines such as transforming growth factor β (TGF-β) and IL-10 (Fadok et al., 2001), inhibiting the further recruitment of macrophages. Thus, apoptosis does not induce inflammation and is immunologically silent.

In contrast, necrosis was defined as accidental cell death, wherein cells sustain injury resulting in swelling, rupture of the plasma membrane, and leakage of cell contents (Majno and Joris, 1995). Intracellular molecules, such as HMG protein, uric acid, heat shock proteins, single-stranded RNA, and genomic DNA, that are secreted into the extracellular space are subsequently recognized as "foreign" by immune cells (Oppenheim and Yang, 2005; Scaffidi et al., 2002; Shi et al., 2003). Inflammation occurs as these released molecules bind and stimulate receptors on neutrophils, macrophages, and natural killer cells (Chen et al., 2007; Oppenheim and Yang, 2005). Furthermore, when necrotic cells are engulfed by phagocytic cells, the phagocyte is induced to produce proinflammatory cytokines such as TNFα and IL-8, further amplifying inflammation (Fadok et al., 2001). Events that trigger necrosis, such as burns, cuts, and compression injury, lead to active inflammation, scarring, and tissue damage.

Incredible progress has been made in the biochemical and genetic understanding of cell death (see sections 2–5). Biochemical and genetic measurements of cell death can be quantified and lead to an understanding that depending on the stimulus and cell type, the two classifications of apoptosis versus necrosis was too simple. Leaders in the field of cell death formed the Nomenclature Committee on Cell Death (NCCD) to address confusion in the field related to definitions of cell death and made recommendations in 2005, 2009, and 2012 (Galluzzi et al., 2012; Kroemer et al., 2005; Kroemer et al., 2009). The NCCD stated, "in view of the substantial progress in the biochemical and genetic exploration of cell death, time has come to switch from morphological to molecular definitions of cell death modalities" (Galluzzi et al., 2012). The committee outlines specific biochemical features that define 13 cell death modes (Table 1). This review will discuss cell death that is classified as apoptotic, and the reader is referred to other excellent reviews that elaborate on non-apoptotic modes of cell death (Degterev and Yuan, 2008; Galluzzi et al., 2012).

TABLE 1: Functional classification of regulated cell death modes

	MAIN BIOCHEMICAL FEATURES	CASPASE DEPENDENCE	EXAMPLES OF INHIBITORY INTERVENTIONS[a]
Anoikis	Downregulation of EGFR Inhibition of ERK1 signaling Lack of β1-integrin engagement Overexpression of BIM Caspase-3 (-6,-7) activation	++	BCL-2 overexpression Z-VAD-fmk administration
Authophagic cell death	MAP1LC3 lipidation SQSTM1 degradation	−−	VPS34 inhibitors AMBRA1, ATG5, ATG7, ATG12 or BCN1 genetic inhibition
Caspase-dependent intrinsic apoptosis	MOMP Irreversible $\Delta\psi_m$ dissipation	++	BCL-2 overexpression Z-VAD-fmk administration

TABLE 1 (*continued*)

	MAIN BIOCHEMICAL FEATURES	CASPASE DEPENDENCE	EXAMPLES OF INHIBITORY INTERVENTIONS[a]
Caspase-independent intrinsic apoptosis	Release of IMS proteins Respiratory chain inhibition	−−	BCL-2 overexpression
Cornification	Activation of transglutaminases Caspase-14 activation	+	Genetic inhibition of TG1, TG3 or TG5 Genetic inhibition of caspase-14
Entosis	RHO activation ROCK1 activation	−−	Genetic inhibition of metallothionein 2A Lysosomal inhibitors
Extrinsic apoptosis by death receptors	Death receptor signaling Caspase-8 (-10) activation BID cleavage and MOMP (in type II cells) Caspase-3 (-6,-7) activation	++	CrmA expression Genetic inhibition of caspase (8 end 3) Z-VAD-fmk administration
Extrinsic apoptosis by dependence receptors	Dependence receptor signaling PF2A activation DAFK1 activation Caspase-9 activation Caspase-3 (-6,-7) activation	++	Genetic inhibition of caspases (9 and 3) Genetic inhibition of PP2A Z-VAD-fmk administration
Mitotic catastrophe	Caspase-2 activation (in some instances) TP53 or TP73 activation (in some instances) Mitotic arrest	−−	Genetic inhibition of TP53 (in some instances) Pharmacological or genetic inhibition of caspase-2 (in some instances)

TABLE 1 (*continued*)

	MAIN BIOCHEMICAL FEATURES	CASPASE DEPENDENCE	EXAMPLES OF INHIBITORY INTERVENTIONS[a]
Necroptosis	Death receptor signaling Caspase inhibition RIP1 and/or RIP3 activation	−−	Administration of necrostatin(s) Genetic inhibition of RIP1/RIP3
Netosis	Caspase inhibition NADPH oxidase activation NET release (in some instances)	−−	Autophagy inhibition NADPH oxidase inhibition Genetic inhibition of PAD4
Parthanatos	PARP1-mediated PAR accumulation Irreversible $\Delta\psi_m$ dissipation ATP and NADH depleton PAR binding to AIF and AIF nuclear translocation	−−	Genetic inhibition of AIF Pharmacological or genetic inhibition of PARP1
Pyroptsis	Caspase-1 activation Caspase-7 activation Secretion of IL-1β and IL-18	++	Administration of Z-YVAD-fmk Genetic inhibition of caspase-1

TABLE 1: Cell death classifications. Abbreviations: ATG, autophagy; BCN1, beclin 1; $\Delta\psi_m$, mitochondrial transmembrane potential; CrmA, cytokine response modifier A; DAPK1, death-associated protein kinase 1; EGFR, epidermal growth factor receptor, ERK1, extracellular-regulated kinase 1; IL, interleukin; MAP1LC3, microtubule-associated protein 1 light chain 3; MOMP, mitochondrial outer membrane permeabilization; NET, neutrophil extracellular trap; PAD4, peptidylarginine deiminase 4; PAR, poly(ADP-ribose); PARP1, poly(ADP-ribose) polymerase 1; PP2A, protein phospatase 2A; ROCK1, RHO-associated, coiled-oil containing protein kinase 1; SQSTM1, sequestosome 1; TG, transglutaminase; Z-VAD-fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; Z-YVAD-fmk, *N*-benzyloxycarbonyl-Tyr-Val-Ala-DL-Asp-fluoromethylketone. Taken with permission from (Galluzzi et al. 2012).

1.4 ROLES OF APOPTOSIS

Why study apoptosis? If apoptosis was critical for proper development, then we would expect that organisms with defective apoptosis would have severe phenotypes. For the most part, this is true. PCD-deficient worms have 15% more cells than normal, yet have a normal lifespan (Ellis et al., 1991b). On the other hand, PCD-deficient flies (White et al., 1994) and mice (Kuida et al., 1996) show extraneous cell accumulation and die early in development. These differing results likely reflect increasing organism complexity. Within higher organisms, it is clear that apoptosis is critical for multiple functions.

Apoptosis is important for the deletion of cells in the broad categories of sculpting organs and structures, deleting unnecessary structures, maintenance of adult tissue homeostasis, and eradication of cells that represent a threat to the integrity of the organism. For review, see Jacobson et al. (1997). Dysfunctional apoptosis is the root cause of many human diseases, and the study of apoptosis has revealed the molecular pathogenesis of human diseases. Ongoing discoveries in this field will identify targets for therapeutic manipulation.

1.4.1 Apoptosis in Developing Tissue

Apoptosis occurs in developing tissue (Figures 6A–D) (Jacobson et al., 1997). Apoptosis is critical to sculpt structures such as digits, where inhibition of apoptosis results in the persistence of tissue between digits (Jacobson et al., 1994; Milligan et al., 1995). PCD is involved in the hollowing out of solid structures to form lumens, such as the formation of the preamniotic cavity in early mouse embryos (Coucouvanis and Martin, 1995) and the lumens of organs in morphogenesis. PCD mediates the deletion of vestigial structures during development, such as the tadpole tail during amphibian metamorphosis (Jacobson et al., 1997). As well, there are many examples whereby excess cells are produced and then eliminated during normal development. For example, it is estimated that over half of the neurons produced during mammalian CNS development are eliminated by PCD (Barres and Raff, 1999). As another example, approximately 80% of human oocytes are eliminated prior to birth (Reynaud and Driancourt, 2000). Why does an organism go to the trouble of producing excess cells that then need to be eliminated? It is possible that a limited supply of extracellular survival signals leads to a competition between developing cells. For example, with respect to mammalian CNS development, cellular trimming ensures that the number of neurons matches the number of target cells that they innervate (Barde, 1989). PCD also eliminates cells that are potentially harmful to the organism. In the vertebrate immune system, developing T and B lymphocytes that are either non-functional or reactive against self antigens must be deleted or they have the potential to cause

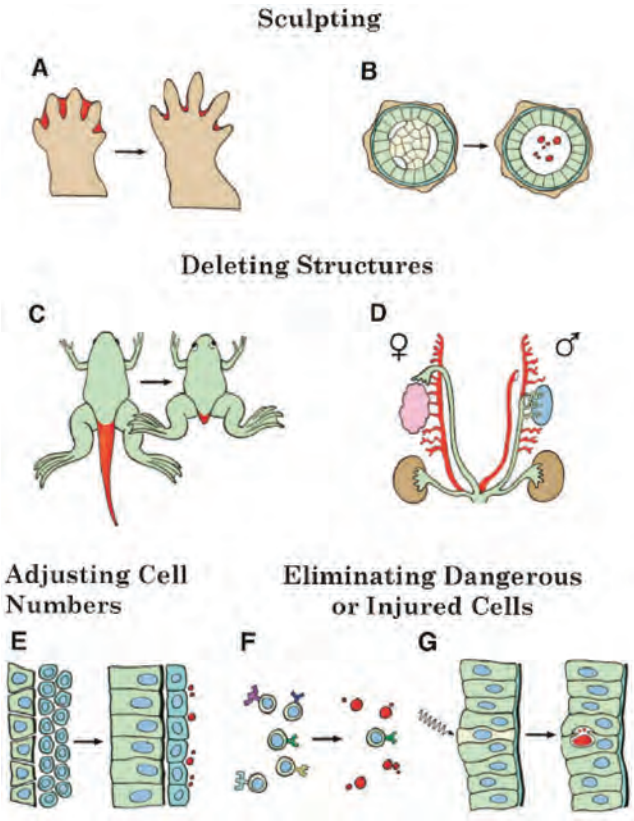


FIGURE 6: Functions of apoptosis and PCD. (A and B) Sculpting. (C and D) Deleting unwanted structures. (E) Controlling cell numbers. (F and G) Eliminating nonfunctional, harmful, abnormal or misplaced cells. Figure and modified legend taken with permission from Jacobson et al. (1997).

autoimmune disease. Thus, the reason for excessive cell production may be to generate a large pool of cellular candidates that through a screening process selects only the best cells for survival (Raff, 1992).

1.4.2 Apoptosis Maintains Adult Tissue Homeostasis

In adult tissues, rates of apoptosis and cell division must be balanced in order to establish a stable homeostatic environment. For example, the thymus of an adult mouse is composed of (1–2) $\times 10^8$ thymocytes. Each day, (2–4) $\times 10^6$ new thymocytes are produced, but only approximately 2–3% of

these cells survive (Chen et al., 1983). An astounding 98% of newly produced thymocytes die and must be cleared away. Humans turn over billions of cells every day at the rate of ~1 million cells per second (Henson, 2005; Nagata et al., 2010). According to Gerry Melino, if there was no apoptosis, by the time a person reached 80 years of age, the culmination of cellular proliferation would produce 2 tons of bone marrow and lymph nodes, and intestines that were 16 km long (Melino, 2001).

1.4.3 Apoptosis Eliminates Damaged Cells

Beyond cell deaths that occur during development or homeostasis, apoptosis also eliminates damaged cells. This non-developmentally regulated cell death occurs after physical damage (ionizing radiation), exposure to toxins (hydrogen peroxide, chemotherapy), and in response to viral infection (Arends and Wyllie, 1991; Cohen, 1991; Hockenbery et al., 1993; Levine et al., 1993; Martikainen et al., 1990). Aged or damaged cells (such as damaged airway epithelial cells, or damaged skin cells) must also be routinely removed. Thus apoptosis is necessary for development, maintaining tissue homeostasis, and as a quality control mechanism for organism integrity. However, how does a cell initiate this process? To gain insights, we must first identify the molecular players.

• • • •

CHAPTER 2

C. elegans and Discovery of the Caspases

2.1 *CAENORHABDITIS ELEGANS* AS A MODEL SYSTEM

Landmark studies that helped elucidate the mechanism of apoptosis came from investigations of PCD in the nematode *C. elegans*. Creation of mutations that affected genes critical for PCD, identification of those genes, and elegant genetic investigations led to the ordering of the genetic pathway. Key gene products identified the core cell death machinery. For review, see Ellis et al. (1991b) and Fuchs and Steller (2011).

In 2002, the Nobel Prize in Physiology or Medicine was awarded to Sydney Brenner, John E. Sulston and H. Robert Horvitz for their "seminal discoveries concerning the genetic regulation of organ development and programmed cell death." Sydney Brenner initiated these studies in the late 1950s, when he became "interested in finding a simple experimental system" to study developmental biology and neurology. He chose to focus on the round worm *Caenorhabditis elegans*. Many practical advantages led Brenner to choose *C. elegans*. The adult worm is small, measuring about 1 mm in length, is composed of exactly 959 somatic cells, and has a short life cycle of only 3 days. Importantly, the organism is transparent, making it easy to observe cell division and subsequent cell fate over time in live animals. Thus, many aspects of this organism have been investigated in great detail. For review, see Kenyon (1988).

During the 1970s and 1980s, the lineage of each individual cell from the zygote to the adult was mapped. Each cell followed a predictable pattern of division and differentiation, and this was the same for every worm (Kimble and Hirsh, 1979; Sulston and Horvitz, 1977; Sulston et al., 1983). Surprisingly, Horvitz and Sulston noticed that more cells were produced than were needed, and these extra cells did not survive to the adult worm (Sulston and Horvitz, 1977; Sulston et al., 1983). In his Nobel lecture, Horvitz stated: "One aspect of the cell lineage particularly caught my attention: in addition to the 959 cells generated during worm development and found in the adult, another 131 cells are generated but are not present in the adult. These cells are absent because they undergo programmed cell death" (Figure 7). Remarkably, the cell deaths were precisely controlled with the same cells dying at the same time in every worm. The dying cells appeared apoptotic, as they became round and refractile, were engulfed by neighboring cells, after which the engulfed cell corpses underwent degradation (Ellis et al., 1991b). Thus, there must be a genetic program that

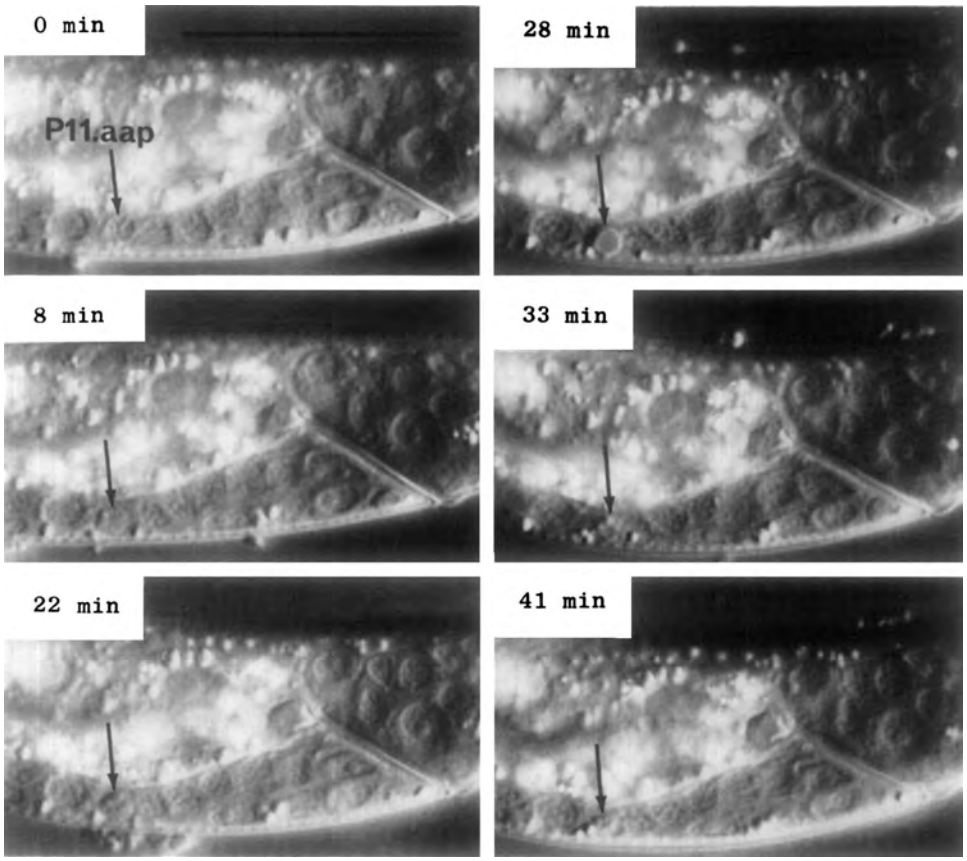


FIGURE 7: PCD in *C. elegans* development. Sequential photographs of an L1 hermaphrodite, lateral view; Nomarski optics. The arrow points to the dying cell, P11.aap. Bar = 20 μm. Legend and figures taken with permission from Sulston and Horvitz (1977).

marked these extraneous 131 cells for death. What were the genes that controlled this process and how could they be identified?

To identify these "death genes," Horvitz and colleagues created worm mutants, looked for alterations in cell death patterns and identified the mutated genes that were responsible for the aberrant phenotype. *C. elegans* were treated with ethyl methanesulphonate (EMS), which is an alkylating agent that induces random DNA point mutations (Sega, 1984). Using this methodology, over 300 EMS-induced mutations were identified (Ankeny, 2001). As this was a non-specific approach, many phenotypes were observed ranging from behavioral effects, defective movement, and alterations in size and shape. One hundred mutations affected programmed cell death (Ellis and Horvitz, 1986; Ellis and Horvitz, 1991; Ellis et al., 1991a; Hedgecock et al., 1983; Hengartner

et al., 1992; Sulston, 1976). Thus, the stage was set for the identification of key players of *C. elegans* programmed cell death.

2.2 IDENTIFICATION OF *C. ELEGANS* CELL DEATH GENES

More than a dozen genes were identified that affected programmed cell deaths. Two genes *ced-3* and *ced-4* (cell death abnormal) were identified that were required for cell death. Mutations in either of these genes led to the survival of almost all of the cells that are normally destined to die during worm development (Ellis and Horvitz, 1986) (Figure 8). Mutations that completely inactivated these genes (designated as *Ced-3* and *Ced-4* mutations) prevented all programmed cell deaths, while

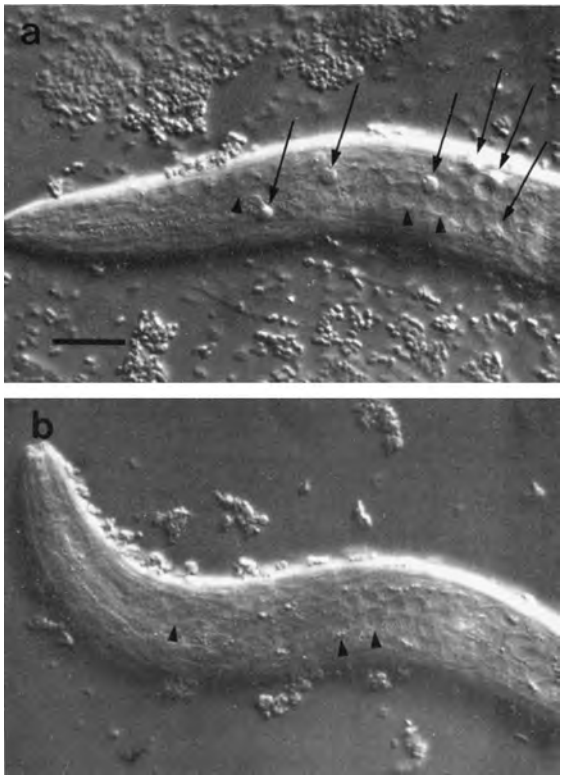


FIGURE 8: Absence of cell death in *ced-3* animals. (a) Nomarski photomicrograph of a newly hatched *ced-1* larva. Arrows indicate dying cells. (b) Nomarski photomicrograph of a newly hatched *ced1*; *ced3* larva. Plane of focus is approximately that shown in (a). Arrowheads indicate several of the nuclei that can be seen in both (a) and (b). No cell deaths are seen in the *ced1*; *ced3* larva. Bar = 10 μm. Legend and figure taken with permission from Ellis and Horvitz (1986).

weaker mutations (*ced-3* and *ced-4*) resulted in a partial suppression of death. Horvitz and colleagues suggested that the products of the *ced-3* and *ced-4* genes encoded toxins that killed cells.

Once the sequence of the *ced-3* and *ced-4* genes were determined, it was possible to investigate the function of the gene products. At the time, the *ced-4* protein was novel in sequence, so no insight could be gained with homologues in other systems. On the other hand, the *ced-3* protein shared 28% identity with a human protein, ICE (Interleukin-1 β-converting enzyme, now known as caspase 1). ICE is a cysteine protease that functions during inflammation by cleaving inactive pro-IL-1 β into functional interleukin-1β (Cerretti et al., 1992; Thornberry et al., 1992). Although it is now known that caspase-1 is not involved in apoptosis, it was nevertheless the first identified member of a family of cysteine-dependent proteases (caspases—cysteine aspartic acid-specific proteases) that have critical roles in inflammation and apoptosis.

Many more cell death genes were identified and the fundamental mechanism of PCD was elucidated. The mechanism of PCD in the worm was discovered to be a simple yet efficient pathway. Apoptosis in mammalian cells utilizes the same core machinery but has evolved into a more complex process (Figure 9). The single death genes of *C. elegans* are represented as expanded large protein families. This indicates that redundancy and specialized signaling are important aspects of mammalian apoptosis.

2.3 DEFINITION AND FUNCTION OF CASPASES IN APOPTOSIS

Loss of CED3 protein activity resulted in loss of PCD in *C. elegans*. This demonstrated the necessity of caspases in PCD and apoptosis. Caspase activity is responsible for most of the morphologies of the apoptotic cell (cell rounding, detachment from neighbors or extracellular matrix, nuclear condensation, DNA fragmentation, membrane blebbing, and phagocytic engulfment). Thornberry et al. (1998) proposed that "caspases participate in apoptosis in a manner reminiscent of a well-planned and executed military operation. They cut off contacts with surrounding cells, reorganize the cytoskeleton, shut down DNA replication and repair, interrupt splicing, destroy DNA, disrupt the nuclear structure, induce the cell to display signals that mark it for phagocytosis and disintegrate the cell into apoptotic bodies." How do caspases do so many things? Identification of downstream targets has revealed how caspases control this wide array of cellular responses.

Caspases cleave substrates after the aspartic acid residue of a tetrapeptide sequence (P4–P3–P2–Asp). Caspases are grouped into initiator caspases and effector caspases and substrate cleavage recognition motifs of residues P4–P3–P2 define caspase subclasses. Effector caspases proteolyse hundreds of intracellular substrates (for comprehensive review, see Taylor et al., 2008). Figure 10 shows examples of caspase substrates that affect apoptotic morphology. Depending on the caspase

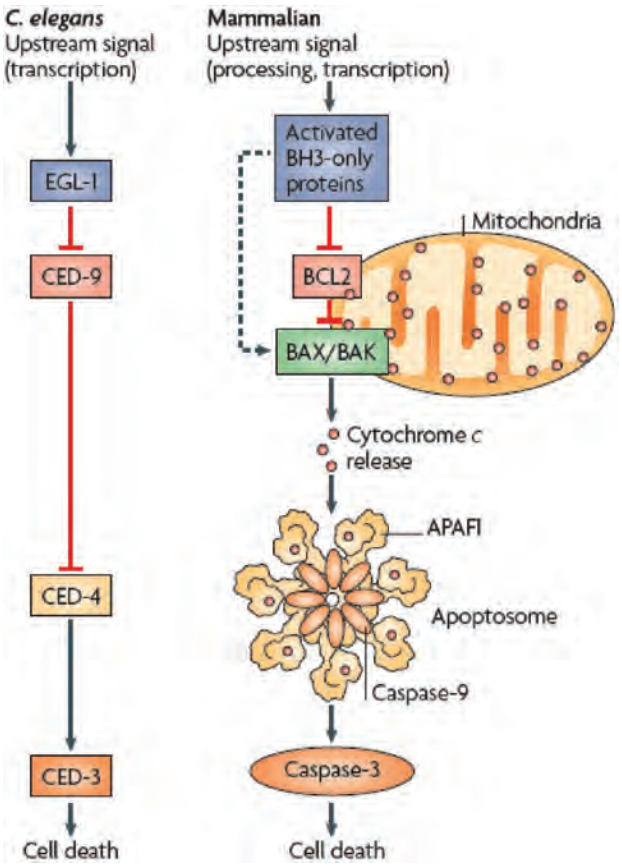


FIGURE 9: Evolutionary expansion of *C. elegans* apoptotic machinery in mammalian cells. Side-by-side comparison of the *Caenorhabditis elegans* CED protein pathway and the core apoptotic machinery in mammalian cells shows the conservation of the general outline of the pathway. Extension of the apoptotic machinery can also be observed at every step of the pathway, including multiple B-cell lymphoma protein-2 (BCL2) homology-3 (BH3)-only protein activating signals, complex regulation of the BCL2 family and the addition of mitochondrial cytochrome c release, which drives the formation of an apoptosome and activation of the upstream caspases (first caspase-9 and then the executioner caspases, such as caspase-3 and caspase-7). Added complexity is provided by the existence of multiple family members in each class of the apoptotic regulators, with both redundant and non-redundant functions. These regulators provide 'fail-safe' apoptosis machinery that can generate specialized responses to various upstream stimuli. Possible direct activation of BAX and BAK by BH3-only proteins is indicated by a dotted line. APAF1, apoptotic protease-activating factor-1; BAK, BCL2-antagonist/killer-1; BAX, BCL2-associated X protein. Legend and figure taken with permission from Degeterev and Yuan (2008).

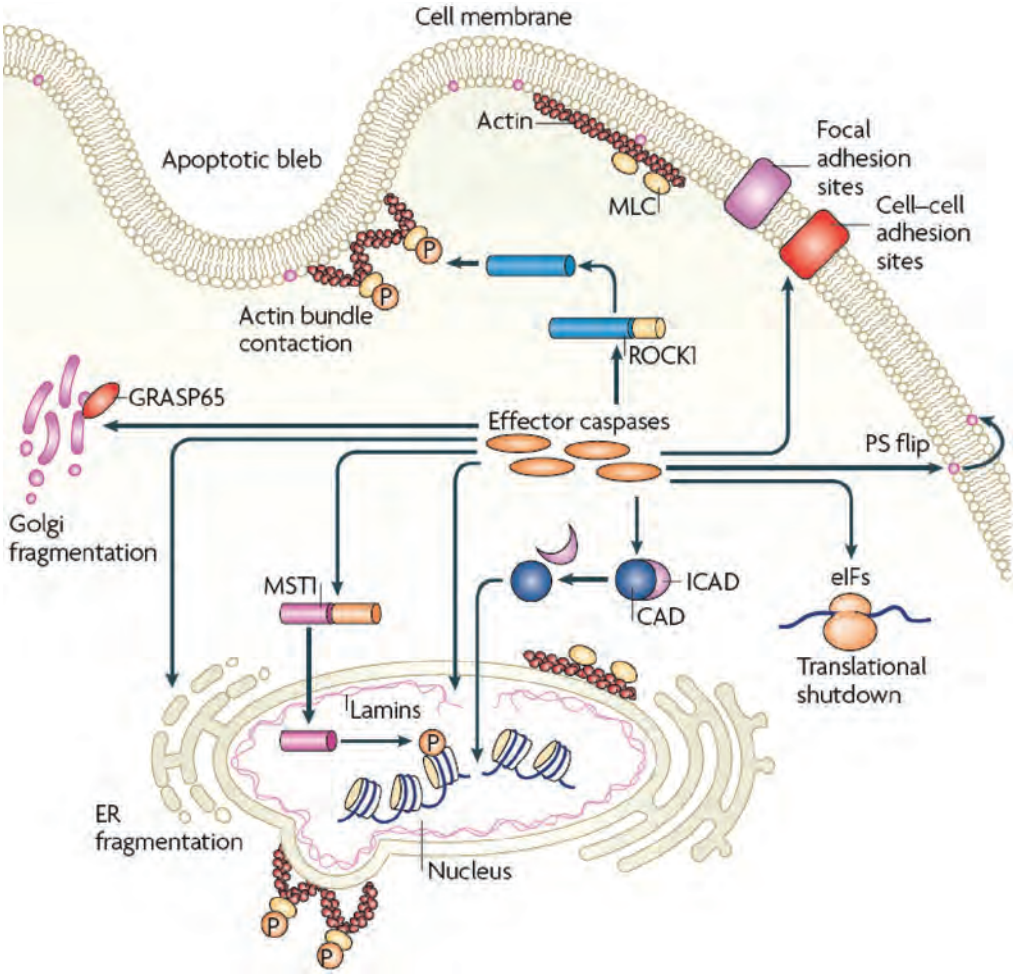


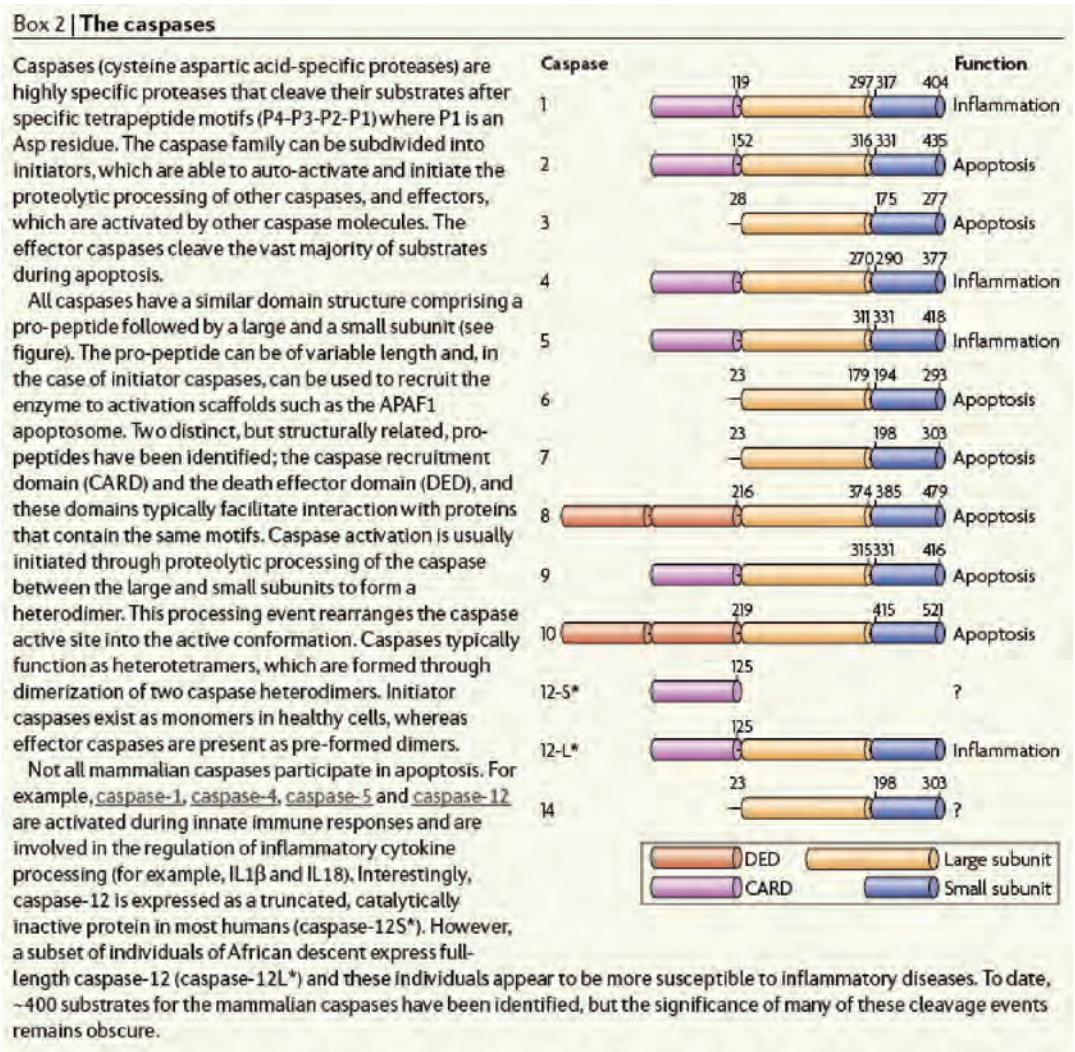
FIGURE 10: Caspases coordinate demolition of key cellular structures and organelles. Effector caspases (such as caspases 3, 6, and 7 in mammals) orchestrate the dismantling of diverse cell structures through cleavage of specific substrates. Collectively, these proteolytic events produce the phenotypic changes to the cell that are characteristic of apoptosis, and some examples are shown here. Cleavage of ICAD (inhibitor of caspase-activated DNase) releases CAD (caspase activated DNase), which can then catalyze inter-nucleosomal DNA cleavage. Caspase-mediated cleavage of nuclear lamins weakens the nuclear lamina, allowing nuclear fragmentation, and nuclear envelope proteins are also proteolysed. Proteolysis of proteins at focal adhesion sites and cell–cell adhesion sites allows cell detachment and retraction. Caspase activity is required for the exposure of phosphatidylserine (PS) and other phagocytic signals on the cell surface. Proteolysis of the Rho effector ROCK1 leads to contraction of the actin cytoskeleton and plasma membrane blebbing as well as nuclear fragmentation, whereas cleavage of tubulins and microtu-

bule associated and motor proteins leads to changes in the microtubule cytoskeleton that may contribute to apoptotic body formation (not shown). Caspases also cleave the Golgi-stacking protein GRASP65 and other Golgi proteins, causing fragmentation of the Golgi apparatus. Proteolysis of the mammalian sterile-20 kinase MST1 results in translocation of a catalytically active fragment of this kinase to the nucleus where it phosphorylates histone H2B to provoke chromatin condensation. Finally, important cellular functions such as translation are disrupted through caspase-mediated proteolysis of multiple translation initiation factors (eIFs). ER, endoplasmic reticulum; MLC, myosin light chain. Legend and figure taken with permission from Taylor et al. (2008).

target, cleavage can either inhibit or stimulate substrate function. Cell rounding and retraction is a result of caspase cleavage and inactivation of cytoskeletal constituents such as components of actin microfilaments (actin, myosin, gelsolin, filamen), microtubular proteins (tubulins, tau) and intermediate filament proteins (vimentin, lamins) (Taylor et al., 2008). Caspases trigger cell blebbing through proteolysis that activates the downstream target ROCK1 (Rho-associated kinase 1). In this case, caspase cleavage removes the inhibitory C-terminus resulting in constitutive kinase activation, phosphorylation of myosin light chain, and contraction of actin bundles leading to membrane blebbing (Coleman et al., 2001; Sebbagh et al., 2001). Detachment from neighboring cells or extracellular matrix is associated with caspase-mediated destruction of cell–matrix focal adhesion sites (focal adhesion kinase) (Levkau et al., 1998), and cell–cell adherens junctions (catenins) (Bannerman et al., 1998; Brancolini et al., 1997). Fragmentation of the nucleus and chromatin condensation occurs when caspases cleave and disassemble the rigid nuclear laminar network (Rao et al., 1996). Caspases trigger DNA fragmentation by indirectly activating the nuclease CAD/DFF-40 (caspase-activated DNase/DNA fragmentation factor 40) (Enari et al., 1998; Liu et al., 1997). In healthy cells, CAD/DFF-40 is found in a complex with its inhibitor/chaperone, ICAD/DFF-45. Caspase 3 cleaves ICAD/DFF-45, resulting in release of CAD/DFF-40, homodimerization of CAD/DFF-40 and migration to the nucleus. The CAD/DFF-40 catalytic site is located between two "blades of scissors" that cannot access nucleosomal DNA, but can cleave spacer DNA resulting in the characteristic DNA ladder pattern (Sakahira et al., 2001). Caspases cleave targets that alter cell surface markers and release specific factors that stimulate phagocytic engulfment of the apoptotic bodies (to be discussed in detail). As well, caspases cleave and inactivate numerous other proteins that mediate transcription, translation, splicing, metabolism and other vital cellular functions (Taylor et al., 2008; Thornberry and Lazebnik, 1998). Thus caspases orchestrate a controlled dismantling of the cell and facilitate its silent disposal.

2.4 THE CASPASE FAMILY

To date, there are at least 11 known functioning members of the caspase family in humans (caspases 1–10, 14) (Figure 11). Not all caspases contribute to apoptosis with some caspases functioning during the innate immune response. Genetic studies demonstrated that mice deficient for specific caspase gene expression were either resistant to apoptosis or had diminished cytokine maturation



Box 2 | **The caspases**

Caspases (cysteine aspartic acid-specific proteases) are highly specific proteases that cleave their substrates after specific tetrapeptide motifs (P4-P3-P2-P1) where P1 is an Asp residue. The caspase family can be subdivided into initiators, which are able to auto-activate and initiate the proteolytic processing of other caspases, and effectors, which are activated by other caspase molecules. The effector caspases cleave the vast majority of substrates during apoptosis.

All caspases have a similar domain structure comprising a pro-peptide followed by a large and a small subunit (see figure). The pro-peptide can be of variable length and, in the case of initiator caspases, can be used to recruit the enzyme to activation scaffolds such as the APAF1 apoptosome. Two distinct, but structurally related, pro-peptides have been identified; the caspase recruitment domain (CARD) and the death effector domain (DED), and these domains typically facilitate interaction with proteins that contain the same motifs. Caspase activation is usually initiated through proteolytic processing of the caspase between the large and small subunits to form a heterodimer. This processing event rearranges the caspase active site into the active conformation. Caspases typically function as heterotetramers, which are formed through dimerization of two caspase heterodimers. Initiator caspases exist as monomers in healthy cells, whereas effector caspases are present as pre-formed dimers.

Not all mammalian caspases participate in apoptosis. For example, caspase-1, caspase-4, caspase-5 and caspase-12 are activated during innate immune responses and are involved in the regulation of inflammatory cytokine processing (for example, IL1β and IL18). Interestingly, caspase-12 is expressed as a truncated, catalytically inactive protein in most humans (caspase-12S*). However, a subset of individuals of African descent express full-length caspase-12 (caspase-12L*) and these individuals appear to be more susceptible to inflammatory diseases. To date, ~400 substrates for the mammalian caspases have been identified, but the significance of many of these cleavage events remains obscure.

FIGURE 11: The caspase family. Figure taken with permission from Taylor et al. (2008).

(Wang and Lenardo, 2000). Caspases 2, 3, 6, 7, 8, 9, and 10 contribute to apoptosis while caspases 1, 4, and 5 are involved in the inflammatory processes.

The apoptotic caspases are grouped into either initiator or executioner caspases. Initiator caspases (caspase 2, 8, 9, and 10) have longer pro-domain segments of two types—Death Effector Domain (DED) or Caspase Recruitment Domain (CARD). As shall be addressed in detail later, theses pro-domains facilitate proximity-induced oligomerization that is required for subsequent enzymatic activation. Initiator caspases are the apical caspases of the signaling pathway, and auto-activate in response to an apoptotic signal (specific apoptotic signals will be discussed later). This triggers a "caspase cascade" through cleavage and activation of downstream executioner caspases (caspase 3, 6, and 7). Targets of initiator caspase cleavage are mostly the initiator caspases themselves and executioner caspases. In contrast, executioner caspases cleave hundreds of downstream targets responsible for the apoptotic morphology. Why are there so many caspases? Having a two-step mechanism of caspase activation (initiator caspases→executioner caspases) allows the amplification of an apoptotic signal. Within each group, similar caspases can serve as "back up" molecules for the other and/or may signify specialized function dependent on apoptotic stimulus and cell type.

2.5 MECHANISM OF CASPASE ACTIVATION

Caspases are present as pro-enzymes with limited enzymatic activity. The procaspases are comprised of three domains: the N-terminal pro-domain and a large and small subunit (Figure 12). Initiator procaspases exist in the cell as monomers that form oligomers in response to an apoptotic signal. Clustering allows the basal enzymatic activity of one pro-caspase molecule to cleave between the domains of another pro-caspase molecule. This results in activation and stabilization of the active initiator caspase. Executioner caspases are present as inactive dimers. Upstream initiator caspases cleave the interdomain regions, leading to rearrangements. These rearrangements form the active site that is comprised of key residues from both the large and small subunits. Structural studies of executioner caspases demonstrated that two heterodimers associate to form a tetramer resulting in an active enzyme with two catalytic sites (Rotonda et al., 1996; Walker et al., 1994; Wilson et al., 1994).

In summary, caspases are present as inactive pro-enzymes in the cells, and their activation is tightly controlled. Apoptotic signals activate initiator caspases that then cleave and activate downstream executioner caspases. Caspase cleavage is required for protein conformational change that creates the catalytic active site. Thus, this two-step process functions to amplify a signal as well as to act as a safe-guard mechanism to ensure that death-inducing caspase activity is strictly regulated. Another mechanism to control caspases occurs through activity of specific inhibitors known as Inhibitor-of-Apoptosis-Proteins (IAPs) that will be discussed in Section 4.2.4. The consequence of

caspase activation such as DNA fragmentation, chromatin condensation, cell blebbing and shrinking are all the early hallmarks that first defined this mode of cell death as "apoptosis." The second defining feature of apoptosis that was summarized by Kerr et al. (1972) was phagocytosis of apoptotic bodies. Caspases also play a major role in the engulfment of apoptotic cells and genetic studies defined the critical role that this process has in preventing autoimmunity (Discussed in Section 3).

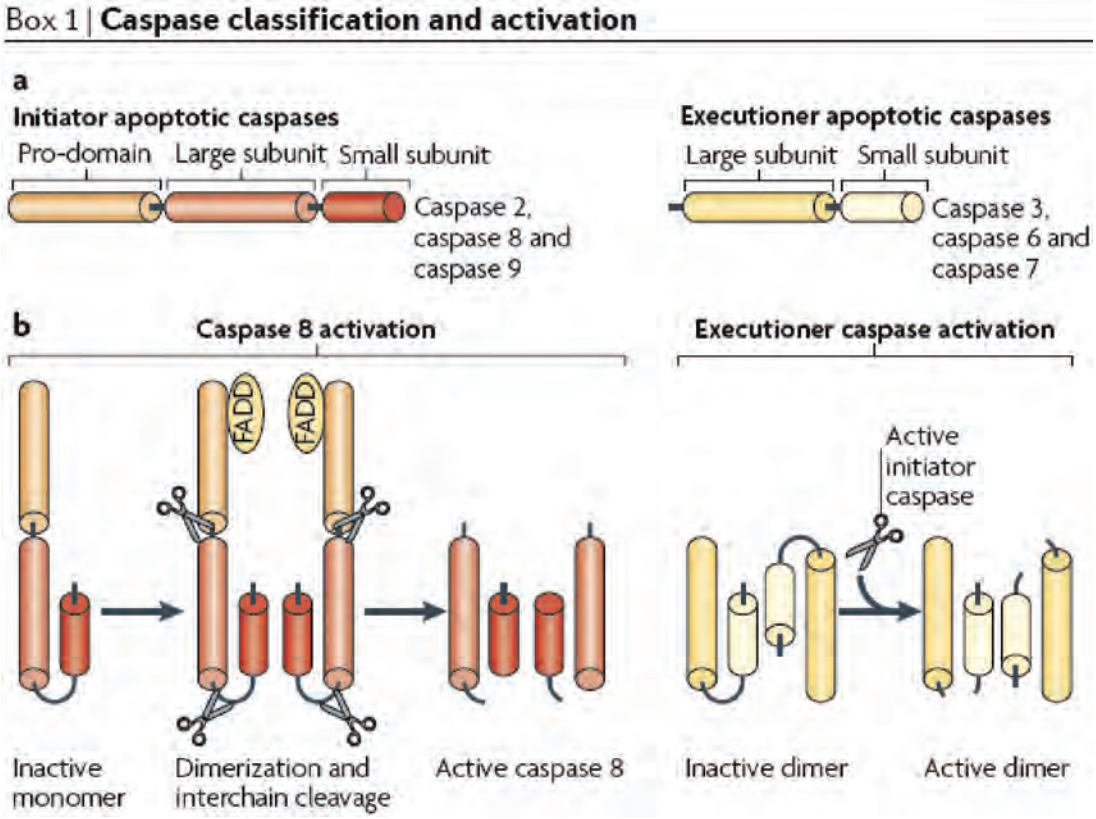


FIGURE 12: Caspases (Cys Asp acid proteases) cleave substrates in a highly specific manner after the Asp residue in short tetrapeptide (X–X–X–Asp) motifs. Besides apoptotic roles, some caspase family members have non-apoptotic functions in processes such as cytokine maturation, inflammation, and differentiation. Additionally, apoptotic caspases can have non-apoptotic roles in certain circumstances. Apoptotic caspases can be divided into two classes: initiator and executioner caspases (see the figure, part **a**). Initiator caspases (caspase 2, caspase 8, and caspase 9) are the apical caspases in apoptosis signaling cascades and their activation is normally required for executioner caspase (cas-

pase 3, caspase 6, and caspase 7) activation. The repertoire of initiator caspase substrates is limited and includes self-cleavage, BCL-2 homology 3 (BH3)-interacting domain death agonist (BID) and executioner caspases. By contrast, executioner caspases cleave hundreds of different substrates and are largely responsible for the phenotypic changes seen during apoptosis. Initiator caspase activation first involves dimerization of inactive caspase monomers (see the figure, part **b**). In the case of caspase 8, following death receptor ligation, dimers are formed by the recruitment of caspase 8 monomers through their pro-domains to the adaptor molecule FAS-associated death domain protein (FADD). Dimerization and interdomain cleavage are required for the activation and stabilization of mature caspase 8. Although dimerization is required for caspase 9 activation and interdomain cleavage occurs, cleavage is involved in the attenuation rather than promotion of caspase 9 activity. The activation mechanism of executioner caspases differs from that of initiator caspases (see the figure, part **b**). Executioner caspases are present as dimers in cells and are activated by cleavage, leading to intramolecular rearrangements and the formation of an enzymatically active dimer. Modified legend and figure taken with permission from Tait and Green (2010).

CHAPTER 3

Engulfment of Apoptotic Cells: "Find Me" and "Eat Me" Signals

It is rare to observe dead cell corpses in vivo, even in tissues with high apoptotic rates. This indicates that apoptotic clearance is efficient and rapid. Apoptotic cell corpses are quickly recognized and removed by neighboring cells or professional phagocytes. However, when these processes are inhibited, apoptotic cells are readily seen (Nagata et al., 2010). As an analogy, Ravichandran (2011) commented that "we do not appreciate the garbage collector until there is a disruption in trash collection and we begin to notice the unpleasant odor." In the same vein, without clearance, apoptotic bodies accumulate and undergo secondary necrosis with the associated leakage of cellular components into the extracellular space. The release of these intracellular materials is most often immunogenic and pro-inflammatory. We know this because deficient cell clearance leads to non-resolving inflammation and autoimmunity toward self-antigens associated with diseases such as systemic lupus erythematosus or arthritis (Franz et al., 2006; Hanayama et al., 2006; Munoz et al., 2010; Nagata et al., 2010; Nathan and Ding, 2010). For extensive review, see Nagata et al. (2010) and Ravichandran (2011).

Multiple steps are required for the phagocytic engulfment and clearance of apoptotic cells. First, the apoptotic cell releases "find-me" signals that bring phagocytes into close proximity to the dying cell (Gardai et al., 2006; Peter et al., 2010). Second, the dying cell exposes "eat-me" signals that interact with "eat-me receptors" on the phagocytic cells. Interaction between the apoptotic corpse and phagocytic cell stimulates phagocytic cell cytoskeletal reorganization that facilitates corpse engulfment (Ravichandran and Lorenz, 2007). Engulfed apoptotic bodies are then directed to lysosomes within the phagocyte for degradation into amino acids, nucleotides, fatty acids, and monosaccharides that are released from the lysosome and recycled as basic building blocks for the cell (Kinchen and Ravichandran, 2008) (Figure 13).

3.1 "FIND-ME" SIGNALS

In *C. elegans*, dead cells are engulfed by healthy neighboring cells (Hoeppner et al., 2001; Reddien et al., 2001). However, in mammalian cells, the engulfing cell is usually a professional phagocytic

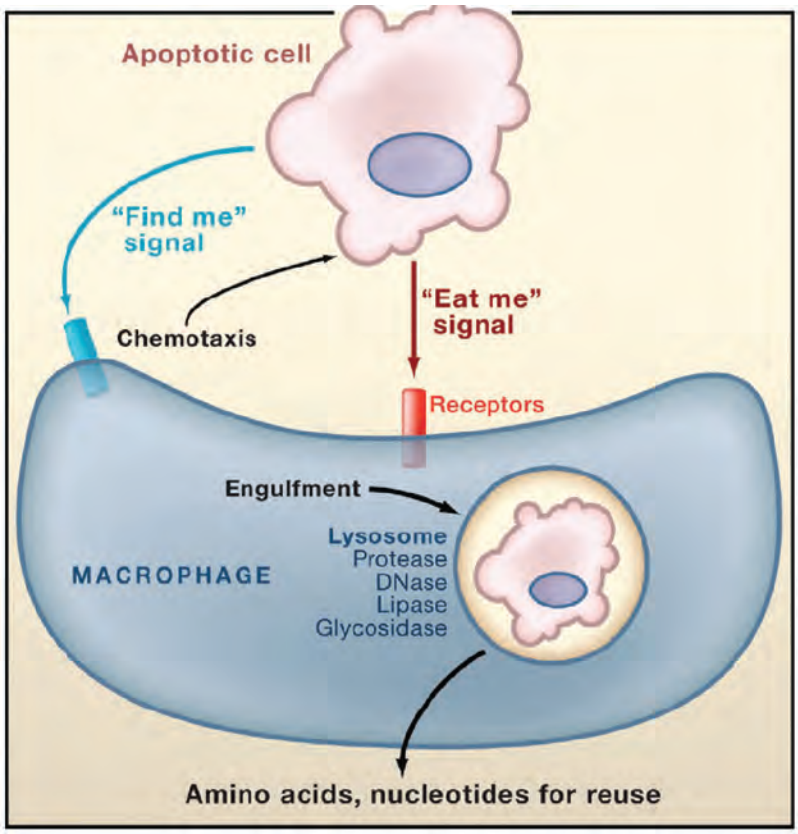


FIGURE 13: Engulfment of apoptotic cells by macrophages. When cells undergo apoptosis, they release "find me" signals to recruit macrophages, and expose "eat me" signals on their surface. In response to the "find me" signal, macrophages approach the dead cells, and they engulf them by recognizing the "eat me" signal. The engulfed dead cells are transferred to lysosomes, where all their components are degraded into amino acids, nucleotides, fatty acids, and monosaccharides by lysosomal enzymes. Legend and figure taken with permission from Nagata et al. (2010).

cell such as a circulating monocyte or its differentiated macrophage or dendritic cell. Because these circulating cells are not immediately beside the cell that is destined to die, they must first be actively recruited to the site of cell death (Ravichandran, 2011). How do these phagocytic cells find their target? The dying cell must somehow advertise its condition. It is now known that dying cells release "find-me" signals. These soluble mediators interact with receptors on the surface of the phagocytic cell triggering intracellular signaling that induces phagocytes to migrate toward areas of apoptotic cells (chemotaxis) (Figure 14). Four specific "find me" signals have been identified. Two

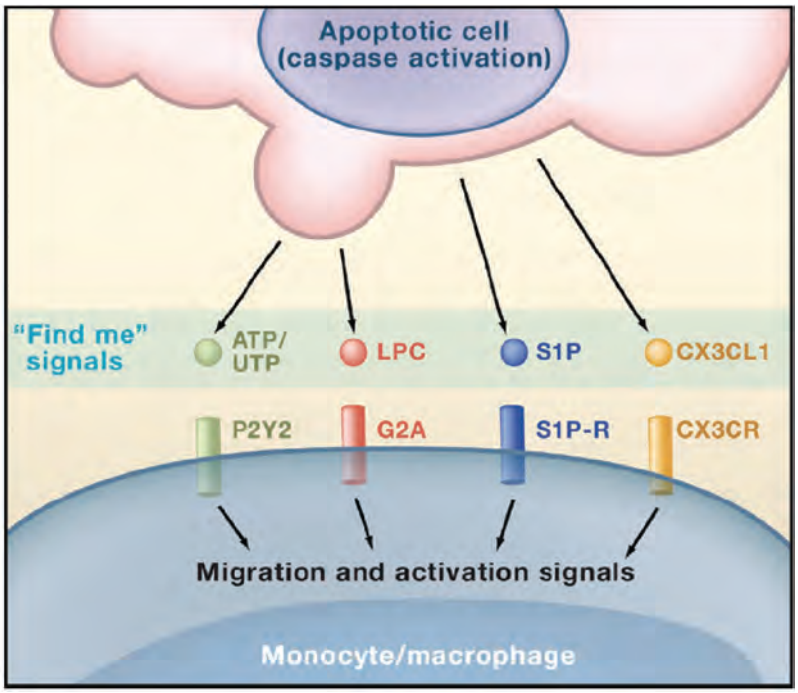


FIGURE 14: Proposed "find me" signals. As "find me" signals, ATP/UTP, lysophosphatidylcholine (LPC), sphingosine-1-phosphate (S1P), and fractalkine CX3CL1 have been proposed. These molecules bind specific receptors on macrophages, all of which are G protein-coupled seven-transmembrane receptors, and activate them for chemotaxis. Legend and figure taken with permission from Nagata et al. (2010).

are the lipid mediators lysophosphatidylcholine (LPC) and sphingosine-1-phosphate (S1P), one is a nucleotide (ATP/UTP) and one is a protein product (fractalkine CX3CL1). These diverse mediators bind specific G protein-coupled receptors on the macrophage cell surface (P2Y2, G2Z, SIP-R, and CX3CR, respectively) and stimulate chemotaxis.

LPC was the first "find me" signal identified (Lauber et al., 2003). Within the apoptotic cell, caspase-3 activates phospholipase A2, which mediates phosphatidylcholine conversion to LPC. LPC is released from the apoptotic cell and establishes a putative concentration gradient, wherein binding to surface receptors on circulating macrophages (G2A—G2 accumulation protein) stimulates chemotaxis toward the apoptotic cell (Peter et al., 2008). Of note, the relevance of this model is in question, as the concentration of LPC required to attract phagocytic cells is high (20–30 μM) and may not be physiologically relevant (Nagata et al., 2010).

S1P is another "find me" signal and a well-known chemotaxis signal for immune responses. Apoptotic cell production of S1P is proposed to occur through two mechanisms. Gude et al. (2008) suggest that apoptosis upregulates intracellular levels of the kinase that produces S1P (SphK1), which in turn increases S1P levels that are released. Weigert et al. (2010) suggest that an S1P concentration gradient is indirectly established when caspase-1 cleaves the S1P kinase, SphK2, causing SphK2 release and subsequent elevation of S1P levels in the extracellular space. In either case, it is proposed that S1P stimulates macrophage recruitment through binding and activation to the macrophage S1P-R receptors. At this present time, the function of neither LPC nor S1P as bonafide "find me" signals has been validated in vivo.

Fractalkine (CX3CL1) is an inter-cellular plasma membrane adhesion molecule. In apoptotic cells, fractalkine is cleaved to a 60-kDa product and is released as microparticles to function as a "find me" signal (Truman et al., 2008). CX3CL1 cleavage is caspase-dependent, but may not be a direct substrate of caspases, but is instead produced through caspase-activated extracellular ADAM protease activity. CX3CL1 interacts with the monocyte receptor CX3CR1 and induces monocyte migration. Mice lacking CX3CR1 showed a decrease in monocyte migration to apoptotic areas validating CX3CL1 in vivo as a "find me" signal (Truman et al., 2008).

Nucleotides are the fourth known "find me" signal (Elliott et al., 2009). ATP and UTP are released through a caspase-dependent opening of a channel (pannexin channel) on the plasma membrane (Chekeni et al., 2010). Mechanistically, the C-terminal tail of the pannexin family member, PANX1, is removed by caspases 3 and 7 triggering channel opening that allow molecules of up to 1 kDa (including nucleotides) to pass through the plasma membrane. Mice with genetic ablation of PANX1 are defective in nucleotide release during apoptosis and show diminished macrophage recruitment (Qu et al., 2011). Released nucleotides attract monocytes in both in vitro and in vivo experimental conditions (Elliott et al., 2009). ATP/UTP interact with the monocyte P2Y2 receptor leading to migration of monocytes to apoptotic cells. Importantly, studies with P2Y2-null mice have demonstrated the importance of this signaling pathway for monocyte migration.

Interestingly, apoptotic epithelial cells of the airway release nucleotides, yet are engulfed by adjacent healthy epithelial cells instead of circulating macrophages (Elliott et al., 2009). As it turns out, "find me" signals also "prime" cells for phagocytosis. In *D. melanogaster*, apoptotic cells induce the upregulation of the engulfment machinery of neighboring cells (MacDonald et al., 2006; Ziegenfuss et al., 2008). In mammalian cells CXCL1 induces macrophage production of MFG-E8 which is a bridging molecule involved in engulfment (Miksa et al., 2007). Thus, in addition to establishing a chemotactic gradient for phagocytic cells, "find me" signals also increase the engulfment activity of nearby cells thereby facilitating two related yet independent means to ensure their own clearance.

A hallmark feature of apoptosis is that it is non-immunogenic, specifically recruiting monocytes and macrophages, as opposed to inflammatory cells of the immune system (Fadok et al., 1998; Voll et al., 1997). However, LPC, S1P and ATP/UTP are also capable of activating inflammatory neutrophils and lymphocytes (Florey and Haskard, 2009; Lecut et al., 2009) and ATP is known as a universal inflammatory "danger signal" (Trautmann, 2009). So for example, how would an organism determine whether ATP should stimulate monocyte (non-immunogenic) or neutrophil (inflammatory) recruitment? A possible differentiating factor may be overall ATP levels in the extracellular space. Apoptotic cells release less ATP than necrotic cells (Elliott et al., 2009) and only higher levels of ATP induce inflammation and recruitment of neutrophils (Aymeric et al., 2010). Additionally, Bournazou et al. (2009) discovered that apoptotic cell supernatants also contain "keep out" signals. Through a caspase-dependent mechanism, lactoferrin is released from apoptotic cells and specifically inhibits neutrophil, but not monocyte chemotaxis (Bournazou et al., 2009). How "find me" and "keep out" signals regulate immunogenic versus non-immunogenic responses is an expanding area of research.

3.2 "EAT ME" SIGNALS

Once professional phagocytes are recruited to the site of the dying cell, it must be able to differentiate the apoptotic corpse from the surrounding healthy cells. This is accomplished through interaction between "eat me" signals displayed on the surface of the apoptotic cell and specific receptors on the phagocyte. Once engaged, the phagocytic receptor initiates a series of signal transduction events that lead to cytoskeletal changes that enable engulfment of the apoptotic corpse (Figure 15).

The first, and most widely recognized "eat me" signal is the surface exposure of phosphatidylserine (PS) (Fadok et al., 1992). In healthy cells, PS is found on the inner leaflet of the lipid bilayer (Balasubramanian and Schroit, 2003). Early in the apoptotic process, in a caspase-dependent manner, PS exposure to the outer membrane surface of the dying cell increases more than 280-fold (Borisenko et al., 2003). Surface-exposed PS quickly became a widely-used experimental identifier of apoptotic cells based on binding of externalized PS to fluorescent annexinV molecules. PS externalization is a critical inducer of phagocytosis. Blocking PS exposure inhibits phagocytosis of apoptotic bodies (Asano et al., 2004; Krahling et al., 1999) and artificially inserting PS into the membrane of healthy cells promotes their engulfment (Tanaka and Schroit, 1983), indicating that PS is an essential "eat me" signal. However, PS exposure alone is not sufficient to trigger engulfment suggesting that other "eat me" signals contribute to the process. As yet, these accessory signals are not clearly defined. Furthermore, it is surprising that the mechanism for PS externalization is not well defined. The current model is that in healthy cells, ATP-dependent translocases maintain PS

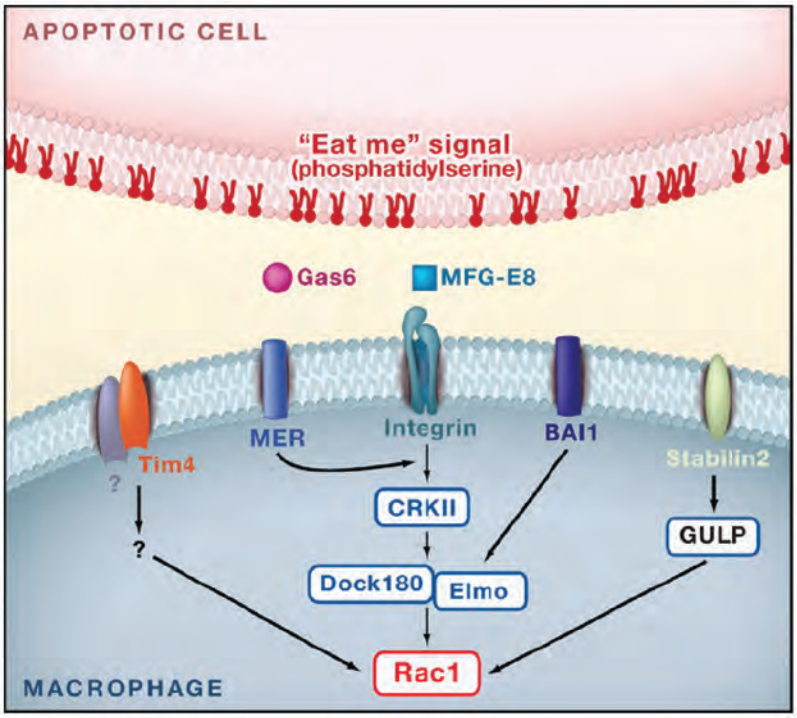


FIGURE 15: Molecules proposed to recognize phosphatidylserine. The most likely "eat me" signal is phosphatidylserine. MFG-E8 and Gas6 are secreted proteins that bind phosphatidylserine and work as bridging molecules between apoptotic cells and macrophages. Tim-4, BAI1, and Stabilin-2 are type I-membrane proteins that are proposed phosphatidylserine receptors. Molecules that activate Rac1 (CrkII, Dock180, Elmo, and GULP) are involved in the engulfment of apoptotic cells. Legend and figure taken with permission from Nagata et al. (2010).

on the inside of the cell, but during apoptosis, ATP levels decrease and diminish translocase activity. Additionally, increasing cytosolic calcium levels activate a Ca2+-dependent phospholipid scramblase flipping phospholipids between the inner and outer leaflet (Balasubramanian and Schroit, 2003; Sahu et al., 2007). This model, however, has not been fully validated with in vivo systems (Darland-Ransom et al., 2008; Zullig et al., 2007).

Once PS is exposed on the apoptotic cell, it must be recognized by phagocytic cell surface receptors. Two distinct types of engulfment receptors are known. Both are membrane proteins but one class directly binds PS and the second type indirectly recognizes PS through intermediate soluble bridging molecules. Direct PS binding receptors are the T cell immunoglobulin and mucin (TIM) family (Kobayashi et al., 2007; Miyanishi et al., 2007; Santiago et al., 2007), brain angiogenesis

inhibitor 1 (BAI1) (Park et al., 2007a), and Stabilin-2 (Park et al., 2008). Integrins indirectly recognize PS through the bridging molecule milk fat globule EGF factor 8 (MFG-E8). MFG-E8 is a secreted soluble protein that has two distinct binding sites that can simultaneously engage PS on the apoptotic cell and integrin αvβ3 on the phagocytic cell (Akakura et al., 2004). Interestingly, MFG-E8 is upregulated in macrophages in response to "find me" signals, establishing a link between "find me" and "eat me" signals. Tyro-3-Axl-Mer receptors (TAM/MER) also indirectly bind PS through the bridging molecule Gas6 and protein S. Loss of TAM/MER receptors inhibits clearance of apoptotic cells in mouse tissues demonstrating in vivo significance of these receptors for apoptotic cell engulfment (Prasad et al., 2006).

Upon receptor stimulation, either directly or indirectly, the phagocytic cell cytoskeleton undergoes physical changes that facilitate corpse engulfment. Currently, the molecular details of the signaling pathway are not clear. However, it is likely that signals converge on the Rho family GTPases such as Rac1 (Nakaya et al., 2006). Upon recognition of an apoptotic cell by a phagocyte, within the phagocyte, activated Rac1 and integrin are recruited to the interface and induce the formation of phagocytic cups containing actin patches. Upon engulfment initiation, Rac1 is inactivated and actin is depolymerized. Rab5 facilitates the transport of apoptotic bodies to lysosomes (Kitano et al., 2008).

3.3 IMPAIRED ENGULFMENT OF APOPTOTIC CORPSES LEADS TO AUTOIMMUNE DISEASE

Deficiency in the clearance of apoptotic bodies leads to autoimmunity. A deficiency in clearance of apoptotic corpses is proposed to be one of the causes for Systemic Lupus Erythematosus (SLE) (Gaipl et al., 2006). The evidence for this model is that some SLE patients show abnormally high numbers of un-engulfed apoptotic cells in their lymph nodes, and have macrophages with a reduced ability to engulf apoptotic cells. This lack of apoptotic cell clearance is proposed to lead to elevated numbers of secondary necrotic cells spilling their intracellular components into the extracellular space leading to production of self-antibodies. Theoretically, this would account for the high levels of circulating antibodies against nuclear components such as DNA and ribonucleoproteins that is characteristic of SLE (Rumore and Steinman, 1990). Experimental validation of this model is seen with MFG-E8 deficient mice. Macrophages from these mice were deficient for engulfment, their sera contained anti-DNA and anti-nuclear antibodies, and they developed an SLE-type of autoimmune disorder (Hanayama et al., 2004).

After apoptotic cells are engulfed by phagocytes, their components are trafficked to the phagocytic lysosome for re-cycling. Acidic lysosomal DNase II catalyzes complete hydrolysis of nucleosomal DNA into nucleotide building blocks. Mice that lack DNase II accumulate undigested

DNA in their macrophages. This induces the macrophages to secrete various cytokines. One such cytokine IFNβ is toxic to erythroblasts and lymphocytes causing the knock-out embryos to die of severe anemia (Kawane et al., 2001). If these same DNaseII knock-out mice are also deficient for the IFN receptor, the mice survive (Kawane et al., 2001). However, these mice develop polyarthritis later in life, because the undigested DNA continues to induce the production of proinflammatory molecules (Kawane et al., 2006). In particular, the macrophages containing undigested DNA are induced to secrete TNFα, which leads to a cytokine storm involving enhanced production of IL-1β, IL-6, and TNFα by synovial cells; all causative agents of polyarthritis. All together, these in vivo studies demonstrate that the presence of genomic DNA in the extracellular space (through necrosis) or accumulation of undigested DNA in the lysosomes of macrophages (through reduced macrophage activity) activates the innate immune response and inflammation.

CHAPTER 4

Extrinsic and Intrinsic Apoptotic Signals

Apoptotic signals are classified as either extrinsic or intrinsic signals. While these two pathways have distinct elements, they both ultimately lead to activation of the downstream core apoptotic machinery. See Figure 16 for overall outline of extrinsic and intrinsic apoptotic signals.

4.1 EXTRINSIC SIGNALS

The extrinsic signal is critical for the development and homeostasis of the immune system as exemplified in the extensive apoptosis of mouse thymocytes. As well, extrinsic signals mediate the apoptosis of cells recognized as dangerous to the organism, such as viral infected or transformed cells. As the name indicates, the extrinsic signal originates from the outside of the cell. In many cases, a death message is transmitted through cell–cell contact between surface bound ligands on a cytotoxic cell with death receptors on the surface of the target cell. The initiating extrinsic signaling molecules are members of the tumor necrosis factor (TNF) family of cytokines named FasL (CD95L/APO1-L), TNF-related apoptosis inducing ligand TRAIL (APO-2L), TNF and TL1A. These death-inducing ligands, bind to plasma membrane receptors of the TNF-R1 family. These death receptors are FAS (CD95, APO-1), TRAIL-R1 (DR4), TRAIL-R2 (DR5), TNF receptor 1 (TNFR1), and TRAMP (DR3). All death receptors contain a conserved 80 amino acid sequence known as the "death domain" (DD). Upon ligand binding to receptor, the DD facilitates protein–protein interactions and transmission of the death signal from the outside of the cell to the apoptotic machinery on the inside of the cell. For review, see Galluzzi et al. (2012), Strasser et al. (2009) and Danial and Korsmeyer (2004). (Figure 17).

The FASL-FAS pathway is the prototypic extrinsic apoptotic pathway and is particularly important for immune homeostasis and function (Siegel et al., 2000). Interestingly, FAS was pulled out of a screen as the target of monoclonal antibodies that induced apoptosis in cell lines (Itoh et al., 1991). Its ligand FASL was discovered soon after (Suda et al., 1993). FASL is expressed both as a membrane-bound surface protein on cytotoxic T cells and natural killers cells, and/or is released as a soluble factor. Both membrane-bound and soluble FASL can activate the target receptor

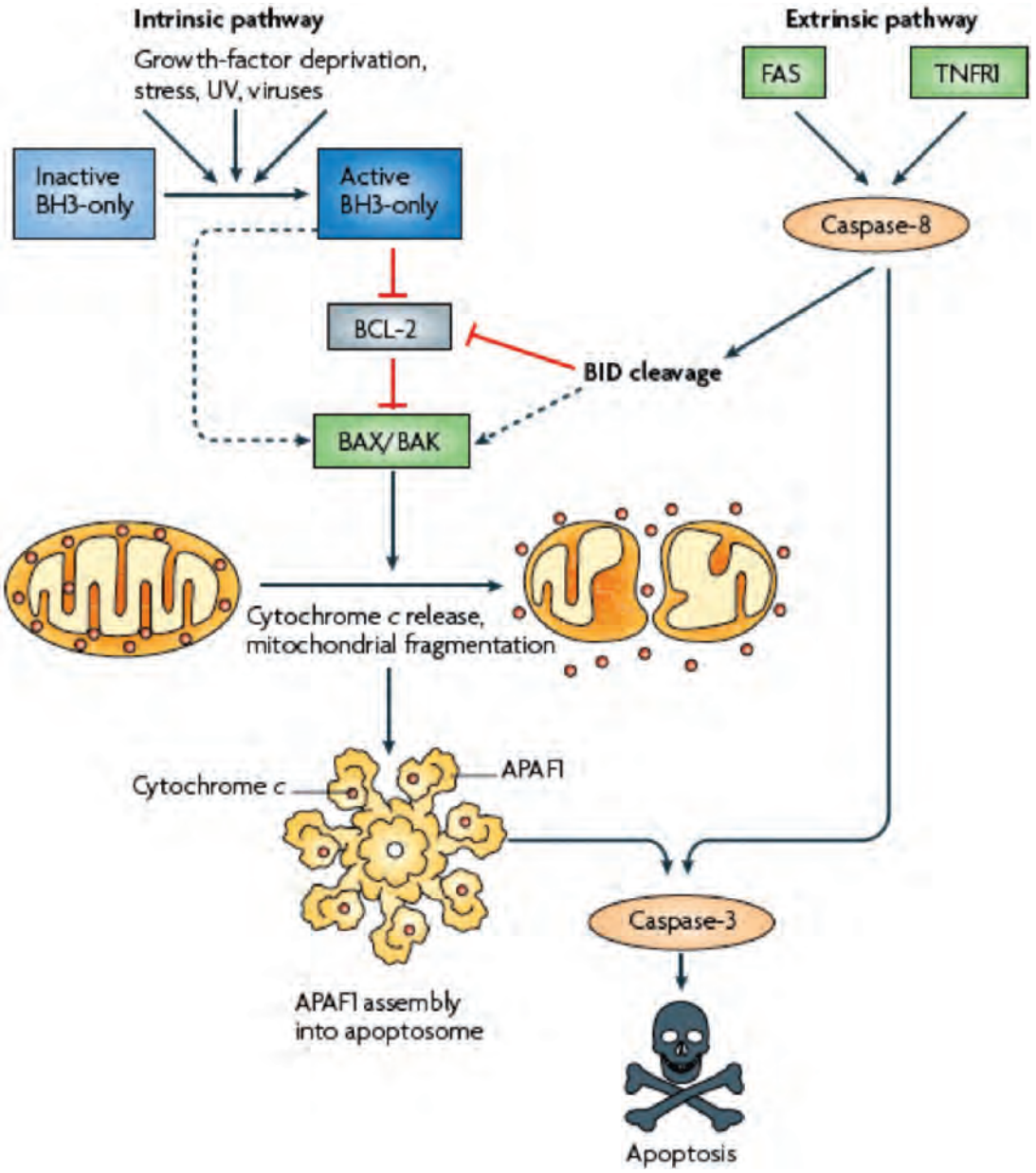


FIGURE 16: Overall model for extrinsic and intrinsic apoptotic signals. Apoptotic pathways. The execution of apoptosis downstream of the death stimuli is governed by two molecular programs known as the intrinsic and the extrinsic apoptotic pathways that ultimately terminate in caspase activation. The intrinsic pathway is activated by multiple cellular stresses, including growth factor deprivation, DNA damage, and misfolded proteins. Mitochondria participate in the intrinsic pathway by releasing apoptogenic

FAS (Tanaka et al., 1995). The FASL-FAS system plays important roles in the immune response. Mice carrying spontaneous mutations in the genes for FAS (*Fas*$^{lpr/lpr}$) or FASL (*Fasl*$^{gld/gld}$) develop SLE-like autoimmune disease (Takahashi et al., 1994; Watanabe-Fukunaga et al., 1992). As well, *FAS* gene mutations in humans are strongly associated with the autoimmune lympho-proliferative syndrome, ALPS (Fisher et al., 1995; Rieux-Laucat et al., 1995) demonstrating that FASL-FAS functions as a barrier for autoimmunity. Furthermore, lymphoma is associated with both ALPS patients and FASL-FAS deficient mice indicating that FASL-FAS is also a barrier to carcinogenesis. Most likely integrated with those functions, FASL-FAS mediates clearance of virus-infected and damaged cells. All together, in vivo and in vitro studies indicate that FASL-FAS is critical for induction of apoptosis and immune cell regulation.

Biochemical studies have led to mechanistic insights of the signaling pathway (Figure 16). FAS is present as an inactive trimer on the surface of healthy cells. FASL binding to FAS induces conformational changes to FAS. This facilitates the recruitment of multiple proteins to the intracellular cytosolic domain of FAS. The assembled multiprotein complex is called the "death-inducing signaling complex" (DISC) (Kischkel et al., 1995). Proteins that were first discovered to make up the DISC are the adaptor FADD (FAS-associated protein with a DD), caspase 8 and the caspase 8-modulator c-FLIP. FAS and FADD both contain "death domains" (DD) that facilitate homotypic protein-protein interactions. FAS-bound FADD then recruits caspase 8 through another

---

factors such as cytochrome c. Upon release, cytochrome c assembles into a molecular platform known as the apoptosome leading to the activation of initiator caspase-9. The BCL-2 protein family consists of antiapoptotic and proapoptotic molecules that function immediately upstream of mitochondria and control the release of apoptogenic factors. The BH3-only subclass of proapoptotic BCL-2 proteins respond to cellular stress and activate the mitochondrial apoptotic program by neutralizing the antiapoptotic members of the family and activating proapoptotic BAX and BAK. Upon activation, BAX and BAK undergo allosteric activation to permeabilize the mitochondrial outer membrane leading to cytochrome c release. The second apoptotic program, the extrinsic pathway, operates downstream of death receptors, including FAS. Activated death receptors then signal to recruit specialized protein complexes to activate initiator caspase-8. For example, a death-inducing signaling complex (DISC) is formed upon the recruitment of adaptor proteins, such as FADD, to activated FAS and the subsequent recruitment and activation of caspase-8. In certain cell types, the extrinsic and intrinsic pathways are linked through a mitochondrial amplification loop, involving caspase-8-mediated cleavage and activation of the BH3-only protein BID, which triggers the intrinsic pathway. Activation of effector caspases by initiator caspase-8 and caspase-9 leads to the cleavage of key cellular substrates and cell demise. Legend and figure taken with permission from Benz et al. (2007).

interaction domain called the "death effector domain" that is present on both FADD and the prodomain of caspase 8. Binding to FADD induces caspase 8 dimerization and conformational change. Caspase 8 oligomerization leads to cross-dimer processing between the large and small subunit (Chang et al., 2003). Active caspase 8 then undergoes auto-cleavage separating the active enzyme from the FADD-bound prodomain, allowing caspase 8 to leave the DISC and access downstream substrates (Boatright et al., 2003). C-FLIP also contains a DED and heterodimerizes with caspase 8, which, depending on the concentration of c-FLIP can either inhibit or stimulate caspase 8 activity. The importance of FADD, caspase 8, and c-FLIP signaling for FAS-induced apoptosis has been demonstrated both biochemically and in whole animal studies (Newton et al., 2000; Varfolomeev et al., 1998; Yeh et al., 2000; Zhang et al., 1998; Zhang and He, 2005). Thus, the extrinsic path-

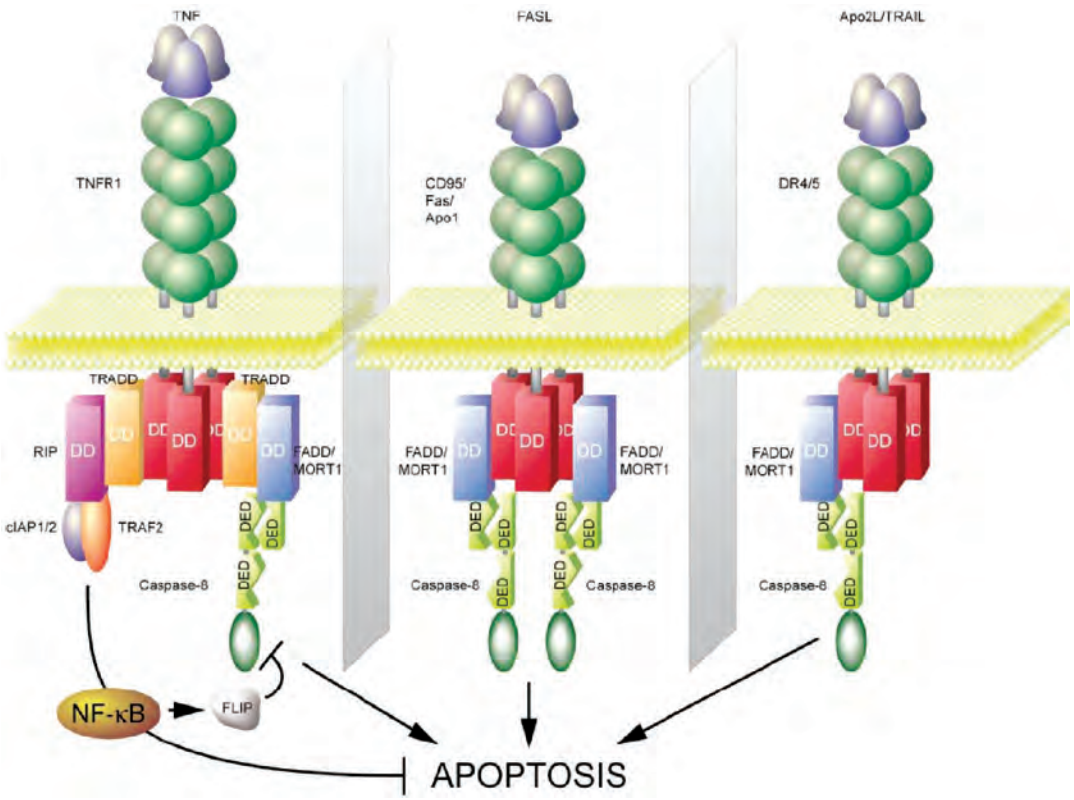


FIGURE 17: Extrinsic death receptor pathways. The distinct composition of the *D*eath-*I*nducing-*S*ignaling *C*omplex (DISC) downstream of the various death receptors TNFR1, CD95, and DR4/5 is illustrated. Legend and figure taken with permission from Danial and Korsmeyer (2004).

way transduces an external apoptotic signal through death receptor stimulation triggering protein-protein interactions that activate caspase 8. Caspase 8 can then cleave downstream caspases and induce apoptosis.

It is now known that the extrinsic pathway is more complex than first described. For example, additional proteins are recruited to the FASL-FAS signaling DISC. These proteins are RIPK1 (receptor-interacting protein kinase 1), cIAPs (cellular inhibitor of apoptosis proteins), and E3 ubiquitin ligases that individually modulate the activity of caspase 8 (Galluzzi et al., 2012). Additionally, TNFR receptors require recruitment of an intermediary protein TRADD (TNFR-associated DD) prior to binding FADD and caspase 8 (Schutze et al., 2008) and TNFR1 also recruits TRAF2/5 (TNFR-associated factor), which initiates activation of the transcription factor NF-kB (Strasser et al., 2009) (Figure 17). Thus, differences in the composition of the protein complexes assembled in response to receptor activation can initiate a death program or alternatively stimulate survival pathways.

The extrinsic pathway triggers a caspase cascade through DISC-mediated caspase 8 activation leading to activation of downstream caspases 3 and 7. While initiator caspases mainly cleave downstream executioner caspases, initiator caspase 8 can also cleave the pro-apoptotic Bcl-2 family member Bid (Li et al., 1998; Luo et al., 1998) (Bid will be discussed in more detail in Section 5.6). Cleavage of Bid initiates caspase activation through an indirect pathway that requires mitochondria. This pathway is known as the "intrinsic apoptotic pathway."

4.2 INTRINSIC SIGNALS

While extrinsic apoptotic signals are delivered from the outside of the cell, intrinsic signals originate from the inside of the cell (Figure 16). Diverse cellular insults, such as DNA damage, growth factor withdrawal, and calcium overload in response to misfolded proteins, among others, independently activate diverse signaling pathways that converge on the mitochondria. These death signals lead to mitochondrial outer membrane permeabilization (MOMP). Pro-apoptotic proteins that are normally contained within the mitochondria leak into the cytoplasm and initiate two distinct apoptotic pathways that are caspase-dependent or -independent. MOMP is considered to be the "point of no return" for cell death (Chipuk et al., 2006). For review, see Vaux (2011).

How was it determined that mitochondria play a central signaling role in apoptosis? Hockenbery et al. (1990) were the first to suggest the involvement of mitochondria when they reported that a regulator of apoptosis protein, Bcl-2, was localized to this organelle. A few years later, using *Xenopus* oocyte extracts, Newmeyer et al. (1994) identified a mitochondrial component that stimulated in vitro DNA fragmentation. Following this observation, Xiadong Wang's group conducted a

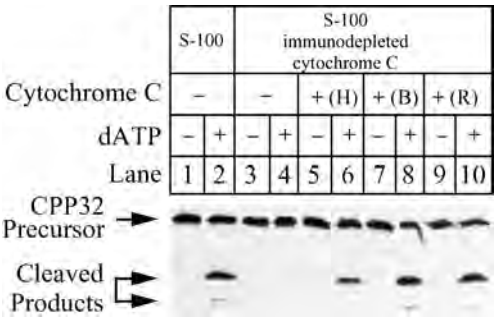


FIGURE 18: Identification of cytochrome c as the active component of apoptotic extracts. (A) HeLa S-100 (lanes 1 and 2), or HeLa S-100 immunodepleted of cytochrome c (lanes 3 and 4), or HeLa S-100 immunodepleted of cytochrome c supplemented with Apaf-2 purified through the Mono S column (H) (lanes 5 and 6), bovine heart cytochrome c (B) (lanes 7 and 8), or rat liver cytochrome c (R) (lanes 9 and 10), were incubated with ^{35}S-labeled CPP32 (caspase 3) in the absence (lanes 1, 3, 5, 7, and 9) or presence (lanes 2, 4, 6, 8, and 10) of dATP. Samples were subjected to SDS–PAGE and transferred to a nitrocellulose filter, which was then exposed to film. Modified legend and figure was taken with permission from Liu et al. (1996).

series of biochemical purification and in vitro reconstitution apoptosis assays. Wang's identification of mitochondrial cytochrome c as an apoptogenic factor cemented the functional contribution of mitochondria to apoptosis.

Identification of a role for cytochrome c in apoptosis was done through an in vitro apoptotic assay wherein addition of dATP to HeLa cytosolic extracts obtained after high-speed centrifugation (S100) resulted in cleavage of caspase 3 (Liu et al., 1996). Biochemical fractionation of S100 revealed that cytochrome c was present in the active fraction. Immunodepletion of cytochrome c from cellular extracts resulted in a loss of caspase activation that was rescued by addition of purified cytochrome c, indicating that cytochrome c was both necessary and sufficient for apoptosis of their in vitro assay (Figure 18). The question remained as to how cytochrome c, which is normally sequestered in the mitochondria, could stimulate cytosolic-localized caspases.

4.2.1 Cytochrome c Induces a Caspase Cascade

Liu et al. demonstrated that cytochrome c leaked out of mitochondria in cells treated with the apoptotic agent staurosporine (Liu et al., 1996), and it is now known that myriad cellular stressors converge on mitochondria, mediating MOMP and release of cytochrome c from the mitochondria.

However, how does cytochrome c lead to caspase activation? Further fractionation studies from the Wang laboratory identified another caspase-activating factor in S100. This factor, Apaf-1, was the mammalian homologue of *C. elegans* CED-4 (Zou et al., 1997). In the presence of cytochrome c and dATP, Apaf-1 formed an oligomeric structure labeled as the "apoptosome," which facilitated caspase activation (Li et al., 1997). Similar to the role of the DISC for extrinsic signaling, Apaf-1 oligomers formed a platform for caspase activation, pointing to the emergence of a common theme of proximity-induced caspase clustering.

Apaf-1 is composed of three domains: a CARD domain, a CED-4-like domain and a WD-40 domain (Figure 19). In healthy cells, Apaf-1 exists as a monomeric molecule. Under conditions of cellular stress when cytochrome c is released into the cytosol, cytochrome c binds to the WD40 domain and dATP binds to the nucleotide-binding region within the CED-4-like domain. This binding induces Apaf-1 to undergo conformational changes (Li et al., 1997) that leads to

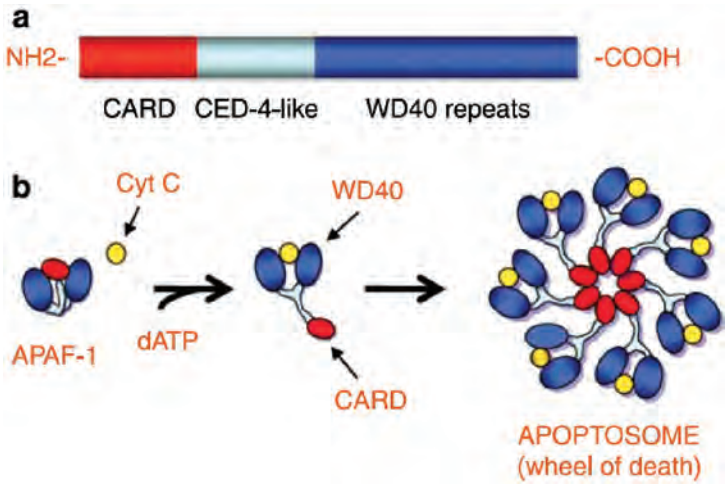


FIGURE 19: The wheel of death: formation of the apoptosome. (a) The Apaf-1 protein consists of three functional units: the CARD, which is responsible for recruiting caspase-9; a nucleotide-binding and oligomerization domain with sequence homology to CED-4, which is responsible for dATP/ATP-dependent oligomerization; and the C-terminal WD40 domain, which is responsible for binding of cytochrome c. (b) Sequential binding of cytochrome c and dATP/ATP converts Apaf-1 from an autoinhibited monomeric state to a heptameric platform for caspase 9 activation. The functional apoptosome adopts a wheel-like structure with the cytochrome c-binding domains protruding like spokes from the central hub of the wheel, which is comprised of the CED-4 and CARD regions. Legend and figure taken with permission from Fadeel et al. (2008).

oligomerization. Seven Apaf-1 molecules assemble to form a heptameric protein complex labeled the apoptosome. Structural studies revealed that the apoptosome is shaped like a wheel and has been dubbed the "wheel of death" (Acehan et al., 2002) (Figure 19). The CED-4-like domain and WD-40 domains form the radiating spokes of the wheel and the CARD domains cluster in the center to form the central hub of the wheel. Pro-caspase 9 also contains a CARD domain in its prodomain and is recruited to Apaf-1 via CARD–CARD interactions. The close proximity of the Apaf-1 CARD domains brings pro-caspase 9 close together facilitating proximity-induced activation of procaspase 9. Activation of initiator caspase 9 leads to activation of downstream caspase 3 and apoptosis.

Elegant biochemical studies defined the intrinsic apoptotic pathway as cytochrome c→Apaf-1→caspase 9→caspase 3. However, the role of cytochrome c in apoptotic signaling was muddied by the fact that cytochrome c has dual functions. Cytochrome c is essential for electron transport and ATP production during oxidative phosphorylation. Conceivably, apoptosis defects in cytochrome c knock-out mice could be due to either depleted ATP levels, or a loss of apoptosome formation. Insightful genetic studies in mice addressed these uncertainties. In order to determine the in vivo relevance of cytochrome c in apoptosis, Tak Mak's group generated knock-in mice that expressed a mutant form of cytochrome c that retained electron transport ability but could not activate Apaf-1 (Hao et al., 2005). The resulting embryos developed severely malformed brains due to acute loss of apoptosis in the developing central nervous system, and did not survive (recall from Section 1.4 that apoptosis normally eliminates over 50% of neurons during brain development). Evidence that the cytochrome c mutant did indeed lose the ability to bind Apaf-1 was demonstrated because in response to multiple apoptotic insults, cells extracted from the embryo showed no Apaf-1 oligomerization and had inhibited apoptosis. These results demonstrated that the ability of cytochrome c to induce apoptosome formation, and not its role in mitochondrial respiration, was critical for apoptosis in multiple cell types, especially neurons. Genetic studies also demonstrated that the cytochrome c→Apaf-1→caspase 9→caspase 3 pathway was biologically important. Similar to cytochrome c Apaf-1-binding-deficient knock-in mice, Apaf-1-deficiency was embryonic lethal with embryos showing similar accumulation of extraneous neurons and protruding masses in the brain (Cecconi et al., 1998; Yoshida et al., 1998). Apaf-1 deficient thymocytes were resistant to various apoptotic insults, despite the expected release of cytochrome c into the cytosol. Finally, mice deficient for caspase 9 or caspase 3 displayed loss of neuronal apoptosis with associated fetal brain deformities (Ranger et al., 2001). All together, these studies demonstrated that the cytochrome c→Apaf-1→caspase 9→caspase 3 pathway was relevant in vivo and essential for neuronal apoptosis.

We have now seen two examples whereby caspase activation is accomplished through recruitment of caspases to adaptor protein complexes; pro-caspase 8 activation through binding to the DISC and pro-caspase 9 activation through binding to the apoptosome. Two other similar

complexes have been discovered in mammals. The "PIDDosome" facilitates pro-caspase 2 activation. The PIDDosome consists of seven RAIDD (receptor-interacting protein (RIP)-associated ICH-1/CED-3-homologous protein with a death domain (DD) molecules complexed to 5 PIDD (p53-induced protein with death domain) molecules (Park et al., 2007b). As well, the "Inflammasome" facilitates the activation of the inflammatory procaspase 1. The inflammasome consists of pro-caspase 1, NALP1 (NACHT-, LRR-, and PYD-containing protein 1) and ASC (apoptosis-associated speck-like CARD protein) (Martinon et al., 2002). Thus, the general theme of caspase activation through adaptor-mediated clustering is revisited many times.

4.2.2 Other Proteins Released from Mitochondria that Contribute to Caspase Cascade

Cytochrome c is not the only protein that is released from the mitochondria of apoptotic cells. Using similar purification strategies, Xiadong Wang's group identified Smac (second mitochondrial activator of caspases) as a component from HeLa cell mitochondria that enhanced in vitro cleavage of caspase 3 (Du et al., 2000). Using a slightly different approach, David Vaux's group was screening for modulators of an apoptotic inhibitor known as XIAP (X-linked Inhibitor of Apoptosis Protein). XIAP is a cytosolic protein that directly binds to active caspase 3, 7 and 9 and inhibits caspase enzymatic activity. (XIAP will be discussed in more detail in section 6.3). Using a co-immunoprecipitation/purification strategy Verhagen et al. identified Diablo (direct IAP binding protein with low pI) as an interacting partner of XIAP (Verhagen et al., 2000). Smac and Diablo turned out to be the same protein. Smac/Diablo is released from apoptotic mitochondria and enhances caspase activity downstream of Apaf-1. Specifically, Smac/Diablo inhibits an inhibitor, and thus indirectly activates caspases. Through an N-terminal IAP Binding Motif (IBM), Smac/Diablo binds and inhibits XIAP-mediated inhibition of active caspases 3 and 9 (Ekert et al., 2001; Srinivasula et al., 2000; Wu et al., 2000).

Unlike the clear contribution of cytochrome c and Apaf-1 to biological apoptotic pathways, the in vivo function of Smac/Diablo is less clear. Mice lacking Smac/Diablo develop normally with no detectable loss in sensitivity to apoptotic insults (Okada et al., 2002). Similarly, XIAP-deficient mice also develop normally with no phenotypic differences in response to apoptotic stimuli (Harlin et al., 2001). A possible explanation for the lack of an apoptotic phenotype in these genetic studies is that both Smac/Diablo and XIAP are redundant and that other proteins compensate for their loss.

A potential protein that could also inhibit XIAP function is HtrA2/Omi. Like Smac/Diablo, HtrA2/Omi was identified as a XIAP-binding protein (Verhagen et al., 2002). HtrA2/Omi is present in the mitochondrial intermembrane space of healthy cells. It functions as a chaperone

and serine protease that refolds or degrades misfolded mitochondrial proteins (Spiess et al., 1999). HtrA2/Omi also contains an IAP-Binding motif (IBM) and once it is released from mitochondria in apoptotic cells, is predicted to inhibit XIAP function similar to Smac/Diablo. However, genetic studies in mice do not support this role for HtrA2/Omi in apoptosis. HtrA2/Omi-deficiency did not lead to apoptotic resistance. Instead, the mice developed lethal neurodegenerative abnormalities, likely a result of mitochondrial misfolded protein accumulation leading to mitochondrial dysfunction (Martins et al., 2004). Further evidence against the idea that Smac/Diablo and HtrA2/Omi have redundant function for XIAP inhibition came with the report that deletion of both genes produced mice with the same phenotype as HtrA2/Omi deficiency alone (Martins et al., 2004). Therefore, while Smac/Diablo and HtrA2/Omi can increase caspase activity in vitro, it does not appear that these proteins function in this capacity in whole organisms.

4.2.3 Caspase-Independent Apoptotic Pathway

In addition to inducing caspase activation, the mitochondrial apoptotic pathway has also been proposed to release proteins that induce cell death independent of caspase activation. AIF (Apoptosis Initiating Factor) is a mitochondrial protein that was first proposed to cause DNA fragmentation independent of caspase activation (Susin et al., 1996). In apoptotic cells, AIF was released from mitochondria, translocated to the nucleus where it directly bound and digested DNA (Ye et al., 2002). However, the evidence that AIF has a physiological role as a pro-apoptotic protein is controversial. Joza et al. concluded that AIF played an essential role in apoptosis, as AIF deficiency in mice led to cellular accumulation during the first wave of developmental programmed cell death—embryoid body cavitation (Joza et al., 2001). However, an alternative model was proposed when it was shown that AIF is an oxidoreductase/NADH oxidase of the mitochondrial electron transport chain (complex I) (Vahsen et al., 2004). A naturally occurring mutant mouse model (Harlequin), which has reduced AIF levels is phenotypically similar to mice with complex I deficiency and do not display cellular accumulation as would be expected if AIF was pro-apoptotic (Klein et al., 2002). In contrast, Harlequin mice display neuronal degeneration that is rescued by AIF expression indicating that AIF has anti-apoptotic activity in the central nervous system. Thus, the role of AIF as a pro-apoptotic mitochondrial protein is not clear.

Another mitochondrial protein that has been proposed to induce caspase-independent cell death is Endo G (Endonuclease G). Endo G is another protein that was identified by Xiaodong Wang's group. They found that Endo G was released from mitochondria, entered the nucleus and cleaved nuclear DNA (Li et al., 2001). Two mouse genetic studies reported opposing results for the role of Endo G as a physiological pro-apoptotic factor. First, Zhang et al. (2003) showed that

EndoG deficiency was embryonic lethal and that cells from EndoG heterozygous mutants had impaired DNA fragmentation and were resistant to multiple cellular stressors. In addition, spontaneous cell death of spermatogonia, thymocytes, and splenocytes was reduced in mutant mice, suggesting a physiological role of EndoG as a pro-apoptotic factor. However, Irvine et al. (2005) created EndoG knock-out mice that did not disturb an overlapping gene of unknown function that had apparently been disrupted in the Zhang study. Irvine reported that their EndoG knockout mice were viable and showed no defect in apoptosis or DNA fragmentation. Therefore, it appears that EndoG does not contribute to mitochondrial apoptotic pathway.

All together, the data indicate that physiological and developmental signals in whole animals activate an intrinsic apoptotic pathway that includes release of mitochondrial cytochrome c leading to activation of Apaf-1 and downstream caspase activation. Smac/Diablo and HtrA2/Omi may contribute to the amplification of the caspase signal, although the genetic validation for this model is not strong. The existence of an intrinsic pathway that leads to caspase-independent cell death through such factors as AIF and EndoG, however, are in question.

The release of proteins from the mitochondria to activate caspases is not a conserved pathway. For example, as already discussed, in mammalian cells apoptotic stimuli cause cytochrome c to be released from mitochondria wherein it binds to the WD-40 region of Apaf-1 contributing to its activation. In *C. elegans*, the Apaf-1-like molecule, CED-4 does not contain WD-40 motifs and is not bound by cytochrome c. Activation of CED-4 occurs spontaneously following removal of its inhibitor CED-9 (Metzstein et al., 1998). In *D. melanogaster*, the Apaf-1 homolog dARK does have WD40 domains, but does not require binding to cytochrome c to activate the *Drosophila* caspase DRONC (Kanuka et al., 1999). Thus, cytochrome c release is not necessary for Apaf-1 activation in lower organisms. What about the requirement for mitochondrial release of Smac/Diablo or HtrA2/Omi as inhibitors of IAP? In *Drosophila*, the antagonists of the IAP homologs DIAP1/2 are Reaper, HID, Grim and Sickle (Kornbluth and White, 2005). These proteins bind and inhibit IAPs in a similar fashion as Smac/Diablo and HtrA2/Omi. However, unlike their mammalian counterparts, Reaper, HID, Grim, and Sickle are located in the cytosol and therefore do not need to be released from mitochondria. Thus, release of mitochondrial proteins does not appear to be necessary for apoptosis in the worm or fly.

4.2.4 Inhibitor of Apoptosis Proteins (IAPs)

Inhibitor of Apoptosis Proteins (IAPs) are a class of proteins that regulate apoptosis, inflammation and cell survival. For review, see (Gyrd-Hansen and Meier, 2010; LaCasse et al., 2008). The first member, OpIAP, was isolated from insect baculovirus based on its ability to inhibit apoptosis in

virus infected cells (Crook et al., 1993). IAP proteins were originally characterized for their ability to inhibit caspase activity, thereby providing a regulatory step for apoptosis downstream of mitochondrial dysfunction. Now, further studies have identified a more complex role for IAPs that includes many aspects of cellular homeostasis.

There are now eight known IAPs in humans, of which three have been shown to inhibit apoptosis through inhibition of caspase activity (Figure 20). All IAPs contain an approximately 70 residue BIR (baculovirus IAP repeat) domain, which mediates protein–protein interactions. BIR domains have differential binding characteristics. For apoptotic inhibition, BIR domains have a peptide-binding groove with a hydrophobic cleft that binds to IBMs (IAP-binding motifs) on the N-terminus of target proteins (Lin et al., 2007; Liu et al., 2000). The target protein IBM is a tetrapeptide domain with an N-terminal alanine residue followed by conserved residues. These sequences are found on caspases 3, 7, and 9 and Smac/Diablo. Additionally, some IAPs contain RING finger domains that confer ubiquitin E3 ligase activity, UBA domains that enable interactions with ubiquitylated proteins and CARD (CAspase Recruitment Domains) domains.

The IAPs, XIAP, cIAP1, and cIAP2 inhibit apoptosis. XIAP is the only mammalian IAP that is a direct inhibitor of caspases (Eckelman et al., 2006). XIAP binds and inhibits caspases 3/7

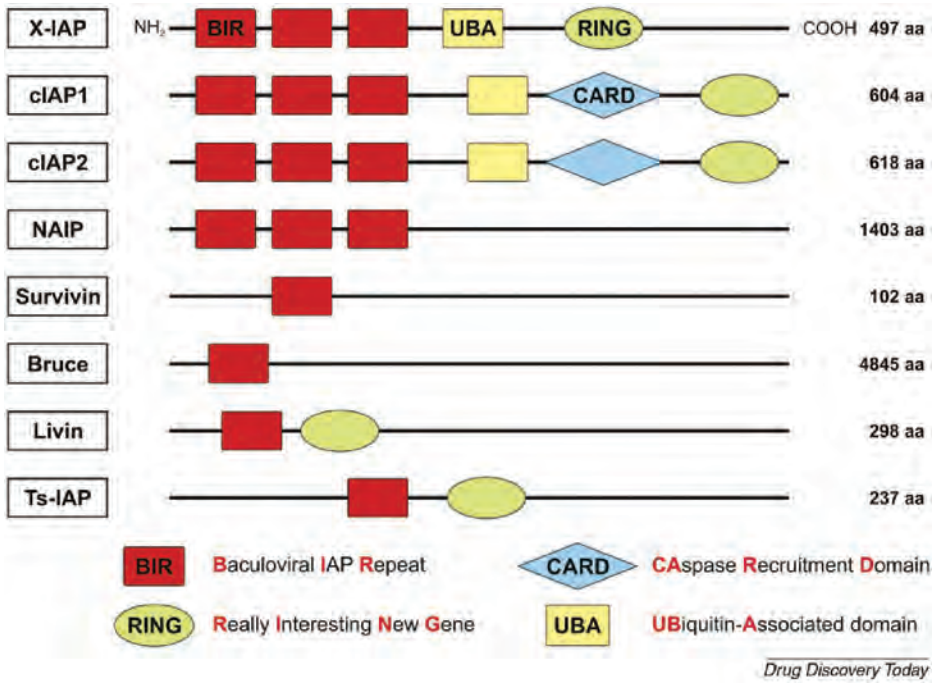


FIGURE 20: The IAP-family. Figure taken with permission from Mannhold et al. (2010).

and caspase 9, although the mechanism of inhibition differs for the executioner versus initiator caspases. For caspase 3/7 inhibitions, two distinct regions of XIAP are important. The BIR2 domain of XIAP interacts with caspase 3 at the newly generated caspase 3 N-terminus of the small subunit that is created upon cleavage by upstream initiator caspases (Riedl et al., 2001; Scott et al., 2005). Additionally, the XIAP linker region between the BIR1 and BIR2 domains binds the catalytic site of caspases 3 or 7, sterically hindering substrate entry (Sun et al., 1999). As one recalls, the caspase active site is created through procaspase cleavage and rearrangement, meaning that XIAP inhibits "active" caspase 3/7 heterotetramers (Figure 12). XIAP-mediated inhibition of caspase 9 is distinctly different. The XIAP BIR3 domain binds to caspase 9 monomers at the region that is required for homodimerization (Shiozaki et al., 2003; Srinivasula et al., 2001). Thus, XIAP-caspase 9 heterodimers prevent formation of the caspase 9 catalytic site.

Investigations into the physiological role of XIAP have not led to a clear picture of XIAP function. As mentioned previously, XIAP knock-out mice were not phenotypically different from wild-type mice with respect to caspase activation and apoptosis (Harlin et al., 2001). However, these mice did show increased levels of c-IAP1/2, which could conceivably compensate for XIAP loss and mask apparent XIAP function. More specific gene-targeting knock-in of an endogenous XIAP gene lacking the RING finger showed that these mice had increased caspase activity and apoptosis in response to death signals (Schile et al., 2008). XIAP and cIAP1/2 have been shown to mono and polyubiquitylate caspases 3 and 7. The functional consequence of monoubiquitylation is unclear, but polyubiquitylation has been linked to degradation and inactivation (Schile et al., 2008; Suzuki et al., 2001). These results suggest that XIAP ubiquitylation activity may contribute to anti-apoptotic function.

• • • •

CHAPTER 5

The Bcl-2 Family

The Bcl-2 family of proteins is a major regulator of apoptosis. Bcl-2 proteins interpret cellular stress signals and coordinate the release of apoptogenic factors from mitochondria. Thus, they are considered as gatekeepers of the mitochondrial apoptotic pathway. The first member of this family, Bcl-2, was identified as an oncogene in follicular lymphomas. Subsequent studies uncovered a direct link between apoptosis and carcinogenesis. This novel concept attracted the interest of many investigators and their combined work has elucidated the basic biology of the apoptotic pathway. The emerging picture is that of a complex cellular machine, that employs novel cell signaling concepts and may hold the key for the development of novel cancer therapeutics. For review, see Chipuk et al. (2010), Danial (2007), Taylor et al. (2008), and Youle and Strasser (2008).

5.1 THE DISCOVERY OF BCL-2

The *t*(14;18) reciprocal chromosomal translocation is a common cytogenetic abnormality of human lymphoma, present in about 85% of follicular lymphoma and up to 30% of diffuse lymphomas (Rowley, 1982; Yunis, 1983). Thus, the development of this form of cancer is likely driven by aberrations in the genes affected by this physical translocation. In 1985, three research groups reported the molecular characterization of this chromosomal rearrangement (Bakhshi et al., 1985; Cleary and Sklar, 1985; Tsujimoto et al., 1985). The translocation placed the immunoglobulin gene enhancer from chromosome 14 close to a gene on chromosome 18. The gene was given the name of *BCL-2* (B-cell lymphoma-2). Because of the strong association of *BCL-2* translocation with lymphoma, *BCL-2* was considered as a putative oncogene. Oncogenes as defined by Michael Bishop and Harold Varmus (Nobel Laureates, 1998) are cancer-causing genes whose protein products cause cellular proliferation and cancer. By all accounts then, *BCL-2* expression should induce cell division. However, Bcl-2 was not able to stimulate cellular proliferation and hence did not easily fit into the classical model of an oncogene. Was Bcl-2 an oncogene that contributed to cancer?

The answer to this question was found in two seminal reports that were published a few years later. In 1988, David Vaux, then a graduate student in the laboratory of Jerry Adams, was exploring the biological role of Bcl-2. He ectopically expressed *bcl-2* in cultured hematopoietic

cells that were dependent on the growth factor IL-3 (interleukin 3) for survival (Vaux et al., 1988). Vaux et al. found that *bcl-2* promoted the survival of cells after growth factor withdrawal, whereas non-*bcl-2* expressing cells died. Notably, while *bcl-2* expressing cells remained viable, they did not proliferate. This result was distinctly different from the effect of ectopic expression of the classical oncogene *myc*, which did not promote survival in the absence of factor (Figure 21). One year later, Stanley Korsmeyer's group created transgenic mice mimicking the *t*(14:18) translocation in order to directly test Bcl-2 overexpression in an in vivo model (McDonnell et al., 1989). They observed B-cell follicular hyperplasia characterized by extended cell survival. Importantly, over time many of the mice developed diffuse large B-cell lymphoma (McDonnell and Korsmeyer, 1991). This was the first definitive piece of evidence that inhibiting cell death could cause cancer. According to Benz and Nathan (Benz and Nathan, 2007), "For those of us who struggled with the inconsistencies of cancer, Stan's discovery was a bombshell" indicating that "cancer could also be

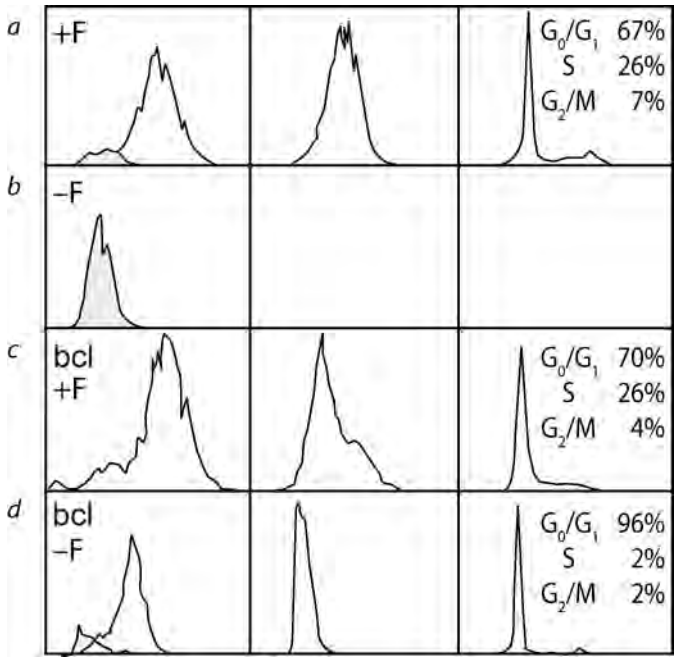


FIGURE 21: Bcl-2 inhibits cell death that normally occurs in response to growth factor withdrawal. Flow cytometric analysis of FDC-P1 cells cultured with or without factor (+/− F). The left column shows cell-size with dead cells (propidium-iodide-stained) shaded. The middle column shows nuclear size and the right column shows DNA content indicative of cell cycle stage, as indicated. Modified legend and modified figure taken with permission from Vaux et al. (1988).

caused by mutations that induce immortality in cells." Furthermore, Korsmeyer's results addressed a long-standing puzzle about follicular lymphoma. Unlike most cancers that were characterized by increased numbers of mitotic cells, follicular lymphoma showed normal mitotic indices (Benz and Nathan, 2007). Korsmeyer's discovery that cancer could arise from a lack of cell death explained the curious lack of mitotic cells in these cancers. This paradigm shift introduced a new concept and class of oncogenes that did not promote proliferation but blocked apoptosis.

Stanley Korsmeyer and his colleagues helped transform cancer research. After identifying the association of Bcl-2 with cancer, Korsmeyer explored the role of mitochondria in apoptosis and identified critical Bcl-2-interacting proteins, Bax, Bad, and Bid. A pioneer in this field, Stanley Korsmeyer died of cancer in 2005 at the age of 54. His legacy is that he defined a connection between apoptosis and cancer and paved the way for investigations into new forms of cancer treatment that were conceived from a solid foundational understanding of basic biological processes.

5.2 BCL-2 FAMILY MEMBERS

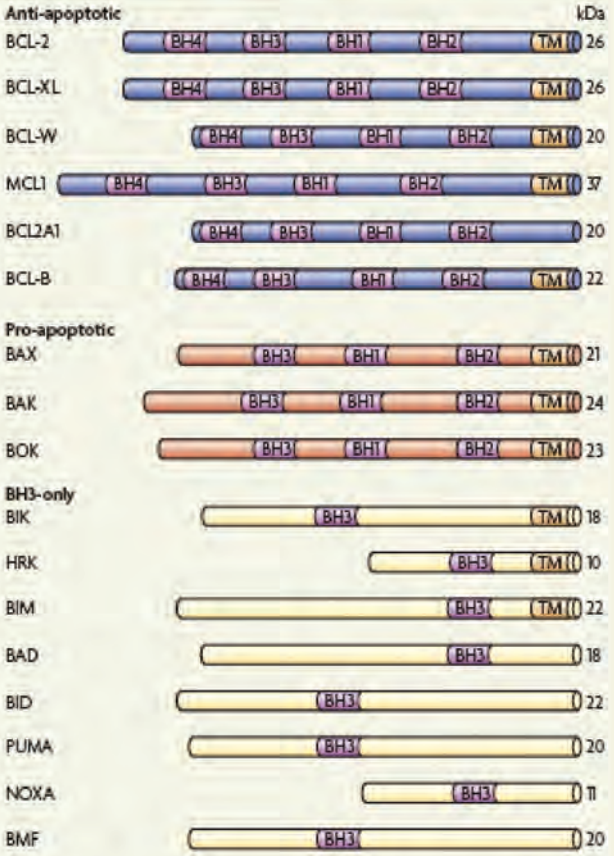
Since the original identification of Bcl-2, the family has grown. Multiple investigators undertook protein interaction screens and homology cloning to identify a growing list of Bcl-2-like proteins. In general, the family is made up of anti-apoptotic and pro-apoptotic proteins. See Figure 22. Within the group of pro-apoptotic proteins, the family is further subdivided into effector multi-domain Bax (BCL-2-associated x protein) or Bak (BCL-2 antagonist killer 1) proteins and BH3-only proteins (proteins that contain only the third Bcl-2 homology-BH-domain).

The defining feature of family members is the possession of conserved sequence homology regions known as BCL-2 homology (BH) domains (from 1 to 4). These BH domains are α-helical segments that define the structure and function of the protein. All anti-apoptotic members and some pro-apoptotic members (Bax, Bak, Bok) contain BH domains 1–3, with some of these members also possessing a BH4 domain. The BH3-only proteins are pro-apoptotic and as their name implies, only contain the BH3 domain, which is regarded as the minimal death domain. All anti-apoptotic and pro-apoptotic multi-domain and a subset of BH3-only (Bim, Bik, HRK) proteins contain a C-terminal transmembrane (TM) domain. This hydrophobic domain facilitates binding to intracellular membranes, such as mitochondria, endoplasmic reticulum, and nuclear membranes.

Why so many family members? Partly because apoptosis is so critical for organism development and tissue homeostasis, multiple members serve as redundant "back-ups" for others. A likely more prominent role is that because of organism complexity, each Bcl-2-family member responds to specific developmental cues, or environmental stressors. Specific intracellular localization, tissue-specific expression and regulated expression during development support this idea of specialized function. Additionally, genetic studies in mice support roles for specification and redundancy.

Box 1 | **The BCL-2 family**

B-cell lymphoma-2 (BCL-2)-family proteins have a crucial role in the regulation of apoptosis through their ability to regulate mitochondrial cytochrome c release. The BCL-2 family comprises three subfamilies that contain between one and four BCL-2 homology (BH) domains (see figure). The anti-apoptotic subfamily comprises proteins that contain four BH domains. Most members of this subfamily also contain transmembrane domains (TM) and are therefore typically associated with membranes. The pro-apoptotic BAX-like subfamily lacks BH4 domains and promotes apoptosis by forming pores in mitochondrial outer membranes. The BH3-only subfamily is a structurally diverse group of proteins that only display homology within the small BH3 motif.



BH3-only proteins

The mammalian BH3-only protein family currently comprises eight members (BID, BAD, BIM, BIK, BMF, NOXA, PUMA and HRK), all of which promote apoptosis when overexpressed. These proteins share little sequence homology apart from the BH3 motif, and are regulated in distinct ways.

NOXA, PUMA and, to a lesser extent, BID are transcriptionally upregulated by p53. DNA damage therefore results in the increased synthesis of these BH3-only proteins in a p53-dependent manner. BID is activated through proteolysis by caspase-8. BAD is inactivated through growth-factor-receptor signals that result in phosphorylation of this BH3-only protein at several sites, leading to sequestration by 14-3-3 proteins. BIM is tethered to microtubules and BMF is tethered to actin microfilaments, possibly through interactions with dynein light chain-1 and dynein light chain-2, respectively, although this remains to be conclusively demonstrated. Disruption of the cytoskeleton can liberate these proteins to promote apoptosis. Growth factors stimulate the ERK-dependent phosphorylation of BIM, and this results in proteasome-mediated degradation of this BH3-only protein. Conversely, growth factor withdrawal results in a decline in ERK signalling and elevated levels of BIM.

Anti-apoptotic BCL-2-family members

BCL-2 and its close relatives (BCL-XL, MCL1, BCL2A1, BCL-W and BCL-B) have four BCL-2 homology (BH) domains and all block apoptosis. The anti-apoptotic BCL-2 proteins block apoptosis by preventing BH3-only protein-induced oligomerization of the pro-apoptotic BCL-2-family members BAX and/or BAK in mitochondrial outer membranes, which would otherwise lead to the efflux of cytochrome c and other mitochondrial intermembrane space proteins. The anti-apoptotic BCL-2 proteins differentially bind to the BH3-only proteins. Some BH3-only proteins (for example, BID and BIM) interact with essentially all anti-apoptotic BCL-2 proteins, whereas others (for example, NOXA) interact only with certain BCL-2-family members.

BAD, BCL-2 antagonist of cell death; BAK, BCL-2-antagonist/killer-1; BAX, BCL-2-associated X protein; BID, BH3-interacting domain death agonist; BIK, BCL-2-interacting killer; BIM, BCL-2-like-11; BMF, BCL-2 modifying factor; BOK, BCL-2-related ovarian killer; ERK, extracellular signal-regulated kinase; HRK, harakiri (also known as death protein-5); PUMA, BCL-2 binding component-3.

FIGURE 22: The Bcl-2 family. Figure taken with permission from (Taylor et al., 2008).

5.3 MOUSE KNOCK-OUT STUDIES DEMONSTRATE SPECIALIZATION OF FUNCTION AND OVERLAPPING FUNCTION

Mouse knock-out studies have given physiological context to the role of individual members of the Bcl-2 family (Table 2). As expected, deletion of anti-apoptotic genes produced animals that showed signs of enhanced cell death. However, the affected organs were different, indicating specialized function of each anti-apoptotic protein. For example, loss of *BCL-2* caused enhanced death of kidney and melanocyte progenitors, and mature B and T lymphocytes. The resulting animals succumbed to fatal polycystic kidney disease, were prematurely grey, and had abnormally low levels of circulating lymphocytes (Veis et al., 1993). Interestingly, all of these effects were rescued by deletion of the BH3-only protein Bim, indicating that Bim and Bcl-2 were linked in a genetic signaling pathway. Loss of *BCL-XL* was embryonic lethal with enhanced death of fetal erythroid progenitors and neuronal cells (Motoyama et al., 1995). *MCL-1* deletion prevented implantation, and conditional knockout animals had premature death of immature B and T lymphoid cells and hematopoietic stem cells (Rinkenberger et al., 2000). All together, these results demonstrated the prevalence of apoptosis regulation in developing systems, especially in hematopoietic and neuronal cells. As described previously, these cell types have some of the highest rates of apoptosis and so it is not surprising that these cells are the most affected by loss of anti-apoptosis proteins. These negative regulators of apoptosis are the "brakes" that keep cell death in check, because, as seen in the knock-out animals, unchecked apoptosis can lead to severe pathologies and death.

In contrast, we would expect that deletion of pro-apoptotic genes would produce animals with diminished cell death and tissue overgrowth. Likely, these defects would be most apparent in hematologic and neuronal cells. Loss of the pro-apoptotic *BAX* gene produced live animals with lymphoid hyperplasia (Knudson et al., 1995). Mice born with deletion of the pro-apoptotic *BAK* gene had no phenotype (Lindsten et al., 2000). The mild phenotypes of the *BAX* and *BAK* deficient mice suggested that the two encoded proteins had overlapping function, and if this was so, the double-deficient mice should show stronger aberrations in cell death. This did turn out to be the case, as most of the *BAX/BAK* double knock-out (DKO) mice were not viable (Lindsten et al., 2000). These mice displayed multiple developmental defects, such as persistence of interdigital webs, and hyperplasia within the central nervous and hematopoietic systems. As well, fibroblasts harvested from mouse embryos were resistant to all apoptotic stimuli that activate the intrinsic pathway, indicating that Bax and Bak are the requisite gatekeepers of MOMP.

Deletions of BH3-only genes were mostly non-lethal and showed tissue-specific and stress-specific phenotypes. These results demonstrated a division of labor among the individual family members of this group. As examples, *BID*-deficient mice were resistant to Fas-induced apoptosis of

TABLE 2: BCL-2 family mouse model phenotypes

GENE	MUTATION	PHENOTYPE	COMBINATION PHENOTYPE	REFERENCE
ANTI-APOPTOTIC BCL-2 PROTEINS				
a1	Knockout	Nuetrophils display enhanced spontaneous apoptosis and lack LPS-induced apoptosis inhibition	-NA-	Hamasaki et al., 1998 Orlofsky et al., 2002
bcl-2	Knockout	Increased postnatal mortality, polycystic kidneys, apoptotic involution of thymus and spleen, graying of hair follicles, reduced number of neurons, small size	**with *bik-/-* :** No additional phenotype **with *bim-/-* :** Heterozygous knockout rescues pigment defect; homozygous knock-out further rescues kidney phenotype (see below)	Coultas et al., 2004 Veis et al., 1993 Boulliet et al., 2001
bcl-w	Knockout	Male sterile due to spermatogenesis defects	-NA-	Print et al., 1998
bcl-x	Knockout	Lethal at embryonic day E13.5 due to massive apotosis of hematopoietic and neuronal cells	-NA-	Motoyama et al., 1995
mcl-1	Knockout	Embryonic lethal due to failure of the blastocyst to implant	-NA-	Rickenberger et al., 2000
PRO-APOPTOTIC EFFECTORS				
bak	Knockout	None	**with *bax-/-* :** Pre-/peri-natal lethality; interdigital webbing; behavioral, he-matopoetic, homeostatic, immune, nervous system and reproductive defects; cell resistant to apotosis **with *bim-/-* :** Increased white blood cells	Lindsten et al., 2000 Hutcheson et al., 2005

TABLE 2: (*continued*)

GENE	MUTATION	PHENOTYPE	COMBINATION PHENOTYPE	REFERENCE
ANTI-APOPTOTIC BCL-2 PROTEINS				
bax	Knockout	B and T cell hyperplasia; abnormal germ cells and gonad morphology; prolonged ovarian lifespan; reduced CNS and PNS cell death	**with *bax-/-* :** See above **with *bim-/-* :** Inter-digital webbing; male infertility; increase white blood cells (see below)	Hutcheson et al., 2005 Knudson et al., 1995 Wei et al., 2001
BH3-ONLY PROTEINS				
bad	Knockin (3S→3A)	Growth factor-dependent survival is reduced in immune and nervous cells	-NA-	Datta et al., 2002
	Knockin (S155A)	Abnormal insulin secretion	-NA-	Danial et al., 2008
	Knockout	Animals develop diffuse large B cell lymphoma	-NA-	Ranger et al., 2003
bid	Knockout	Animals are resistant to anti-Fas ligation	**with *bim-/-* :** No additional phenotype (see below)	Yin et al., 1999 Willis et al., 2001
bik	Knockout	None	**with *bcl-2-/-*** See above **with *bim-/-* :** Male infertility	Coultas et al., 2005
bim	Knockout	Accumulation of lymphoid and myeloid cells; development of autoimmune kidney disease; females display imperforated vaginas	**with *bak-/-* :** See above **with *bax-/-* :** See above **with *bid-/-* :** See above **with *bik-/-* :** See above **with *bcl-2-/-* :** See above **with *noxa-/-* :** Abnormal NK cell morphology	Bouillet et al., 1999 Huntington et al., 2007

TABLE 2: (*continued*)

ANTI-APOPTOTIC BCL-2 PROTEINS				
GENE	**MUTATION**	**PHENOTYPE**	**COMBINATION PHENOTYPE**	**REFERENCE**
bmf	Knockout	Lymphocytes protected from apoptosis induced by glucocorticoids or histone deacetylase inhibitors; mice develop B-cell restricted lymphadenopathy; increased rate of gamma-irradiation-induced thymic lymphomas	-NA-	Labi et al., 2008
bnip3L/nix	Knockout	Abnormal morphology, lack of mitochondrial clearance, decreased numbers and increased fragility of reticulocytes and erythcrocytes	-NA-	Diwan et al., 2007 Schweers et al., 2007
brk	Knockout	Motorneurons are protected from cell death induced by resection of the hypoglossal nerve; delayed cell death of superior cervical ganglia neurons triggered by nerve growth factor withdrawal	-NA-	Imaizumi et al., 2004 Coultas et al., 2007
noxa	Knockout	MEFs and thymocytes display mild resistance to etoposide	**with *bim-/-* :** see above	Villunger et al., 2003
puma	Knockout	Lymphocytes protected from apoptosis; thymocytes, neurons and MEFs resistance to DNA-damage induced apoptosis	-NA-	Jeffers et al., 2003 Villunger et al., 2003

Bcl-2 family mouse model phenotypes. Reference citations correspond to references from the original publication (Chipuk et al. 2010). Taken with permission from (Chipuk et al. 2010).

hepatocytes, although they showed normal lymphoid cell numbers with no increased resistance to apoptotic insults (Kaufmann et al., 2007; Yin et al., 1999). On the other hand, *BAD*-deficient mice were slightly resistant to the apoptotic signal of growth factor deprivation, and had lymphoid hyperplasia that progressed to diffuse lymphoma in aged animals (Ranger et al., 2003). *PUMA*-deficient and also *NOXA*-deficient mice had cell types that were resistant to DNA damage (Jeffers et al.,

2003; Villunger et al., 2003). However, *BIM*-deficient mice were only mildly resistant and *BID*-deficient mice showed no resistance to DNA damage, indicating that individual BH3-only members were responsive to different apoptotic signals. Of the BH3-only knock-out mice, *BIM*-deficient mice had the most severe phenotype with hyperplasia of lymphoid and myeloid cells developing into fatal SLE-like autoimmune disease. This phenotype is similar to what was seen with loss of function of Fas/FasL, validating the critical role of apoptosis in immune cell homeostasis (Bouillet et al., 1999). All together, these results demonstrated the physiological role of BCL-2 family members. Multiple members compensate for the loss of function of others, and also importantly give specialized function.

5.4 PROTEIN–PROTEIN INTERACTIONS BETWEEN BCL-2 FAMILY MEMBERS DICTATE FUNCTION

Many of these Bcl-2 family members were identified through protein–protein interaction screens, so it was clear that each class of proteins must modulate each other's functions. Structural studies delivered insights at the molecular level to how these proteins interacted. In 1996, Stephen Fesik's group published the crystal and solution structure of Bcl-XL (Muchmore et al., 1996). Its structure consisted of two central hydrophobic α-helices surrounded by amphipathic helices. Particularly striking was the close arrangement of the helices of the BH1, BH2 and BH3 domains that created an elongated surface exposed hydrophobic cleft. Fesik's group hypothesized that this hydrophobic face might be an interaction site for other Bcl-2 family proteins. Subsequent structural studies demonstrated that indeed this cleft was a binding site for the BH3 domain of a partner Bcl-2-like protein. These early discoveries set the stage for investigations that unraveled the complex Bcl-2 family signaling network and identified molecular interactions that formed the basis for rational drug design.

It is now known that in vertebrates, the Bcl-2 family forms the requisite gateway through which cell death signals are transduced to the mitochondria. Pro-apoptotic proteins stimulate MOMP (mitochondrial outer membrane permeabilization), while anti-apoptotic proteins inhibit MOMP. The general understanding is that latent BH3-only proteins are activated in response to cellular stress and transmit a death signal to the mitochondria by either inhibiting anti-apoptotic proteins or stimulating pro-apoptotic multi-domain proteins. Depending on the strength of the apoptotic insult and the relative concentrations of pro- versus anti-apoptotic proteins, the multi-domain pro-apoptotic proteins Bax and/or Bak form pores in the mitochondrial outer membrane and induce MOMP. This induces the release of mitochondrial apoptogenic factors such as cytochrome c, eventually leading to caspase activation and death of the cell. For review, see Chipuk et al. (2010) and Danial (2007).

A number of models have been put forth to explain the interaction patterns of the different classes of Bcl-2 family members (Figure 23b). The indirect activation model proposes that the major function of the anti-apoptotic proteins is to inhibit the pore-forming activity of Bax and Bak (Adams and Cory, 2007b). BH3-only proteins initiate apoptotic signaling by binding anti-apoptotic proteins and displacing Bax and Bak. In other words, the BH3-only proteins are inhibitors of inhibitors. Liberated Bax and Bak then induce MOMP. Within this model, the BH3-only family is further subdivided into two classes. Bid, Bim and Puma form one class that can bind to all anti-apoptotic proteins and therefore is the stronger activator of the two classes. On the other hand, Bad, Bmf, and Noxa only bind to a subset of anti-apoptotic proteins. Bad, Bmf, and Bik bind to the anti-apoptotic proteins Bcl-2, Bcl-XL, and Bcl-w, whereas Noxa binds to Mcl-1 and A1

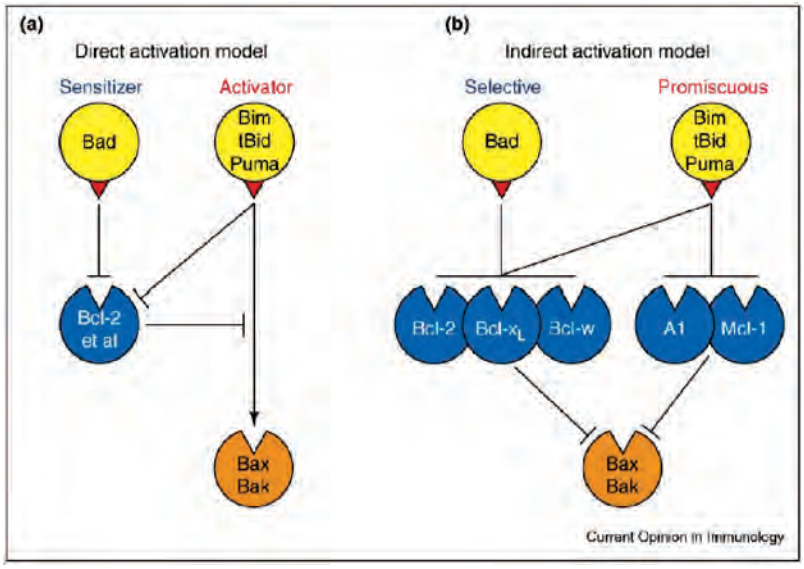


FIGURE 23: The direct and indirect activation models for how BH3-only proteins activate Bax and Bak. (a) In the direct activation model, the indicated activator BH3-only proteins, via their BH3 domain (red triangle), directly engage Bax and Bak and activate them, whereas sensitizer BH3-only proteins (e.g., Bad or Noxa), which can only bind the pro-survival proteins, serve only to displace activators from the pro-survival proteins. (b) In the indirect activation model, the BH3-only proteins only bind the pro-survival proteins. Because the promiscuous binders (Bim, tBid, and Puma) can neutralize all pro-survival proteins, each can readily trigger Bax/Bak activation, whereas any selective binder (e.g., Bad) must be co-expressed with a complementary binder (e.g., Noxa) to do so. Modified legend and figure taken with permission from Adams and Cory (2007a).

(Chen et al., 2005). Thus, apoptosis is ensured with activation of Bid, Bim, and Puma, alone, or, for example, with combined activation of Bad and Noxa (Willis et al., 2005).

The direct activation model also proposes two classes of the BH3-only proteins, however, with distinct mechanisms of activation (Kuwana et al., 2005; Letai et al., 2002) (Figure 2a). Activator BH3-only proteins (Bid, Bim, and Puma) bind not only to the anti-apoptotic proteins, but also to the multi-domain pro-apoptotic Bax and Bak. Unlike the indirect model, this model proposes that binding of activator BH3-only proteins to Bax and Bak can directly induce conformational changes leading to MOMP. The other class of BH3-only proteins (Bad, Bmf Bik, and Noxa), are called the sensitizers or derepressors and function to bind to anti-apoptotic proteins and release activator BH3-only proteins that are free to directly stimulate Bax or Bak.

The stumbling block for the acceptance of the direct activation model was that the interaction between BH3-only proteins and Bax or Bak, was initially difficult to visualize. Thus, proponents of the direct model rationalized that the association was transient—a "hit and run" (Wei et al., 2000). Since then, numerous groups have reported direct binding of Bax and Bak to BH3-only proteins or peptides, leading to a general acceptance of this model (Cartron et al., 2004; Gavathiotis et al., 2008; Kim et al., 2006; Letai et al., 2002; Walensky et al., 2006). Furthermore, triple Bim, Bid, and Puma knock-out mice were phenotypically similar to Bax/Bak DKO mice (Ren et al., 2010). This result suggested that Bax or Bak were dependent on direct activation by Bim, Bid, or Puma, thus supporting the direct activator model. If BH3-only proteins functioned only through an indirect activation model, the presence of other BH3-only proteins should have compensated for the genetic loss in the triple knock-out mice. In all likelihood, apoptotic signaling incorporates aspects of both the indirect and direct activation models. David Andrew's group has suggested further refinement by the "embedded together" model. This group used in vitro reconstitution systems to identify that interactions between Bcl-2 family members occurs both in the cytosol and continue to progress in the lipid bilayer. Thus, these distinct environments confer distinct regulatory mechanisms (Basanez and Hardwick, 2008; Billen et al., 2008). According to Andrews' group, BH3-only proteins directly regulate both anti- and pro-apoptotic Bcl-2 family members. Binding results in conformational change of Bcl-2 associated with insertion of multiple membrane spanning helices, which can inhibit Bax or Bak. Thus, this model has expanded upon and incorporates aspects of both the originally proposed direct and indirect models.

5.5 THE BAX, BAK PORE

Apoptotic signals converge on the mitochondria. Cells that are depleted of Bax and Bak have a severely compromised cell death response to multiple apoptotic stimuli. Specifically, these cells do not induce MOMP, indicating that Bax and Bak play central roles in membrane permeabiliza-

tion. A clue to how Bax could accomplish this task first came from the structure of its inhibitor, Bcl-XL. The structure of Bcl-XL was reminiscent of the membrane translocation domain of bacterial toxins. Surprisingly, the structure of Bax was found to be very similar to Bcl-XL (Petros et al., 2004). Therefore, it was suggested that perhaps Bax had pore-forming properties that contributed to MOMP with the subsequent apoptotic release of mitochondrial proteins. Since those early discoveries, how Bcl-2 family proteins induce MOMP has been the focus of intense study.

In general, there are two mechanisms whereby MOMP occurs. The first is through assembly of pore structures in the mitochondrial outer membrane composed of oligomers of either Bax or Bak. The second occurs through induction of mitochondrial permeability transitions that results in mitochondrial swelling and non-specific rupture of the outer membrane. Both models result in the release of mitochondrial apoptogenic proteins. Bcl-2 family members regulate both activities.

Novel insights into the mechanism of Bax-mediated MOMP have been brought to light by in vitro biophysical and structural analyses. Donald Newmeyer's group was the first to use a stepwise reductionist approach to investigate the putative pore-forming ability of Bax (Kuwana et al., 2002) (Figure 24). Using isolated mitochondria, resealed mitochondrial outer membrane vesicles and defined liposomes, this group demonstrated that a combination of the BH3-only protein Bid and Bax

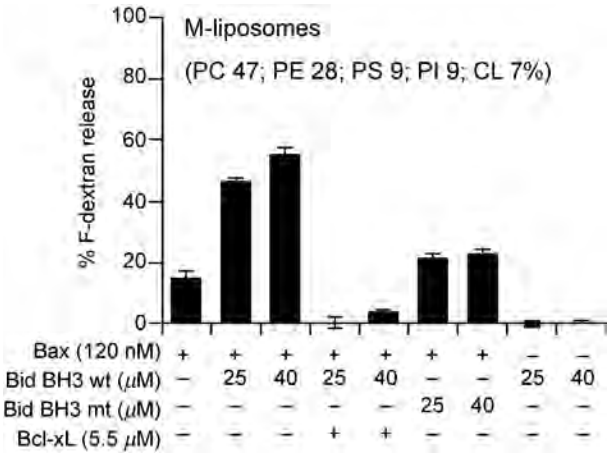


FIGURE 24: In vitro reconstitution assays for analysis of ability of Bax to permeabilize lipid membranes. A Bid BH3 domain peptide can activate Bax to induce permeabilization of liposomes. Dextran-loaded liposomes were treated with the indicated concentrations of wild-type and (less active) mutant Bid BH3 peptide, monomeric Bax, and BclxL. The lipid compositions of liposomes, estimated by thin-layer chromatography, are indicated at the top; and mimic mitochondrial membranes. Modified legend and figure taken with permission from Kuwana et al. (2002).

could induce membrane permeabilization that was inhibited by Bcl-XL. Thus, this reconstituted system faithfully reproduced the effects of Bcl-2 family proteins. Their results demonstrated that Bcl-2 family proteins by themselves could permeabilize lipid bilayers, and that BH3 domains could directly activate Bax. Furthermore, permeabilization was measured by the release of fluorescently conjugated dextran molecules (larger than cytochrome c), suggestive of the formation of a multimeric pore. While an oligomeric Bax pore has not been directly demonstrated in cells, Bax pores have been induced in lipid bilayers in the presence of supraphysiological Ca^{2+} concentrations (Epand et al., 2002). While Bax has been demonstrated to permeabilize membranes, how do apoptotic signals activate Bax?

Upstream apoptotic signals activate Bax and Bak by inducing protein conformational change that leads to protein homo-oligomerization. The specific mechanism of activation is different for either protein. In healthy cells, Bax is a monomeric protein that is either soluble in the cytosol, or loosely attached to the mitochondrial outer membrane (Goping et al., 1998; Wolter et al., 1997). A surface-exposed hydrophobic cleft of Bax interacts with its own C-terminal tail thus preventing Bax from integrating into mitochondrial membranes in the absence of apoptotic stimuli (Suzuki et al., 2000). Structural studies demonstrate that the α-helical BH3-domain peptide of Bim binds to the opposite face of the Bax monomer and induces a conformational change that exposes an N-terminal epitope (Figure 25). It is predicted that these conformational changes releases the C-terminal tail allowing Bax insertion into mitochondrial membranes. This is followed by the membrane insertion of additional α-helices facilitating Bax oligomerization and pore formation in the mitochondrial outer membrane (Annis et al., 2005) (Figure 26). In addition to protein–protein interactions protein–lipid interactions also regulate Bax pore-forming ability (Chipuk et al., 2012; Kuwana et al., 2002).

Bak activation differs. In healthy cells, Bak exists as an inactive monomer that is already integrated into the mitochondrial outer membrane. The mitochondrial protein VDAC (voltage-dependent-anion channel) binds and maintains Bak in an inactive state (Cheng et al., 2003). Apoptotic signals induce BH3-only proteins to bind to Bak, dislodging VDAC, facilitating Bak oligomerization and pore formation (Kim et al., 2006). Other groups have shown that Bak is also inhibited by binding to the anti-apoptotic proteins Mcl-1 and Bcl-XL, which are displaced in response to BH3-only protein binding (Willis et al., 2005). In addition to the requirement for BH3-only protein involvement, Bak activation is also stimulated by the sphingolipid product sphingosine-1-phosphate, implicating involvement of ER lipid metabolism in apoptotic signaling (Chipuk et al., 2012).

Homotypic oligomerization of Bax or Bak molecules requires a series of steps (Figure 26). Conformational changes within their N-termini expose previously buried BH3-domains that can now interact with the surface hydrophobic grooves of neighboring Bax or Bak molecules. Additional "back-to-back" interactions between dimers facilitates the formation of higher-order structures

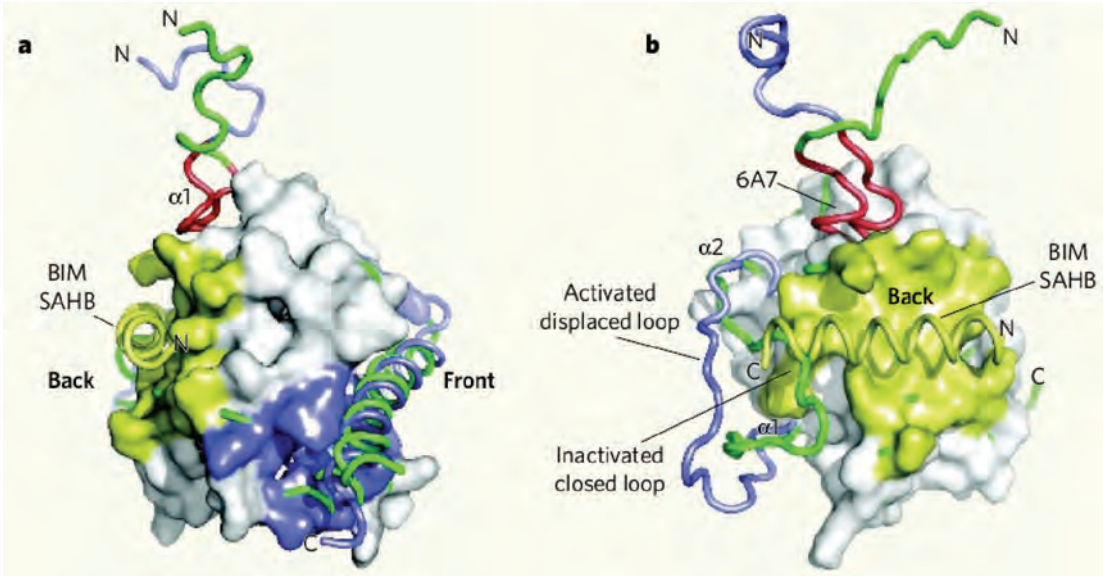


FIGURE 25: Activation of the BAX protein by the BIM BH3 domain. a, The structure of BAX based is shown with BIM SAHB binding at a site on the 'back' of the molecule (yellow) (SAHB, stabilized α-helices of BCL-2 domains). This contrasts with the expected binding of a BH3-domain helix in the conventional (at least for antiapoptotic proteins) hydrophobic BH pocket located at the 'front' of the molecule (blue). b, Gavathiotis et al. (2008) find that conformational changes accompany the binding of a BIM SAHB peptide to BAX. The chain containing α-helix 1 (α1) and the loop between α1 and α2 is shown before (green) and after (blue) binding of BIM SAHB. The BAX amino-terminal region 6A7, which becomes exposed on association with BIM SAHB, is shown in red. (Images prepared by T. Moldoveanu, St. Jude Children's Research Hospital.) Modified legend and figure taken with permission from Green and Chipuk (2008).

that form membrane pores allowing the efflux of mitochondrial apoptogenic proteins (Dewson and Kluck, 2009). Anti-apoptotic Bcl-2 proteins have been shown to be capable of inhibiting Bak at this step (Ruffolo and Shore, 2003). Thus, the activation of the multi-domain Bax and Bak proteins follows a multi-step process that is initiated by upstream BH3-only proteins, inhibited by anti-apoptotic proteins and culminates in the formation of membrane pores that induce MOMP and cue the next step of the apoptotic pathway.

An alternative pathway for MOMP involves a mitochondrial conductance channel known as the Permeability Transition Pore (PTP). Mobilization of ER Ca2+ stores induces mitochondrial uptake of Ca2+ that triggers mitochondrial PTP opening (Rizzuto and Pozzan, 2006). Opening of the PTP causes mitochondria swelling and rupturing of the MOM (Szalai et al., 1999). Bcl-2

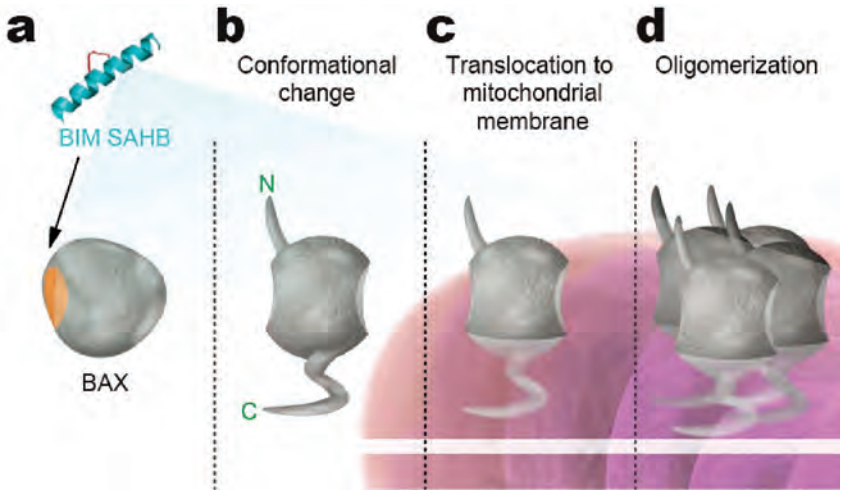


FIGURE 26: Direct activation of BAX triggers a continuum of events that leads to mitochondrial apoptosis. The interaction between BIM SAHB and BAX at the α1-α6 interaction site (a) triggers a conformational change in BAX (b) that leads to its translocation to mitochondria (c), oligomerization within the mitochondrial outer membrane (d), and the release of key apoptogenic factors. Legend and figure taken with permission from Gavathiotis et al. (2008).

family members regulate this process not by acting on mitochondria, but by controlling Ca2+ efflux at the ER (Nutt et al., 2002; Pinton et al., 2001; Scorrano et al., 2003). Ectopic expression of Bcl-2 or loss of both Bax and Bak results in lower ER Ca2+ levels and consequently less Ca2+ uptake by mitochondria (Lam et al., 1994; Scorrano et al., 2003). Apoptotic stimuli, such as C2-ceramide, arachidonic acid and oxidative stress, have been shown to cause Ca2+ efflux from the ER and induce cell death via PTP activation (Scorrano et al., 2003).

5.6 BH3-ONLY PROTEINS TRANSMIT DEATH SIGNALS

What are some of the triggers for apoptosis and how do they activate the pro-apoptotic members of the Bcl-2 family? This job falls to the BH3-only proteins. These molecules are upstream sentinels that relay the death message to Bax or Bak (Figure 27). Their activity is tightly controlled. Also, as we recall from the mouse genetic models of Bcl-2 family proteins, loss of BH3-only genes resulted in varied phenotypes. Together these observations indicate that each BH3-only protein is "kept in

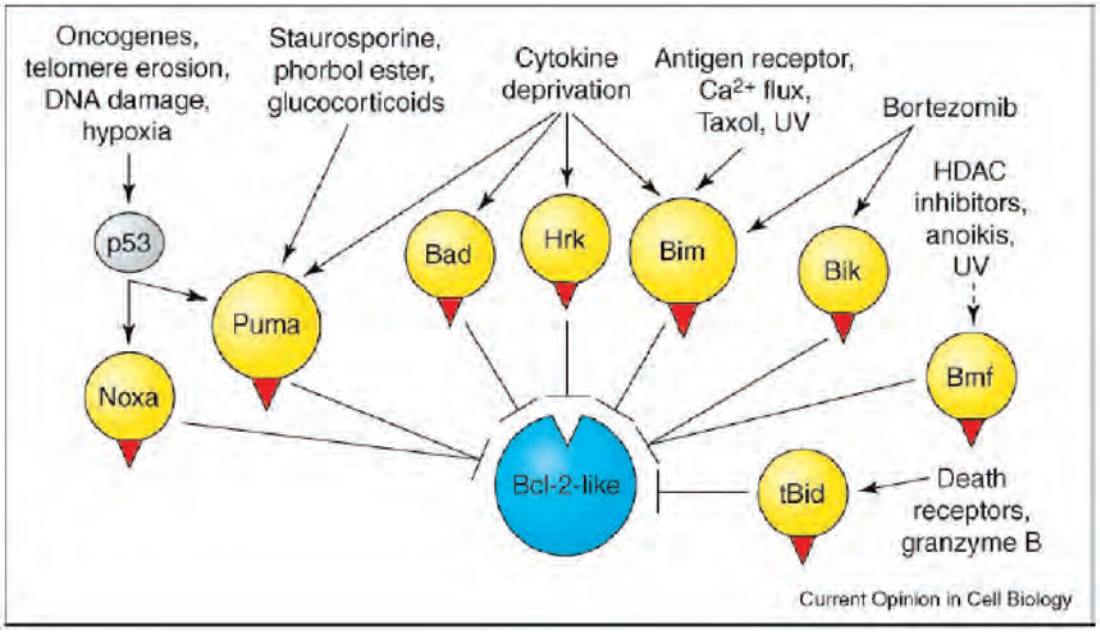


FIGURE 27: BH3-only proteins monitor cellular well being. BH3-only proteins are activated by a variety of cellular stresses. Once activated, they initiate apoptosis by binding and neutralizing Bcl-2 pro-survival proteins via their BH3 domain (red triangle). Bid, which is typically activated following caspase cleavage, amplifies the apoptotic response by engaging the Bcl-2 pro-survival proteins. Potentially tBid may also engage Bax (or Bak). Modified legend and figure taken with permission from Willis and Adams (2005).

check" by multiple mechanisms and once activated, play tissue-specific roles in response to specialized death signals.

Specific roles for BH3-only proteins have been elucidated by elegant cell biology and genetic approaches. For the most part, BH3-only proteins are inactive until apoptotic insults result in transcriptional expression or post-translational modifications. For example, in healthy cells, Bid is localized in the cytosol. Upon delivery of a death signal through Fas ligation, subsequent activation of caspase 8 results in Bid cleavage. This cleaved Bid then migrates to mitochondria where it can bind and inactivate anti-apoptotic Bcl-2 proteins, and/or activate pro-apoptotic Bax (Gavathiotis et al., 2008; Li et al., 1998; Luo et al., 1998). The in vivo relevance of this pathway is demonstrated by the Bid knock-out mice that show resistance to Fas-induced apoptosis in hepatocytes (Yin et al., 1999).

As another example, Bad intracellular localization is also altered in response to apoptotic stimuli, however, in this case, the trigger for Bad activation is de-phosphorylation (Datta et al., 2000; Zha et al., 1996). In healthy cells, survival signaling pathways (e.g., PI3-K/AKT and Ras/MEK pathways) maintain Bad in a phosphorylated state with three conserved serine residues playing a major role. In particular, phosphorylation of S136 is a critical binding site for 14-3-3 proteins and this interaction sequesters Bad in the cytosol. When growth factor is depleted, subsequent loss of survival kinase activity leads to Bad de-phosphorylation and migration to mitochondria where it binds and inhibits anti-apoptotic Bcl-2 proteins. The analysis of Bad^{3SA} knock-in mice, wherein the three critical phosphorylation sites were mutated to non-phosphorylatable alanines, supported the regulatory role of Bad phosphorylation. The resulting mice were viable, showed enhance cell death with reduced lymphocyte and neuronal cells due to the inability of survival kinases to phosphorylate and inactivate mutant Bad (Datta et al., 2002). Thus, Bad phosphorylation serves as a sensor for survival pathway signaling and titrates the strength of an apoptotic signal.

As a last example, the activation of Noxa and Puma requires transcriptional activation. In response to DNA damage, p53 levels increase and lead to expression of a number of genes including NOXA and PUMA. Noxa and Puma then inhibit anti-apoptotic proteins to induce cell death. NOXA and PUMA knock-out mice demonstrated resistance to DNA-damage-induced cell death verifying a specific role of Noxa and Puma in the DNA damage response (Nakano and Vousden, 2001; Oda et al., 2000; Yu et al., 2001). Thus, in summary, unique intracellular localization, interactions with regulatory molecules and specific mechanisms of activation indicate that each BH3-only protein is a sensor for distinct apoptotic signals.

• • • •

CHAPTER 6

Apoptosis and Cancer

Since the first association of Bcl-2 with human follicular lymphoma, there has been a clear link between loss of apoptotic signaling and cancer. This paradigm is supported by multiple mouse models wherein genetic inhibition of apoptosis leads to cancer development. Diminished apoptosis also contributes to resistance to anti-cancer treatments because these therapies rely on activating apoptosis. Through the process of developing a better understanding of the apoptotic pathway, a number of groups have rationally explored new molecular targeted therapies designed to "re-boot" the cancer cell's weakened apoptotic machinery. For review, see Fulda (2009) and Lessene et al. (2008).

6.1 DIMINISHED APOPTOSIS IN CANCER

There are many reports in various cancer types wherein apoptotic regulators are dysfunctional (Figure 28). Mediators of the extrinsic apoptotic pathway can be deregulated in cancer. For example, downregulation or mutation of the Fas death receptor has been reported in hematological cancers as well as solid tumors (Friesen et al., 1997; Fulda et al., 1998; Lee et al., 1999). Aberrant expression of anti-apoptotic FLIP prevents death receptor-mediated activation of pro-caspase 8 (Fulda, 2009). Bcl-2 family protein alterations also exist. For example, *BAX* mutations are found in ~50% of colorectal cancers (Miquel et al., 2005) and mutations in *BAD* and *BIK* have been found in colon cancers and B cell lymphomas (Arena et al., 2003; Lee et al., 2004). Bcl-2 overexpression is found not only in B-cell lymphomas but also in Hodgkin's lymphoma where it is associated with poor overall survival (Gascoyne et al., 1997) and breast cancer where it is associated with reduced survival and resistance to endocrine therapy (Tawfik et al., 2012). Mcl-1 expression is a negative prognostic indicator of disease progression and outcome in chronic lymphocytic leukemia (Pepper et al., 2008). Bcl-XL expression levels in the NCI-60 cell lines have been shown to correlate with resistance to 122 different chemotherapeutic agents (Amundson et al., 2000). Clearly, downregulation of pro-apoptotic and upregulation of anti-apoptotic Bcl-2 family members is associated with cancer progression and treatment response. The scenario with IAP overexpression is more complex. A variety of human cancers show increase in IAP expression that is associated with poor prognosis and therapy resistance, however in some cases, loss of IAPs is associated with tumor progression

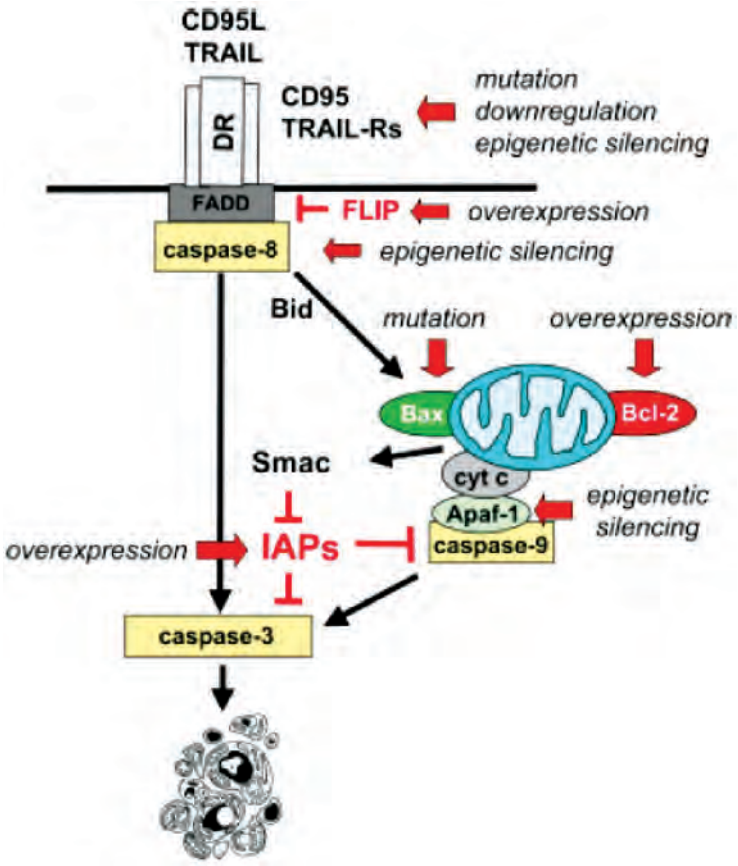


FIGURE 28: Defects in apoptosis pathways in human cancers. Signaling via the death receptor pathway can be inhibited by mutations, downregulation or epigenetic silencing of death receptors (DR) such as CD95 or TRAIL receptors (TRAIL-Rs), overexpression of FLIP or epigenetic silencing of caspase-8. The mitochondrial pathway may be impaired by overexpression of anti-apoptotic Bcl-2 proteins, Bax mutations or epigenetic silencing of Apaf-1. Activation of downstream caspases can be blocked by high levels of "Inhibitor of Apoptosis Proteins" (IAPs). Legend and figure taken with permission from Fulda (2009).

(Favaloro et al., 2012). These effects are likely due to the dual roles of IAPs as both caspase inhibitors and regulators of the NF-kB pathway. Nevertheless, altogether these studies demonstrate that defects in apoptosis are common in cancer (Hanahan and Weinberg, 2011). Based on the current molecular understanding of apoptotic signaling, novel ideas have been proposed and are being tested that aim to stimulate apoptosis of cancer cells for clinical benefit.

6.2 BCL-2 FAMILY ANTAGONISTS

A simple approach to inactivating anti-apoptotic Bcl-2 proteins is through targeted downregulation. This approach led to the development of the antisense Bcl-2 molecule oblimersen. Positive early stage results in the clinical setting, led to multiple randomized phase II clinical trials in chronic lymphocytic leukemia, metastatic melanoma, multiple myeloma, and acute myelogenous leukemia. Oblimersen had no single agent activity (Benz et al., 2007), although combination therapy with cyclophosphamide and fludarabine showed promising results (O'Brien et al., 2007). Trials are ongoing and the story of oblimersen underscores the difficulty in identifying effective clinical trials with which to test new drug efficacy.

Based on structural insights, the search began for compounds that could bind to the hydrophobic groove of anti-apoptotic proteins and thus raise apoptotic potential of cancer cells. Using a SAR (structure–activity relationship) approach that is a reiterative approach combining NMR based screening of small molecules followed by structure-based design, a large consortium of researchers discovered ABT-737—a small molecule inhibitor that had been screened for Bcl-XL-binding (Oltersdorf et al., 2005) (Figure 29). ABT-737 induced apoptosis in malignant cells, but not normal cells, suggesting that toxic side-effects would be favorable in the clinical setting. Further modifications led to the production of ABT-263, which is orally available and thus a superior clinical candidate (Tse et al., 2008). ABT263/737 binds with high affinity to Bcl-2, Bcl-XL, and Bcl-w, revealing a similar binding specificity as the BH3-only protein Bad. Accordingly, ABT263/737 was toxic to cells that expressed Bcl-2, Bcl-XL or Bcl-w but not expressing Mcl-1 (van Delft et al., 2006). The compound showed single-agent efficacy in models of small cell lung cancer, lymphoma and leukemia (Oltersdorf et al., 2005). Not surprisingly, resistance to ABT263/737 is associated with cells overexpressing Mcl-1(van Delft et al., 2006), thus potentially limiting the effectiveness of this compound for therapy.

An independent screen of a natural products library identified GX15-070 (obatoclax), which binds Bcl-2, Bcl-XL, Bcl-w, and Mcl-1 (Nguyen et al., 2007). GX15-070 has single agent activity in chronic lymphocytic leukemia, mantle cell lymphoma, breast, and non-small cell lung cancer cells (Campas et al., 2006; Shore and Viallet, 2005). Ongoing studies include clinical trials of hematological malignancies (Lessene et al., 2008).

Modified peptides mimicking the BH3 domain have also been explored. Unmodified peptides have relatively poor binding affinity to anti-apoptotic Bcl-2 proteins because of unstable helix formation (Petros et al., 2000). Replacement of two non-essential amino acids with non-natural amino acids enables "stapling" of the peptide, which enhances the helicity of 23 mer peptides of the Bid and Bim BH3 domain (Walensky et al., 2004).

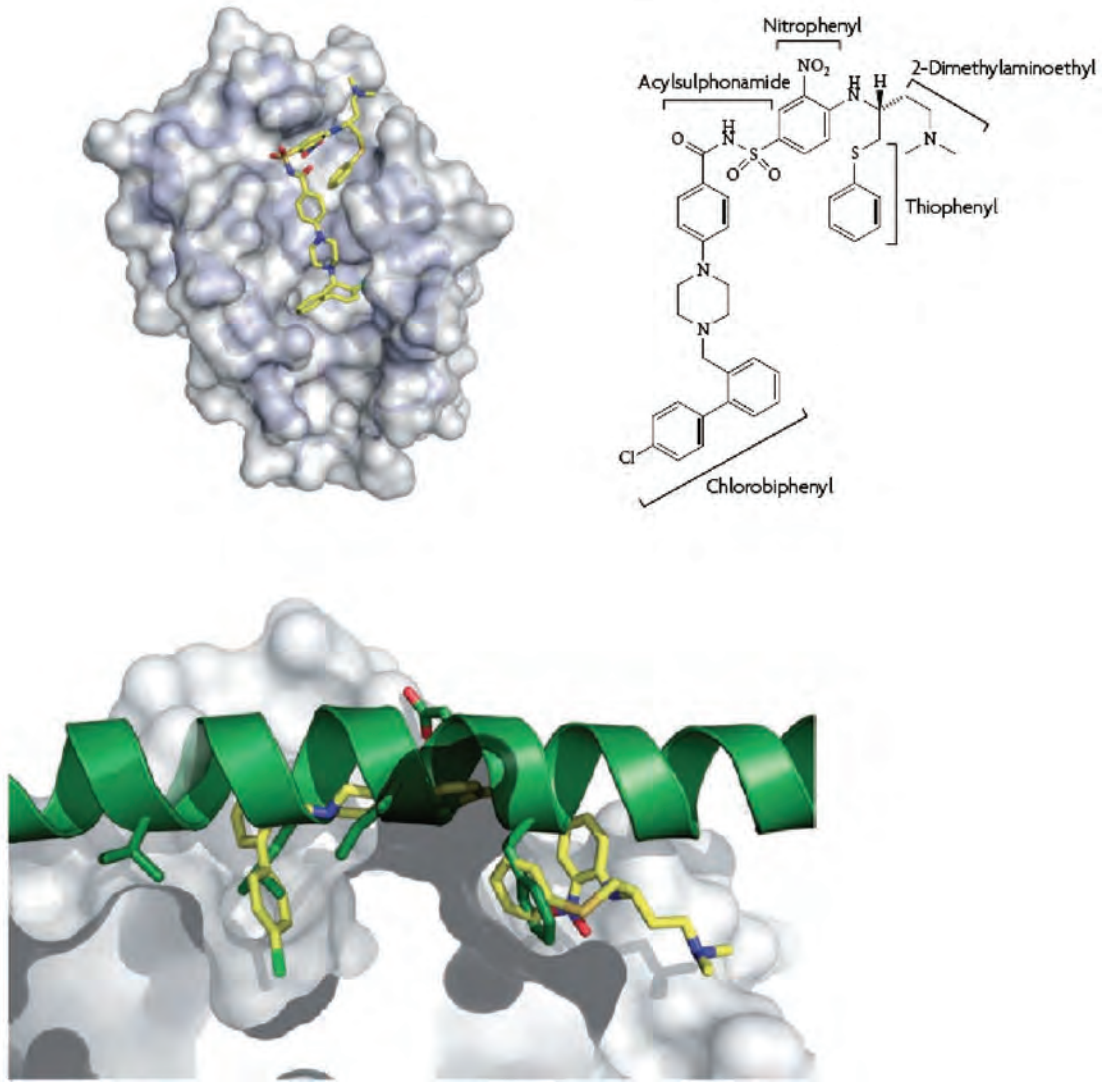


FIGURE 29: Structural studies related to the discovery of ABT-737. (b) BCL-XL bound to ABT-737 (PDB: 2YXJ). (c) Overlay of BCL-XL–ABT-737 complex with BCL-XL–BIM BH3 complex (PDB: 1PQ1). For clarity BCL-XL from the BCL-XL–BIM BH3 structure is not displayed in the overlay. Legend and figure taken with permission from Lessene et al. (2008).

Another small molecule inhibitor of anti-apoptotic Bcl-2 family proteins is the cotton plant product, gossypol. This molecule binds Bcl-2, Bcl-XL, and Mcl-1 with higher affinity than GX15-070 (Tang et al., 2008). It shows single agent activity against cell lines of multiple myeloma and chronic lymphocytic leukemia (Lessene et al., 2008) and promising clinical trial data.

6.3 IAP ANTAGONISTS

Small molecule inhibitors have also been generated to target IAP activity (Mannhold et al., 2010). The first molecule was modeled after the 4-residue amino-terminus of Smac that interacts with XIAP-BIR3 (Sun et al., 2006; Zobel et al., 2006). Higher affinity peptides were acquired after screening of several peptide libraries. Bivalent Smac mimetics containing two binding sites bind to XIAP-BIR2-BIR3. These compounds relieve XIAP-mediated inhibition of caspases as well as trigger XIAP autoubiquitination and degradation. Smac mimetics have single agent activity in addition to activity in combination with other therapeutic agents. Currently, these compounds are in pre-clinical and clinical development.

CHAPTER 7

Summary

Apoptosis is a fundamental biological process. It is vital for the development, the maintenance of tissue homeostasis, and the eradication of damaged cells that pose a risk to the whole organism. While publication records suggest that apoptosis was discovered in the late twentieth century, tissue regression was recognized and recorded at least 250 years before that. Increased interest in apoptosis was a result of technical advances and genetic insights that facilitated the merging of different disciplines that had been independently studying the same phenomenon. Use of simple experimental model systems, in vitro reconstitution and cell biology studies produced hypotheses that were validated (or disproven) through mouse genetics approaches. The elucidation of complex signaling pathways and structural insights of the core signaling molecules resulted in characterization of the apoptotic pathway at the molecular level. Understanding how loss or enhanced apoptosis leads to human pathologies is a current challenge that will be met by ongoing investigations of apoptotic pathways in concert with rational approaches to modulate this critical cellular program.

• • • •

References

Acehan, D., X. Jiang, D.G. Morgan, J.E. Heuser, X. Wang, and C.W. Akey. 2002. Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. *Mol Cell*. 9: pp. 423–32.

Adams, J.M., and S. Cory. 2007a. Bcl-2-regulated apoptosis: mechanism and therapeutic potential. *Curr Opin Immunol*. 19: pp. 488–96. doi: 10.1016/j.coi.2007.05.004

Adams, J.M., and S. Cory. 2007b. The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene*. 26: pp. 1324–37. doi: 10.1038/sj.onc.1210220

Akakura, S., S. Singh, M. Spataro, R. Akakura, J.I. Kim, M.L. Albert, and R.B. Birge. 2004. The opsonin MFG-E8 is a ligand for the alphavbeta5 integrin and triggers DOCK180-dependent Rac1 activation for the phagocytosis of apoptotic cells. *Exp Cell Res*. 292: pp. 403–16. doi: 10.1016/j.yexcr.2003.09.011

Amundson, S.A., T.G. Myers, D. Scudiero, S. Kitada, J.C. Reed, and A.J. Fornace, Jr. 2000. An informatics approach identifying markers of chemosensitivity in human cancer cell lines. *Cancer Res*. 60: pp. 6101–10.

Ankeny, R.A. 2001. The natural history of *Caenorhabditis elegans* research. *Nat Rev Genet*. 2: pp. 474–9. doi: 10.1038/35076538

Annis, M.G., E.L. Soucie, P.J. Dlugosz, J.A. Cruz-Aguado, L.Z. Penn, B. Leber, and D.W. Andrews. 2005. Bax forms multispanning monomers that oligomerize to permeabilize membranes during apoptosis. *EMBO J*. 24: pp. 2096–103.

Arena, V., M. Martini, M. Luongo, A. Capelli, and L.M. Larocca. 2003. Mutations of the BIK gene in human peripheral B-cell lymphomas. *Genes Chromosomes Cancer*. 38: pp. 91–6.

Arends, M.J., and A.H. Wyllie. 1991. Apoptosis: mechanisms and roles in pathology. *Int Rev Exp Pathol*. 32: pp. 223–54.

Asano, K., M. Miwa, K. Miwa, R. Hanayama, H. Nagase, S. Nagata, and M. Tanaka. 2004. Masking of phosphatidylserine inhibits apoptotic cell engulfment and induces autoantibody production in mice. *J Exp Med*. 200: pp. 459–67. doi: 10.1084/jem.20040342

Aymeric, L., L. Apetoh, F. Ghiringhelli, A. Tesniere, I. Martins, G. Kroemer, M.J. Smyth, and L. Zitvogel. 2010. Tumor cell death and ATP release prime dendritic cells and efficient anticancer immunity. *Cancer Res*. 70: pp. 855–8. doi: 10.1158/0008-5472.CAN-09-3566

Bakhshi, A., J.P. Jensen, P. Goldman, J.J. Wright, O.W. McBride, A.L. Epstein, and S.J. Korsmeyer. 1985. Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. *Cell*. 41: pp. 899–906. doi: 10.1016/S0092-8674(85)80070-2

Balasubramanian, K., and A.J. Schroit. 2003. Aminophospholipid asymmetry: a matter of life and death. *Annu Rev Physiol*. 65: pp. 701–34.

Bannerman, D.D., M. Sathyamoorthy, and S.E. Goldblum. 1998. Bacterial lipopolysaccharide disrupts endothelial monolayer integrity and survival signaling events through caspase cleavage of adherens junction proteins. *J Biol Chem*. 273: pp. 35371–80. doi: 10.1074/jbc.273.52.35371

Barde, Y.A. 1989. Trophic factors and neuronal survival. *Neuron*. 2: pp. 1525–34. doi: 10.1016/0896-6273(89)90040-8

Barfurth, D. 1887. Die Rückbildung des Froschlarvenschwanzes und die sogenannten Sarcoplasten. *Arch Mikrosk Anat*. 29: pp. 35–60. doi: 10.1007/BF02955493

Barres, B.A., and M.C. Raff. 1999. Axonal control of oligodendrocyte development. *J Cell Biol*. 147: pp. 1123–8. doi: 10.1083/jcb.147.6.1123

Basanez, G., and J.M. Hardwick. 2008. Unravelling the Bcl-2 Apoptosis Code with a Simple Model System. *PLoS Biol*. 6: p. e154. doi: 10.1371/journal.pbio.0060154

Benz, E.J., Jr., D.G. Nathan, R.K. Amaravadi, and N.N. Danial. 2007. Targeting the cell death-survival equation. *Clin Cancer Res*. 13: pp. 7250–3. doi: 10.1158/1078-0432.CCR-07-2227

Benz, E.J.J., and D.G. Nathan. 2007. Dedication. *Clin Cancer Res*. 13. p. 7248.

Berry, D.L., and E.H. Baehrecke. 2007. Growth arrest and autophagy are required for salivary gland cell degradation in *Drosophila*. *Cell*. 131: pp. 1137–48. doi: 10.1016/j.cell.2007.10.048

Billen, L.P., C.L. Kokoski, J.F. Lovell, B. Leber, and D.W. Andrews. 2008. Bcl-XL inhibits membrane permeabilization by competing with Bax. *PLoS Biol*. 6: p. e147. doi: 10.1371/journal.pbio.0060147

Boatright, K.M., M. Renatus, F.L. Scott, S. Sperandio, H. Shin, I.M. Pedersen, J.E. Ricci, W.A. Edris, D.P. Sutherlin, D.R. Green, and G.S. Salvesen. 2003. A unified model for apical caspase activation. *Mol Cell*. 11: pp. 529–41. doi: 10.1016/S1097-2765(03)00051-0

Borisenko, G.G., T. Matsura, S.X. Liu, V.A. Tyurin, J. Jianfei, F.B. Serinkan, and V.E. Kagan. 2003. Macrophage recognition of externalized phosphatidylserine and phagocytosis of apoptotic Jurkat cells—existence of a threshold. *Arch Biochem Biophys*. 413: pp. 41–52. doi: 10.1016/S0003-9861(03)00083-3

Bouillet, P., D. Metcalf, D.C. Huang, D.M. Tarlinton, T.W. Kay, F. Kontgen, J.M. Adams, and A. Strasser. 1999. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science*. 286: pp. 1735–8. doi: 10.1126/science.286.5445.1735

Bournazou, I., J.D. Pound, R. Duffin, S. Bournazos, L.A. Melville, S.B. Brown, A.G. Rossi, and

C.D. Gregory. 2009. Apoptotic human cells inhibit migration of granulocytes via release of lactoferrin. *J Clin Invest*. 119: pp. 20–32. doi: 10.1172/JCI36226

Brancolini, C., D. Lazarevic, J. Rodriguez, and C. Schneider. 1997. Dismantling cell-cell contacts during apoptosis is coupled to a caspase-dependent proteolytic cleavage of beta-catenin. *J Cell Biol*. 139: pp. 759–71. doi: 10.1083/jcb.139.3.759

Campas, C., A.M. Cosialls, M. Barragan, D. Iglesias-Serret, A.F. Santidrian, L. Coll-Mulet, M. de Frias, A. Domingo, G. Pons, and J. Gil. 2006. Bcl-2 inhibitors induce apoptosis in chronic lymphocytic leukemia cells. *Exp Hematol*. 34: pp. 1663–9. doi: 10.1016/j.exphem.2006.07.008

Cartron, P.F., T. Gallenne, G. Bougras, F. Gautier, F. Manero, P. Vusio, K. Meflah, F.M. Vallette, and P. Juin. 2004. The first alpha helix of Bax plays a necessary role in its ligand-induced activation by the BH3-only proteins Bid and PUMA. *Mol Cell*. 16: pp. 807–18. doi: 10.1016/j.molcel.2004.10.028

Cecconi, F., G. Alvarez-Bolado, B.I. Meyer, K.A. Roth, and P. Gruss. 1998. Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development. *Cell*. 94: pp. 727–37. doi: 10.1016/S0092-8674(00)81732-8

Cerretti, D.P., C.J. Kozlosky, B. Mosley, N. Nelson, K. Van Ness, T.A. Greenstreet, C.J. March, S.R. Kronheim, T. Druck, L.A. Cannizzaro, and et al. 1992. Molecular cloning of the interleukin-1 beta converting enzyme. *Science*. 256: pp. 97–100. doi: 10.1126/science.1373520

Chang, D.W., Z. Xing, V.L. Capacio, M.E. Peter, and X. Yang. 2003. Interdimer processing mechanism of procaspase-8 activation. *EMBO J*. 22: pp. 4132–42. doi: 10.1093/emboj/cdg414

Chekeni, F.B., M.R. Elliott, J.K. Sandilos, S.F. Walk, J.M. Kinchen, E.R. Lazarowski, A.J. Armstrong, S. Penuela, D.W. Laird, G.S. Salvesen, B.E. Isakson, D.A. Bayliss, and K.S. Ravichandran. 2010. Pannexin 1 channels mediate 'find-me' signal release and membrane permeability during apoptosis. *Nature*. 467: pp. 863–7. doi: 0.1038/nature09413

Chen, C.J., H. Kono, D. Golenbock, G. Reed, S. Akira, and K.L. Rock. 2007. Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. *Nat Med*. 13: pp. 851–6.

Chen, L., S.N. Willis, A. Wei, B.J. Smith, J.I. Fletcher, M.G. Hinds, P.M. Colman, C.L. Day, J.M. Adams, and D.C. Huang. 2005. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell*. 17: pp. 393–403. doi: 10.1016/j.molcel.2004.12.030

Chen, W.F., R. Scollay, I. Clark-Lewis, and K. Shortman. 1983. The size of functional T-lymphocyte pools within thymic medullary and cortical cell subsets. *Thymus*. 5: pp. 179–95.

Cheng, E.H., T.V. Sheiko, J.K. Fisher, W.J. Craigen, and S.J. Korsmeyer. 2003. VDAC2 inhibits BAK activation and mitochondrial apoptosis. *Science*. 301: pp. 513–7. doi: 10.1126/science.1083995

Chipuk, J.E., L. Bouchier-Hayes, and D.R. Green. 2006. Mitochondrial outer membrane

permeabilization during apoptosis: the innocent bystander scenario. *Cell Death Differ*. 13: pp. 1396–402. doi: 10.1038/sj.cdd.4401963

Chipuk, J.E., G.P. McStay, A. Bharti, T. Kuwana, C.J. Clarke, L.J. Siskind, L.M. Obeid, and D.R. Green. 2012. Sphingolipid metabolism cooperates with BAK and BAX to promote the mitochondrial pathway of apoptosis. *Cell*. 148: pp. 988–1000. doi: 10.1016/j.cell.2012.01.038

Chipuk, J.E., T. Moldoveanu, F. Llambi, M.J. Parsons, and D.R. Green. 2010. The BCL-2 family reunion. *Mol Cell*. 37: pp. 299–310. doi: 10.1016/j.molcel.2010.01.025

Clarke, P.G., and S. Clarke. 1996. Nineteenth century research on naturally occurring cell death and related phenomena. *Anat Embryol (Berl)*. 193: pp. 81–99. doi: 10.1007/BF00214700

Cleary, M.L., and J. Sklar. 1985. 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. *Proc Natl Acad Sci U S A*. 82: pp. 7439–43. doi: 10.1073/pnas.82.21.7439

Cohen, J.J. 1991. Programmed cell death in the immune system. *Adv Immunol*. 50: pp. 55–85. doi: 10.1016/S0065-2776(08)60822-6

Cohen, J.J., and R.C. Duke. 1984. Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *J Immunol*. 132: pp. 38–42.

Coleman, M.L., E.A. Sahai, M. Yeo, M. Bosch, A. Dewar, and M.F. Olson. 2001. Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. *Nat Cell Biol*. 3: pp. 339–45. doi: 10.1038/35070009

Coucouvanis, E., and G.R. Martin. 1995. Signals for death and survival: a two-step mechanism for cavitation in the vertebrate embryo. *Cell*. 83: pp. 279–87. doi: 10.1016/0092-8674(95)90169-8

Crook, N.E., R.J. Clem, and L.K. Miller. 1993. An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. *J Virol*. 67: pp. 2168–74.

Danial, N.N. 2007. BCL-2 family proteins: critical checkpoints of apoptotic cell death. *Clin Cancer Res*. 13: pp. 7254–63. doi: 10.1158/1078-0432.CCR-07-1598

Danial, N.N., and S.J. Korsmeyer. 2004. Cell death: critical control points. *Cell*. 116: pp. 205–19.

Darland-Ransom, M., X. Wang, C.L. Sun, J. Mapes, K. Gengyo-Ando, S. Mitani, and D. Xue. 2008. Role of *C. elegans* TAT-1 protein in maintaining plasma membrane phosphatidylserine asymmetry. *Science*. 320: pp. 528–31. doi: 10.1126/science.1155847

Datta, S.R., A. Katsov, L. Hu, A. Petros, S.W. Fesik, M.B. Yaffe, and M.E. Greenberg. 2000. 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. *Mol Cell*. 6: pp. 41–51. doi: 10.1016/S1097-2765(00)00006-X; doi: 10.1016/S1097-2765(05)00012-2

Datta, S.R., A.M. Ranger, M.Z. Lin, J.F. Sturgill, Y.C. Ma, C.W. Cowan, P. Dikkes, S.J. Korsmeyer, and M.E. Greenberg. 2002. Survival factor-mediated BAD phosphorylation raises

the mitochondrial threshold for apoptosis. *Dev Cell*. 3: pp. 631–43. doi: 10.1016/S1534-5807(02)00326-X

Degterev, A., and J. Yuan. 2008. Expansion and evolution of cell death programmes. *Nat Rev Mol Cell Biol*. 9: pp. 378–90. doi: 10.1038/nrm2393

Denton, D., B. Shravage, R. Simin, K. Mills, D.L. Berry, E.H. Baehrecke, and S. Kumar. 2009. Autophagy, not apoptosis, is essential for midgut cell death in *Drosophila*. *Curr Biol*. 19: pp. 1741–6. doi: 10.1016/j.cub.2009.08.042

Dewson, G., and R.M. Kluck. 2009. Mechanisms by which Bak and Bax permeabilise mitochondria during apoptosis. *J Cell Sci*. 122: pp. 2801–8. doi: 10.1242/jcs.038166

Du, C., M. Fang, Y. Li, L. Li, and X. Wang. 2000. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell*. 102: pp. 33–42. doi: 10.1016/S0092-8674(00)00008-8

Dugès, A. 1835. Recherches sur l'ostéologie et la myologie des batraciens à leurs différens ages. *Mémoires présentés par divers savans à l'Academie royale des Sciences le l'Institut de France; sciences mathématiques et physiques*. 6: pp. 1–216. doi: 10.5962/bhl.title.5058

Eckelman, B.P., G.S. Salvesen, and F.L. Scott. 2006. Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family. *EMBO Rep*. 7: pp. 988–94. doi: 10.1038/sj.embor.7400795

Ekert, P.G., J. Silke, C.J. Hawkins, A.M. Verhagen, and D.L. Vaux. 2001. DIABLO promotes apoptosis by removing MIHA/XIAP from processed caspase 9. *J Cell Biol*. 152: pp. 483–90. doi: 10.1083/jcb.152.3.483

Elliott, M.R., F.B. Chekeni, P.C. Trampont, E.R. Lazarowski, A. Kadl, S.F. Walk, D. Park, R.I. Woodson, M. Ostankovich, P. Sharma, J.J. Lysiak, T.K. Harden, N. Leitinger, and K.S. Ravichandran. 2009. Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature*. 461: pp. 282–6. doi: 10.1038/nature08296

Ellis, H.M., and H.R. Horvitz. 1986. Genetic control of programmed cell death in the nematode *C. elegans*. *Cell*. 44: pp. 817–29. doi: 10.1016/0092-8674(86)90004-8

Ellis, R.E., and H.R. Horvitz. 1991. Two *C. elegans* genes control the programmed deaths of specific cells in the pharynx. *Development*. 112: pp. 591–603.

Ellis, R.E., D.M. Jacobson, and H.R. Horvitz. 1991a. Genes required for the engulfment of cell corpses during programmed cell death in *Caenorhabditis elegans*. *Genetics*. 129: pp. 79–94.

Ellis, R.E., J.Y. Yuan, and H.R. Horvitz. 1991b. Mechanisms and functions of cell death. *Annu Rev Cell Biol*. 7: pp. 663–98. doi: 10.1146/annurev.cb.07.110191.003311

Enari, M., H. Sakahira, H. Yokoyama, K. Okawa, A. Iwamatsu, and S. Nagata. 1998. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature*. 391: pp. 43–50.

Epand, R.F., J.C. Martinou, S. Montessuit, R.M. Epand, and C.M. Yip. 2002. Direct evidence for membrane pore formation by the apoptotic protein Bax. *Biochem Biophys Res Commun*. 298: pp. 744–9. doi: 10.1016/S0006-291X(02)02544-5

Fadeel, B., A. Ottosson, and S. Pervaiz. 2008. Big wheel keeps on turning: apoptosome regulation and its role in chemoresistance. *Cell Death Differ*. 15: pp. 443–52. doi: 10.1038/sj.cdd.4402265

Fadok, V.A., D.L. Bratton, L. Guthrie, and P.M. Henson. 2001. Differential effects of apoptotic versus lysed cells on macrophage production of cytokines: role of proteases. *J Immunol*. 166: pp. 6847–54.

Fadok, V.A., D.L. Bratton, A. Konowal, P.W. Freed, J.Y. Westcott, and P.M. Henson. 1998. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J Clin Invest*. 101: pp. 890–8. doi: 10.1172/JCI1112

Fadok, V.A., D.R. Voelker, P.A. Campbell, J.J. Cohen, D.L. Bratton, and P.M. Henson. 1992. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol*. 148: pp. 2207–16.

Favaloro, B., N. Allocati, V. Graziano, C. Di Ilio, and V. De Laurenzi. 2012. Role of apoptosis in disease. *Aging (Albany NY)*. 4: pp. 330–49.

Fisher, G.H., F.J. Rosenberg, S.E. Straus, J.K. Dale, L.A. Middleton, A.Y. Lin, W. Strober, M.J. Lenardo, and J.M. Puck. 1995. Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. *Cell*. 81: pp. 935–46. doi: 10.1016/0092-8674(95)90013-6

Flemming, W. 1885. Ueber die Bildung von richtungsfiguren in Säugethiereiern beim Untergang Graaf'scher Follikel. *Arch Anat Physiol*. Jahrgang 1885: pp. 221–4.

Florey, O., and D.O. Haskard. 2009. Sphingosine 1-phosphate enhances Fc gamma receptor-mediated neutrophil activation and recruitment under flow conditions. *J Immunol*. 183: pp. 2330–6.

Franz, S., U.S. Gaipl, L.E. Munoz, A. Sheriff, A. Beer, J.R. Kalden, and M. Herrmann. 2006. Apoptosis and autoimmunity: when apoptotic cells break their silence. *Curr Rheumatol Rep*. 8: pp. 245–7. doi: 10.1007/s11926-006-0001-y

Friesen, C., S. Fulda, and K.M. Debatin. 1997. Deficient activation of the CD95 (APO-1/Fas) system in drug-resistant cells. *Leukemia*. 11: pp. 1833–41. doi: 10.1038/sj.leu.2400827

Fuchs, Y., and H. Steller. 2011. Programmed cell death in animal development and disease. *Cell*. 147: pp. 742–58. doi: 10.1016/j.cell.2011.10.033

Fulda, S. 2009. Tumor resistance to apoptosis. *Int J Cancer*. 124: pp. 511–5. doi: 10.1002/ijc.24064

Fulda, S., M. Los, C. Friesen, and K.M. Debatin. 1998. Chemosensitivity of solid tumor

cells in vitro is related to activation of the CD95 system. *Int J Cancer*. 76: pp. 105–14. doi: 10.1002/(SICI)1097-0215(19980330)76:1<105::AID-IJC

Funder, J. 1994. Letter to nature. *Nature*. 371.

Gaipl, U.S., A. Kuhn, A. Sheriff, L.E. Munoz, S. Franz, R.E. Voll, J.R. Kalden, and M. Herrmann. 2006. Clearance of apoptotic cells in human SLE. *Curr Dir Autoimmun*. 9: pp. 173–87. doi: 10.1159/000090781

Galluzzi, L., I. Vitale, J.M. Abrams, E.S. Alnemri, E.H. Baehrecke, M.V. Blagosklonny, T.M. Dawson, V.L. Dawson, W.S. El-Deiry, S. Fulda, E. Gottlieb, D.R. Green, M.O. Hengartner, O. Kepp, R.A. Knight, S. Kumar, S.A. Lipton, X. Lu, F. Madeo, W. Malorni, P. Mehlen, G. Nunez, M.E. Peter, M. Piacentini, D.C. Rubinsztein, Y. Shi, H.U. Simon, P. Vandenabeele, E. White, J. Yuan, B. Zhivotovsky, G. Melino, and G. Kroemer. 2012. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. *Cell Death Differ*. 19: pp. 107–20. doi: 10.1038/cdd.2011.96

Gardai, S.J., D.L. Bratton, C.A. Ogden, and P.M. Henson. 2006. Recognition ligands on apoptotic cells: a perspective. *J Leukoc Biol*. 79: pp. 896–903. doi: 10.1189/jlb.1005550

Gascoyne, R.D., S.A. Adomat, S. Krajewski, M. Krajewska, D.E. Horsman, A.W. Tolcher, S.E. O'Reilly, P. Hoskins, A.J. Coldman, J.C. Reed, and J.M. Connors. 1997. Prognostic significance of Bcl-2 protein expression and Bcl-2 gene rearrangement in diffuse aggressive non-Hodgkin's lymphoma. *Blood*. 90: pp. 244–51.

Gavathiotis, E., M. Suzuki, M.L. Davis, K. Pitter, G.H. Bird, S.G. Katz, H.C. Tu, H. Kim, E.H. Cheng, N. Tjandra, and L.D. Walensky. 2008. BAX activation is initiated at a novel interaction site. *Nature*. 455: pp. 1076–81. doi: 10.1038/nature07396

Glücksmann, A. 1951. Cell deaths in normal vertebrate ontogeny. *Biol Rev Camb Philos Soc*. 26: pp. 59–86. doi: 10.1111/j.1469-185X.1951.tb00774.x

Goping, I.S., A. Gross, J.N. Lavoie, M. Nguyen, R. Jemmerson, K. Roth, S.J. Korsmeyer, and G.C. Shore. 1998. Regulated targeting of BAX to mitochondria. *J Cell Biol*. 143: pp. 207–15. doi: 10.1083/jcb.143.1.207

Green, D.R., and J.E. Chipuk. 2008. Apoptosis: stabbed in the BAX. *Nature*. 455: pp. 1047–9. doi: 10.1038/4551047a

Gude, D.R., S.E. Alvarez, S.W. Paugh, P. Mitra, J. Yu, R. Griffiths, S.E. Barbour, S. Milstien, and S. Spiegel. 2008. Apoptosis induces expression of sphingosine kinase 1 to release sphingosine-1-phosphate as a "come-and-get-me" signal. *FASEB J*. 22: pp. 2629–38. doi: 10.1096/fj.08-107169

Gyrd-Hansen, M., and P. Meier. 2010. IAPs: from caspase inhibitors to modulators of NF-kappaB, inflammation and cancer. *Nat Rev Cancer*. 10: pp. 561–74. doi: 10.1038/nrc2889

Hanahan, D., and R.A. Weinberg. 2011. Hallmarks of cancer: the next generation. *Cell*. 144: pp. 646–74. doi: 10.1016/j.cell.2011.02.013

Hanayama, R., K. Miyasaka, M. Nakaya, and S. Nagata. 2006. MFG-E8-dependent clearance of apoptotic cells, and autoimmunity caused by its failure. *Curr Dir Autoimmun*. 9: pp. 162–72. doi: 10.1159/000090780

Hanayama, R., M. Tanaka, K. Miyasaka, K. Aozasa, M. Koike, Y. Uchiyama, and S. Nagata. 2004. Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. *Science*. 304: pp. 1147–50. doi: 10.1126/science.1094359

Hao, Z., G.S. Duncan, C.C. Chang, A. Elia, M. Fang, A. Wakeham, H. Okada, T. Calzascia, Y. Jang, A. You-Ten, W.C. Yeh, P. Ohashi, X. Wang, and T.W. Mak. 2005. Specific ablation of the apoptotic functions of cytochrome C reveals a differential requirement for cytochrome C and Apaf-1 in apoptosis. *Cell*. 121: pp. 579–91. doi: 10.1016/j.cell.2005.03.016

Harlin, H., S.B. Reffey, C.S. Duckett, T. Lindsten, and C.B. Thompson. 2001. Characterization of XIAP-deficient mice. *Mol Cell Biol*. 21: pp. 3604–8. doi: 10.1128/MCB.21.10.3604-3608.2001

Harvey, W. 1628. Exercitatio Anatomica de Motu Cordis et Sanguinis in Animalibus.

Hedgecock, E.M., J.E. Sulston, and J.N. Thomson. 1983. Mutations affecting programmed cell deaths in the nematode *Caenorhabditis elegans*. *Science*. 220: pp. 1277–9. doi: 10.1126/science.6857247

Hengartner, M.O., R.E. Ellis, and H.R. Horvitz. 1992. *Caenorhabditis elegans* gene ced-9 protects cells from programmed cell death. *Nature*. 356: pp. 494–9. doi: 10.1038/356494a0

Henson, P.M. 2005. Dampening inflammation. *Nat Immunol*. 6: pp. 1179–81. doi: 10.1038/ni1205-1179

Hewish, D.R., and L.A. Burgoyne. 1973. Chromatin sub-structure. The digestion of chromatin DNA at regularly spaced sites by a nuclear deoxyribonuclease. *Biochem Biophys Res Commun*. 52: pp. 504–10. doi: 10.1016/0006-291X(73)90740-7

Hockenbery, D., G. Nunez, C. Milliman, R.D. Schreiber, and S.J. Korsmeyer. 1990. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature*. 348: pp. 334–6. doi: 10.1038/348334a0

Hockenbery, D.M., Z.N. Oltvai, X.M. Yin, C.L. Milliman, and S.J. Korsmeyer. 1993. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell*. 75: pp. 241–51. doi: 10.1016/0092-8674(93)80066-N

Hoeppner, D.J., M.O. Hengartner, and R. Schnabel. 2001. Engulfment genes cooperate with ced-3 to promote cell death in *Caenorhabditis elegans*. *Nature*. 412: pp. 202–6.

Irvine, R.A., N. Adachi, D.K. Shibata, G.D. Cassell, K. Yu, Z.E. Karanjawala, C.L. Hsieh, and M.R. Lieber. 2005. Generation and characterization of endonuclease G null mice. *Mol Cell Biol*. 25: pp. 294–302. doi: 10.1128/MCB.25.1.294-302.2005

Itoh, N., S. Yonehara, A. Ishii, M. Yonehara, S. Mizushima, M. Sameshima, A. Hase, Y. Seto, and S. Nagata. 1991. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell*. 66: pp. 233–43. doi: 10.1016/0092-8674(91)90614-5

Jacobson, M.D., J.F. Burne, and M.C. Raff. 1994. Programmed cell death and Bcl-2 protection in the absence of a nucleus. *EMBO J*. 13: pp. 1899–910.

Jacobson, M.D., M. Weil, and M.C. Raff. 1997. Programmed cell death in animal development. *Cell*. 88: pp. 347–54. doi: 10.1016/S0092-8674(00)81873-5

Jeffers, J.R., E. Parganas, Y. Lee, C. Yang, J. Wang, J. Brennan, K.H. MacLean, J. Han, T. Chittenden, J.N. Ihle, P.J. McKinnon, J.L. Cleveland, and G.P. Zambetti. 2003. Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. *Cancer Cell*. 4: pp. 321–8. doi: 10.1016/S1535-6108(03)00244-7

Joza, N., S.A. Susin, E. Daugas, W.L. Stanford, S.K. Cho, C.Y. Li, T. Sasaki, A.J. Elia, H.Y. Cheng, L. Ravagnan, K.F. Ferri, N. Zamzami, A. Wakeham, R. Hakem, H. Yoshida, Y.Y. Kong, T.W. Mak, J.C. Zuniga-Pflucker, G. Kroemer, and J.M. Penninger. 2001. Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. *Nature*. 410: pp. 549–54.

Kanuka, H., K. Sawamoto, N. Inohara, K. Matsuno, H. Okano, and M. Miura. 1999. Control of the cell death pathway by Dapaf-1, a *Drosophila* Apaf-1/CED-4-related caspase activator. *Mol Cell*. 4: pp. 757–69. doi: 10.1016/S1097-2765(00)80386-X

Kaufmann, T., L. Tai, P.G. Ekert, D.C. Huang, F. Norris, R.K. Lindemann, R.W. Johnstone, V.M. Dixit, and A. Strasser. 2007. The BH3-only protein bid is dispensable for DNA damage- and replicative stress-induced apoptosis or cell-cycle arrest. *Cell*. 129: pp. 423–33. doi: 10.1016/j.cell.2007.03.017

Kawane, K., H. Fukuyama, G. Kondoh, J. Takeda, Y. Ohsawa, Y. Uchiyama, and S. Nagata. 2001. Requirement of DNase II for definitive erythropoiesis in the mouse fetal liver. *Science*. 292: pp. 1546–9. doi: 10.1126/science.292.5521.1546

Kawane, K., M. Ohtani, K. Miwa, T. Kizawa, Y. Kanbara, Y. Yoshioka, H. Yoshikawa, and S. Nagata. 2006. Chronic polyarthritis caused by mammalian DNA that escapes from degradation in macrophages. *Nature*. 443: pp. 998–1002. doi: 10.1038/nature05245

Kenyon, C. 1988. The nematode *Caenorhabditis elegans*. *Science*. 240: pp. 1448–53. doi: 10.1126/science.3287621

Kerr, J.F. 1965. A histochemical study of hypertrophy and ischaemic injury of rat liver with special reference to changes in lysosomes. *J Pathol Bacteriol*. 90: pp. 419–35. doi: 10.1002/path.1700900210

Kerr, J.F., and J. Searle. 1972a. A mode of cell loss in malignant neoplasms. *J Pathol*. 106: p. xi.

Kerr, J.F., and J. Searle. 1972b. A suggested explanation for the paradoxically slow growth rate of basal-cell carcinomas that contain numerous mitotic figures. *J Pathol*. 107: pp. 41–4. doi: 10.1002/path.1711070107

Kerr, J.F., A.H. Wyllie, and A.R. Currie. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer*. 26: pp. 239–57.

Kim, H., M. Rafiuddin-Shah, H.C. Tu, J.R. Jeffers, G.P. Zambetti, J.J. Hsieh, and E.H. Cheng. 2006. Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. *Nat Cell Biol*. 8: pp. 1348–58. doi: 10.1038/ncb1499

Kimble, J., and D. Hirsh. 1979. The postembryonic cell lineages of the hermaphrodite and male gonads in *Caenorhabditis elegans*. *Dev Biol*. 70: pp. 396–417. doi: 10.1016/0012-1606(79)90035-6

Kinchen, J.M., and K.S. Ravichandran. 2008. Phagosome maturation: going through the acid test. *Nat Rev Mol Cell Biol*. 9: pp. 781–95. doi: 10.1038/nrm2515

Kischkel, F.C., S. Hellbardt, I. Behrmann, M. Germer, M. Pawlita, P.H. Krammer, and M.E. Peter. 1995. Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO J*. 14: pp. 5579–88.

Kitano, M., M. Nakaya, T. Nakamura, S. Nagata, and M. Matsuda. 2008. Imaging of Rab5 activity identifies essential regulators for phagosome maturation. *Nature*. 453: pp. 241–5. doi: 10.1038/nature06857

Klein, J.A., C.M. Longo-Guess, M.P. Rossmann, K.L. Seburn, R.E. Hurd, W.N. Frankel, R.T. Bronson, and S.L. Ackerman. 2002. The harlequin mouse mutation downregulates apoptosis-inducing factor. *Nature*. 419: pp. 367–74.

Knudson, C.M., K.S. Tung, W.G. Tourtellotte, G.A. Brown, and S.J. Korsmeyer. 1995. Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science*. 270: pp. 96–9.

Kobayashi, N., P. Karisola, V. Pena-Cruz, D.M. Dorfman, M. Jinushi, S.E. Umetsu, M.J. Butte, H. Nagumo, I. Chernova, B. Zhu, A.H. Sharpe, S. Ito, G. Dranoff, G.G. Kaplan, J.M. Casasnovas, D.T. Umetsu, R.H. Dekruyff, and G.J. Freeman. 2007. TIM-1 and TIM-4 glycoproteins bind phosphatidylserine and mediate uptake of apoptotic cells. *Immunity*. 27: pp. 927–40. doi: 10.1016/j.immuni.2007.11.011

Kornbluth, S., and K. White. 2005. Apoptosis in *Drosophila*: neither fish nor fowl (nor man, nor worm). *J Cell Sci*. 118: pp. 1779–87. doi: 10.1242/jcs.02377

Kowalevsky, A. 1887. Beiträge zur Kenntnis der nachembryonalen Entwicklung der Musciden. *Z Wiss Zool*. 45: pp. 542–94.

Krahling, S., M.K. Callahan, P. Williamson, and R.A. Schlegel. 1999. Exposure of phosphatidylserine is a general feature in the phagocytosis of apoptotic lymphocytes by macrophages. *Cell Death Differ*. 6: pp. 183–9. doi: 10.1038/sj.cdd.4400473

Kroemer, G., W.S. El-Deiry, P. Golstein, M.E. Peter, D. Vaux, P. Vandenabeele, B. Zhivotovsky, M.V. Blagosklonny, W. Malorni, R.A. Knight, M. Piacentini, S. Nagata, and G. Melino. 2005. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death. *Cell Death Differ*. 12(Suppl 2): pp. 1463–7. doi: 10.1038/sj.cdd.4401724

Kroemer, G., L. Galluzzi, P. Vandenabeele, J. Abrams, E.S. Alnemri, E.H. Baehrecke, M.V. Blagosklonny, W.S. El-Deiry, P. Golstein, D.R. Green, M. Hengartner, R.A. Knight, S. Kumar, S.A. Lipton, W. Malorni, G. Nunez, M.E. Peter, J. Tschopp, J. Yuan, M. Piacentini, B. Zhivotovsky, and G. Melino. 2009. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differ*. 16: pp. 3–11. doi: 10.1038/cdd.2008.150

Kuida, K., T.S. Zheng, S. Na, C. Kuan, D. Yang, H. Karasuyama, P. Rakic, and R.A. Flavell. 1996. Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature*. 384: pp. 368–72. doi: 10.1038/384368a0

Kuwana, T., L. Bouchier-Hayes, J.E. Chipuk, C. Bonzon, B.A. Sullivan, D.R. Green, and D.D. Newmeyer. 2005. BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol Cell*. 17: pp. 525–35. doi: 10.1016/j.molcel.2005.02.003

Kuwana, T., M.R. Mackey, G. Perkins, M.H. Ellisman, M. Latterich, R. Schneiter, D.R. Green, and D.D. Newmeyer. 2002. Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell*. 111: pp. 331–42. doi: 10.1016/S0092-8674(02)01036-X

LaCasse, E.C., D.J. Mahoney, H.H. Cheung, S. Plenchette, S. Baird, and R.G. Korneluk. 2008. IAP-targeted therapies for cancer. *Oncogene*. 27: pp. 6252–75. doi: 10.1038/onc.2008.302

Lam, M., G. Dubyak, L. Chen, G. Nunez, R.L. Miesfeld, and C.W. Distelhorst. 1994. Evidence that BCL-2 represses apoptosis by regulating endoplasmic reticulum-associated Ca2+ fluxes. *Proc Natl Acad Sci U S A*. 91: pp. 6569–73. doi: 10.1073/pnas.91.14.6569

Lauber, K., E. Bohn, S.M. Krober, Y.J. Xiao, S.G. Blumenthal, R.K. Lindemann, P. Marini, C. Wiedig, A. Zobywalski, S. Baksh, Y. Xu, I.B. Autenrieth, K. Schulze-Osthoff, C. Belka, G. Stuhler, and S. Wesselborg. 2003. Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. *Cell*. 113: pp. 717–30. doi: 10.1016/S0092-8674(03)00422-7

Lecut, C., K. Frederix, D.M. Johnson, C. Deroanne, M. Thiry, C. Faccinetto, R. Maree, R.J. Evans, P.G. Volders, V. Bours, and C. Oury. 2009. P2X1 ion channels promote neutrophil chemotaxis through Rho kinase activation. *J Immunol*. 183: pp. 2801–9. doi: 10.4049/jimmunol.0804007

Lee, J.W., Y.H. Soung, S.Y. Kim, S.W. Nam, C.J. Kim, Y.G. Cho, J.H. Lee, H.S. Kim, W.S. Park, S.H. Kim, J.Y. Lee, N.J. Yoo, and S.H. Lee. 2004. Inactivating mutations of proapoptotic Bad gene in human colon cancers. *Carcinogenesis*. 25: pp. 1371–6. doi: 10.1093/carcin/bgh145

Lee, S.H., M.S. Shin, W.S. Park, S.Y. Kim, H.S. Kim, J.Y. Han, G.S. Park, S.M. Dong, J.H. Pi, C.S. Kim, S.H. Kim, J.Y. Lee, and N.J. Yoo. 1999. Alterations of Fas (Apo-1/CD95) gene in non-small cell lung cancer. *Oncogene*. 18: pp. 3754–60. doi: 10.1038/sj.onc.1202769

Lessene, G., P.E. Czabotar, and P.M. Colman. 2008. BCL-2 family antagonists for cancer therapy. *Nat Rev Drug Discov*. 7: pp. 989–1000. doi: 10.1038/nrd2658

Letai, A., M.C. Bassik, L.D. Walensky, M.D. Sorcinelli, S. Weiler, and S.J. Korsmeyer. 2002. Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell*. 2: pp. 183–92. doi: 10.1016/S1535-6108(02)00127-7

Levine, B., Q. Huang, J.T. Isaacs, J.C. Reed, D.E. Griffin, and J.M. Hardwick. 1993. Conversion of lytic to persistent alphavirus infection by the bcl-2 cellular oncogene. *Nature*. 361: pp. 739–42. doi: 10.1038/361739a0

Levkau, B., B. Herren, H. Koyama, R. Ross, and E.W. Raines. 1998. Caspase-mediated cleavage of focal adhesion kinase pp125FAK and disassembly of focal adhesions in human endothelial cell apoptosis. *J Exp Med*. 187: pp. 579–86. doi: 10.1084/jem.187.4.579

Li, H., H. Zhu, C.J. Xu, and J. Yuan. 1998. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell*. 94: pp. 491–501. doi: 10.1016/S0092-8674(00)81590-1

Li, L.Y., X. Luo, and X. Wang. 2001. Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature*. 412: pp. 95–9.

Li, P., D. Nijhawan, I. Budihardjo, S.M. Srinivasula, M. Ahmad, E.S. Alnemri, and X. Wang. 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell*. 91: pp. 479–89. doi: 10.1016/S0092-8674(00)80434-1

Lin, S.C., Y. Huang, Y.C. Lo, M. Lu, and H. Wu. 2007. Crystal structure of the BIR1 domain of XIAP in two crystal forms. *J Mol Biol*. 372: pp. 847–54. doi: 10.1016/j.jmb.2007.07.019

Lindsten, T., A.J. Ross, A. King, W.X. Zong, J.C. Rathmell, H.A. Shiels, E. Ulrich, K.G. Waymire, P. Mahar, K. Frauwirth, Y. Chen, M. Wei, V.M. Eng, D.M. Adelman, M.C. Simon, A. Ma, J.A. Golden, G. Evan, S.J. Korsmeyer, G.R. MacGregor, and C.B. Thompson. 2000. The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. *Mol Cell*. 6: pp. 1389–99. doi: 10.1016/S1097-2765(00)00136-2

Liu, X., C.N. Kim, J. Yang, R. Jemmerson, and X. Wang. 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell*. 86: pp. 147–57. doi: 10.1016/S0092-8674(00)80085-9

Liu, X., H. Zou, C. Slaughter, and X. Wang. 1997. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell*. 89: pp. 175–84. doi: 10.1016/S0092-8674(00)80197-X

Liu, Z., C. Sun, E.T. Olejniczak, R.P. Meadows, S.F. Betz, T. Oost, J. Herrmann, J.C. Wu, and S.W. Fesik. 2000. Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain. *Nature*. 408: pp. 1004–8.

Lockshin, R.A. 1997. The early modern period in cell death. *Cell Death Differ*. 4: pp. 347–51. doi: 10.1038/sj.cdd.4400260

Lockshin, R.A., and C.M. Williams. 1965. Programmed cell death. IV. The influence of drugs on the breakdown of the intersegmental muscles of silkmoths. *J Insect Physiol*. 11: pp. 803–9. doi: 10.1016/0022-1910(65)90159-9

Luo, X., I. Budihardjo, H. Zou, C. Slaughter, and X. Wang. 1998. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell*. 94: pp. 481–90. doi: 10.1016/S0092-8674(00)81589-5

MacDonald, J.M., M.G. Beach, E. Porpiglia, A.E. Sheehan, R.J. Watts, and M.R. Freeman. 2006. The *Drosophila* cell corpse engulfment receptor Draper mediates glial clearance of severed axons. *Neuron*. 50: pp. 869–81. doi: 10.1016/j.neuron.2006.04.028

Majno, G., and I. Joris. 1995. Apoptosis, oncosis, and necrosis. An overview of cell death. *Am J Pathol*. 146: pp. 3–15.

Mannhold, R., S. Fulda, and E. Carosati. 2010. IAP antagonists: promising candidates for cancer therapy. *Drug Discov Today*. 15(5–6): pp. 210–9. doi: 10.1016/j.drudis.2010.01.003

Martikainen, P., N. Kyprianou, and J.T. Isaacs. 1990. Effect of transforming growth factor-beta 1 on proliferation and death of rat prostatic cells. *Endocrinology*. 127: pp. 2963–8.

Martinon, F., K. Burns, and J. Tschopp. 2002. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell*. 10: pp. 417–26.

Martins, L.M., A. Morrison, K. Klupsch, V. Fedele, N. Moisoi, P. Teismann, A. Abuin, E. Grau, M. Geppert, G.P. Livi, C.L. Creasy, A. Martin, I. Hargreaves, S.J. Heales, H. Okada, S. Brandner, J.B. Schulz, T. Mak, and J. Downward. 2004. Neuroprotective role of the Reaper-related serine protease HtrA2/Omi revealed by targeted deletion in mice. *Mol Cell Biol*. 24: pp. 9848–62.

Mayer, S. 1886. Die sogenannten Sarkoplasten. *Anat Anz*. 1: pp. 231–5.

McDonnell, T.J., N. Deane, F.M. Platt, G. Nunez, U. Jaeger, J.P. McKearn, and S.J. Korsmeyer. 1989. bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. *Cell*. 57: pp. 79–88. doi: 10.1016/0092-8674(89)90174-8

McDonnell, T.J., and S.J. Korsmeyer. 1991. Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14; 18). *Nature*. 349: pp. 254–6. doi: 10.1038/349254a0

Melino, G. 2001. The Sirens' song. *Nature*. 412: p. 23.

Metzstein, M.M., G.M. Stanfield, and H.R. Horvitz. 1998. Genetics of programmed cell death in *C. elegans*: past, present and future. *Trends Genet*. 14: pp. 410–6. doi: 10.1016/S0168-9525(98)01573-X

Miksa, M., D. Amin, R. Wu, T.S. Ravikumar, and P. Wang. 2007. Fractalkine-induced MFG-E8 leads to enhanced apoptotic cell clearance by macrophages. *Mol Med*. 13: pp. 553–60.

Milligan, C.E., D. Prevette, H. Yaginuma, S. Homma, C. Cardwell, L.C. Fritz, K.J. Tomaselli, R.W. Oppenheim, and L.M. Schwartz. 1995. Peptide inhibitors of the ICE protease family arrest programmed cell death of motoneurons in vivo and in vitro. *Neuron*. 15: pp. 385–93. doi: 10.1016/0896-6273(95)90042-X

Miquel, C., F. Borrini, S. Grandjouan, A. Auperin, J. Viguier, V. Velasco, P. Duvillard, F. Praz, and J.C. Sabourin. 2005. Role of bax mutations in apoptosis in colorectal cancers with microsatellite instability. *Am J Clin Pathol*. 123: pp. 562–70. doi: 0.1309/JQ2X-3RV3-L8F9-TGYW

Miyanishi, M., K. Tada, M. Koike, Y. Uchiyama, T. Kitamura, and S. Nagata. 2007. Identification of Tim4 as a phosphatidylserine receptor. *Nature*. 450: pp. 435–9. doi: 10.1038/nature06307

Motoyama, N., F. Wang, K.A. Roth, H. Sawa, K. Nakayama, I. Negishi, S. Senju, Q. Zhang, S. Fujii, and et al. 1995. Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. *Science*. 267: pp. 1506–10.

Muchmore, S.W., M. Sattler, H. Liang, R.P. Meadows, J.E. Harlan, H.S. Yoon, D. Nettesheim, B.S. Chang, C.B. Thompson, S.L. Wong, S.L. Ng, and S.W. Fesik. 1996. X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. *Nature*. 381: pp. 335–41. doi: 10.1038/381335a0

Munoz, L.E., K. Lauber, M. Schiller, A.A. Manfredi, and M. Herrmann. 2010. The role of defective clearance of apoptotic cells in systemic autoimmunity. *Nat Rev Rheumatol*. 6: pp. 280–9.

Nagata, S., R. Hanayama, and K. Kawane. 2010. Autoimmunity and the clearance of dead cells. *Cell*. 140: pp. 619–30. doi: 10.1016/j.cell.2010.02.014

Nakano, K., and K.H. Vousden. 2001. PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell*. 7: pp. 683–94. doi: 10.1016/S1097-2765(01)00214-3

Nakaya, M., M. Tanaka, Y. Okabe, R. Hanayama, and S. Nagata. 2006. Opposite effects of rho family GTPases on engulfment of apoptotic cells by macrophages. *J Biol Chem*. 281: pp. 8836–42. doi: 10.1074/jbc.M510972200

Nathan, C., and A. Ding. 2010. Nonresolving inflammation. *Cell*. 140:871–82. doi: 10.1016/j.cell.2010.02.029

Newmeyer, D.D., D.M. Farschon, and J.C. Reed. 1994. Cell-free apoptosis in *Xenopus* egg extracts: inhibition by Bcl-2 and requirement for an organelle fraction enriched in mitochondria. *Cell*. 79: pp. 353–64. doi: 10.1016/0092-8674(94)90203-8

Newton, K., A.W. Harris, and A. Strasser. 2000. FADD/MORT1 regulates the pre-TCR checkpoint and can function as a tumour suppressor. *EMBO J*. 19: pp. 931–41. doi: 10.1093/emboj/19.5.931

Nguyen, M., R.C. Marcellus, A. Roulston, M. Watson, L. Serfass, S.R. Murthy Madiraju, D. Goulet, J. Viallet, L. Belec, X. Billot, S. Acoca, E. Purisima, A. Wiegmans, L. Cluse, R.W.

Johnstone, P. Beauparlant, and G.C. Shore. 2007. Small molecule obatoclax (GX15-070) antagonizes MCL-1 and overcomes MCL-1-mediated resistance to apoptosis. *Proc Natl Acad Sci U S A*. 104: pp. 19512–7. doi: 10.1073/pnas.0709443104

Nutt, L.K., A. Pataer, J. Pahler, B. Fang, J. Roth, D.J. McConkey, and S.G. Swisher. 2002. Bax and Bak promote apoptosis by modulating endoplasmic reticular and mitochondrial Ca2+ stores. *J Biol Chem*. 277: pp. 9219–25. doi: 10.1074/jbc.M106817200

O'Brien, S., J.O. Moore, T.E. Boyd, L.M. Larratt, A. Skotnicki, B. Koziner, A.A. Chanan-Khan, J.F. Seymour, R.G. Bociek, S. Pavletic, and K.R. Rai. 2007. Randomized phase III trial of fludarabine plus cyclophosphamide with or without oblimersen sodium (Bcl-2 antisense) in patients with relapsed or refractory chronic lymphocytic leukemia. *J Clin Oncol*. 25: pp. 1114–20. doi: 10.1200/JCO.2006.07.1191

Oda, E., R. Ohki, H. Murasawa, J. Nemoto, T. Shibue, T. Yamashita, T. Tokino, T. Taniguchi, and N. Tanaka. 2000. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science*. 288: pp. 1053–8. doi: 10.1126/science.288.5468.1053

Okada, H., W.K. Suh, J. Jin, M. Woo, C. Du, A. Elia, G.S. Duncan, A. Wakeham, A. Itie, S.W. Lowe, X. Wang, and T.W. Mak. 2002. Generation and characterization of Smac/DIABLO-deficient mice. *Mol Cell Biol*. 22: pp. 3509–17. doi: 10.1128/MCB.22.10.3509-3517.2002

Oltersdorf, T., S.W. Elmore, A.R. Shoemaker, R.C. Armstrong, D.J. Augeri, B.A. Belli, M. Bruncko, T.L. Deckwerth, J. Dinges, P.J. Hajduk, M.K. Joseph, S. Kitada, S.J. Korsmeyer, A.R. Kunzer, A. Letai, C. Li, M.J. Mitten, D.G. Nettesheim, S. Ng, P.M. Nimmer, J.M. O'Connor, A. Oleksijew, A.M. Petros, J.C. Reed, W. Shen, S.K. Tahir, C.B. Thompson, K.J. Tomaselli, B. Wang, M.D. Wendt, H. Zhang, S.W. Fesik, and S.H. Rosenberg. 2005. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature*. 435: pp. 677–81. doi: 10.1038/nature03579

Oppenheim, J.J., and D. Yang. 2005. Alarmins: chemotactic activators of immune responses. *Curr Opin Immunol*. 17: pp. 359–65. doi: 10.1016/j.coi.2005.06.002

Park, D., A.C. Tosello-Trampont, M.R. Elliott, M. Lu, L.B. Haney, Z. Ma, A.L. Klibanov, J.W. Mandell, and K.S. Ravichandran. 2007a. BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module. *Nature*. 450: pp. 430–4. doi: 10.1038/nature06329

Park, H.H., E. Logette, S. Raunser, S. Cuenin, T. Walz, J. Tschopp, and H. Wu. 2007b. Death domain assembly mechanism revealed by crystal structure of the oligomeric PIDDosome core complex. *Cell*. 128: pp. 533–46. doi: 10.1016/j.cell.2007.01.019

Park, S.Y., M.Y. Jung, H.J. Kim, S.J. Lee, S.Y. Kim, B.H. Lee, T.H. Kwon, R.W. Park, and I.S. Kim. 2008. Rapid cell corpse clearance by stabilin-2, a membrane phosphatidylserine receptor. *Cell Death Differ*. 15: pp. 192–201. doi: 10.1038/sj.cdd.4402242

Pepper, C., T.T. Lin, G. Pratt, S. Hewamana, P. Brennan, L. Hiller, R. Hills, R. Ward, J. Starczynski,

B. Austen, L. Hooper, T. Stankovic, and C. Fegan. 2008. Mcl-1 expression has in vitro and in vivo significance in chronic lymphocytic leukemia and is associated with other poor prognostic markers. *Blood*. 112: pp. 3807–17. doi: 10.1182/blood-2008-05-157131

Peter, C., M. Waibel, C.G. Radu, L.V. Yang, O.N. Witte, K. Schulze-Osthoff, S. Wesselborg, and K. Lauber. 2008. Migration to apoptotic "find-me" signals is mediated via the phagocyte receptor G2A. *J Biol Chem*. 283: pp. 5296–305. doi: 10.1074/jbc.M706586200

Peter, C., S. Wesselborg, and K. Lauber. 2010. Molecular suicide notes: last call from apoptosing cells. *J Mol Cell Biol*. 2: pp. 78–80. doi: 10.1093/jmcb/mjp045

Petros, A.M., D.G. Nettesheim, Y. Wang, E.T. Olejniczak, R.P. Meadows, J. Mack, K. Swift, E.D. Matayoshi, H. Zhang, C.B. Thompson, and S.W. Fesik. 2000. Rationale for Bcl-xL/Bad peptide complex formation from structure, mutagenesis, and biophysical studies. *Protein Sci*. 9: pp. 2528–34. doi: 10.1017/S096183680000331X

Petros, A.M., E.T. Olejniczak, and S.W. Fesik. 2004. Structural biology of the Bcl-2 family of proteins. *Biochim Biophys Acta*. 1644: pp. 83–94. doi: 10.1016/j.bbamcr.2003.08.012

Pinton, P., D. Ferrari, E. Rapizzi, F. Di Virgilio, T. Pozzan, and R. Rizzuto. 2001. The Ca2+ concentration of the endoplasmic reticulum is a key determinant of ceramide-induced apoptosis: significance for the molecular mechanism of Bcl-2 action. *EMBO J*. 20: pp. 2690–701. doi: 10.1093/emboj/20.11.2690

Prasad, D., C.V. Rothlin, P. Burrola, T. Burstyn-Cohen, Q. Lu, P. Garcia de Frutos, and G. Lemke. 2006. TAM receptor function in the retinal pigment epithelium. *Mol Cell Neurosci*. 33: pp. 96–108. doi: 10.1016/j.mcn.2006.06.011

Qu, Y., S. Misaghi, K. Newton, L.L. Gilmour, S. Louie, J.E. Cupp, G.R. Dubyak, D. Hackos, and V.M. Dixit. 2011. Pannexin-1 is required for ATP release during apoptosis but not for inflammasome activation. *J Immunol*. 186: pp. 6553–61. doi: 10.4049/jimmunol.1100478

Raff, M.C. 1992. Social controls on cell survival and cell death. *Nature*. 356: pp. 397–400. doi: 10.1038/356397a0

Ranger, A.M., B.A. Malynn, and S.J. Korsmeyer. 2001. Mouse models of cell death. *Nat Genet*. 28: pp. 113–8.

Ranger, A.M., J. Zha, H. Harada, S.R. Datta, N.N. Danial, A.P. Gilmore, J.L. Kutok, M.M. Le Beau, M.E. Greenberg, and S.J. Korsmeyer. 2003. Bad-deficient mice develop diffuse large B cell lymphoma. *Proc Natl Acad Sci U S A*. 100: pp. 9324–9. doi: 10.1073/pnas.1533446100

Rao, L., D. Perez, and E. White. 1996. Lamin proteolysis facilitates nuclear events during apoptosis. *J Cell Biol*. 135: pp. 1441–55. doi: 10.1083/jcb.135.6.1441

Ravichandran, K.S. 2011. Beginnings of a good apoptotic meal: the find-me and eat-me signaling pathways. *Immunity*. 35: pp. 445–55. doi: 10.1016/j.immuni.2011.09.004

Ravichandran, K.S., and U. Lorenz. 2007. Engulfment of apoptotic cells: signals for a good meal. *Nat Rev Immunol*. 7: pp. 964–74. doi: 10.1038/nri2214

Reddien, P.W., S. Cameron, and H.R. Horvitz. 2001. Phagocytosis promotes programmed cell death in *C. elegans*. *Nature*. 412: pp. 198–202.

Ren, D., H.C. Tu, H. Kim, G.X. Wang, G.R. Bean, O. Takeuchi, J.R. Jeffers, G.P. Zambetti, J.J. Hsieh, and E.H. Cheng. 2010. BID, BIM, and PUMA are essential for activation of the BAX- and BAK-dependent cell death program. *Science*. 330: pp. 1390–3. doi: 10.1126/science.1190217

Reynaud, K., and M.A. Driancourt. 2000. Oocyte attrition. *Mol Cell Endocrinol*. 163: pp. 101–8. doi: 10.1016/S0303-7207(99)00246-4

Riedl, S.J., M. Renatus, R. Schwarzenbacher, Q. Zhou, C. Sun, S.W. Fesik, R.C. Liddington, and G.S. Salvesen. 2001. Structural basis for the inhibition of caspase-3 by XIAP. *Cell*. 104: pp. 791–800. doi: 10.1016/S0092-8674(01)00274-4

Rieux-Laucat, F., F. Le Deist, C. Hivroz, I.A. Roberts, K.M. Debatin, A. Fischer, and J.P. de Villartay. 1995. Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. *Science*. 268: pp. 1347–9. doi: 10.1126/science.7539157

Rinkenberger, J.L., S. Horning, B. Klocke, K. Roth, and S.J. Korsmeyer. 2000. Mcl-1 deficiency results in peri-implantation embryonic lethality. *Genes Dev*. 14: pp. 23–7.

Rizzuto, R., and T. Pozzan. 2006. Microdomains of intracellular Ca2+: molecular determinants and functional consequences. *Physiol Rev*. 86: pp. 369–408. doi: 10.1152/physrev.00004.2005

Rotonda, J., D.W. Nicholson, K.M. Fazil, M. Gallant, Y. Gareau, M. Labelle, E.P. Peterson, D.M. Rasper, R. Ruel, J.P. Vaillancourt, N.A. Thornberry, and J.W. Becker. 1996. The three-dimensional structure of apopain/CPP32, a key mediator of apoptosis. *Nat Struct Biol*. 3: pp. 619–25. doi: 10.1038/nsb0796-619

Rowley, J.D. 1982. Identification of the constant chromosome regions involved in human hematologic malignant disease. *Science*. 216: pp. 749–51. doi: 10.1126/science.7079737

Ruffolo, S.C., and G.C. Shore. 2003. BCL-2 selectively interacts with the BID-induced open conformer of BAK, inhibiting BAK auto-oligomerization. *J Biol Chem*. 278: pp. 25039–45. doi: 10.1074/jbc.M302930200

Rumore, P.M., and C.R. Steinman. 1990. Endogenous circulating DNA in systemic lupus erythematosus. Occurrence as multimeric complexes bound to histone. *J Clin Invest*. 86: pp. 69–74. doi: 10.1172/JCI114716

Sahu, S.K., S.N. Gummadi, N. Manoj, and G.K. Aradhyam. 2007. Phospholipid scramblases: an overview. *Arch Biochem Biophys*. 462: pp. 103–14. doi: 10.1016/j.abb.2007.04.002

Sakahira, H., Y. Takemura, and S. Nagata. 2001. Enzymatic active site of caspase-activated DNase (CAD) and its inhibition by inhibitor of CAD. *Arch Biochem Biophys*. 388: pp. 91–9. doi: 10.1006/abbi.2000.2266

Santiago, C., A. Ballesteros, L. Martinez-Munoz, M. Mellado, G.G. Kaplan, G.J. Freeman, and J.M. Casasnovas. 2007. Structures of T cell immunoglobulin mucin protein 4 show a

metal-Ion-dependent ligand binding site where phosphatidylserine binds. *Immunity*. 27: pp. 941–51. doi: 10.1016/j.immuni.2007.11.008

Saunders, J.W., Jr. 1966. Death in embryonic systems. *Science*. 154: pp. 604–12. doi: 10.1126/science.154.3749.604

Saunders, J.W., Jr., and J.F. Fallon. 1966. Major Problems in Developmental Biology. *In* 25th Symposium of the Society for Developmental Biology. M. Locke, editor. Academic Press, New York. 289.

Scaffidi, P., T. Misteli, and M.E. Bianchi. 2002. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature*. 418: pp. 191–5. doi: 10.1038/nature00858

Schile, A.J., M. Garcia-Fernandez, and H. Steller. 2008. Regulation of apoptosis by XIAP ubiquitin-ligase activity. *Genes Dev*. 22: pp. 2256–66. doi: 10.1101/gad.1663108

Schleiden, M.J. 1842. Grundzüge der wissenschaftlichen Botanik. Engelmann, Leipzig. doi: 10.5962/bhl.title.61283

Schutze, S., V. Tchikov, and W. Schneider-Brachert. 2008. Regulation of TNFR1 and CD95 signalling by receptor compartmentalization. *Nat Rev Mol Cell Biol*. 9: pp. 655–62. doi: 10.1038/nrm2430

Schwann, T. 1839. Mikroskopische Untersuchungen. Sander, Berlin.

Scorrano, L., S.A. Oakes, J.T. Opferman, E.H. Cheng, M.D. Sorcinelli, T. Pozzan, and S.J. Korsmeyer. 2003. BAX and BAK regulation of endoplasmic reticulum Ca2+: a control point for apoptosis. *Science*. 300: pp. 135–9. doi: 10.1126/science.1081208

Scott, F.L., J.B. Denault, S.J. Riedl, H. Shin, M. Renatus, and G.S. Salvesen. 2005. XIAP inhibits caspase-3 and -7 using two binding sites: evolutionarily conserved mechanism of IAPs. *EMBO J*. 24: pp. 645–55. doi: 10.1038/sj.emboj.7600544

Sebbagh, M., C. Renvoize, J. Hamelin, N. Riche, J. Bertoglio, and J. Breard. 2001. Caspase-3-mediated cleavage of ROCK I induces MLC phosphorylation and apoptotic membrane blebbing. *Nat Cell Biol*. 3: pp. 346–52. doi: 10.1038/35070019

Sega, G.A. 1984. A review of the genetic effects of ethyl methanesulfonate. *Mutat Res*. 134: pp. 113–42. doi: 10.1016/0165-1110(84)90007-1

Shi, Y., J.E. Evans, and K.L. Rock. 2003. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature*. 425: pp. 516–21. doi: 10.1038/nature01991

Shiozaki, E.N., J. Chai, D.J. Rigotti, S.J. Riedl, P. Li, S.M. Srinivasula, E.S. Alnemri, R. Fairman, and Y. Shi. 2003. Mechanism of XIAP-mediated inhibition of caspase-9. *Mol Cell*. 11: pp. 519–27. doi: 10.1016/S1097-2765(03)00054-6

Shore, G.C., and J. Viallet. 2005. Modulating the bcl-2 family of apoptosis suppressors for potential therapeutic benefit in cancer. *Hematology Am Soc Hematol Educ Program*. pp. 226–30. doi: 10.1182/asheducation-2005.1.226

Siegel, R.M., F.K. Chan, H.J. Chun, and M.J. Lenardo. 2000. The multifaceted role of Fas signaling in immune cell homeostasis and autoimmunity. *Nat Immunol*. 1: pp. 469–74.

Spiess, C., A. Beil, and M. Ehrmann. 1999. A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. *Cell*. 97: pp. 339–47. doi: 10.1016/S0092-8674(00)80743-6

Srinivasula, S.M., P. Datta, X.J. Fan, T. Fernandes-Alnemri, Z. Huang, and E.S. Alnemri. 2000. Molecular determinants of the caspase-promoting activity of Smac/DIABLO and its role in the death receptor pathway. *J Biol Chem*. 275: pp. 36152–7. doi: 10.1074/jbc.C000533200

Srinivasula, S.M., R. Hegde, A. Saleh, P. Datta, E. Shiozaki, J. Chai, R.A. Lee, P.D. Robbins, T. Fernandes-Alnemri, Y. Shi, and E.S. Alnemri. 2001. A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. *Nature*. 410: pp. 112–6.

Strasser, A., P.J. Jost, and S. Nagata. 2009. The many roles of FAS receptor signaling in the immune system. *Immunity*. 30: pp. 180–92. doi: 10.1016/j.immuni.2009.01.001

Suda, T., T. Takahashi, P. Golstein, and S. Nagata. 1993. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell*. 75: pp. 1169–78. doi: 10.1016/0092-8674(93)90326-L

Sulston, J.E. 1976. Post-embryonic development in the ventral cord of *Caenorhabditis elegans*. *Philos Trans R Soc Lond B Biol Sci*. 275: pp. 287–97. doi: 10.1098/rstb.1976.0084

Sulston, J.E., and H.R. Horvitz. 1977. Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. *Dev Biol*. 56: pp. 110–56. doi: 10.1016/0012-1606(77)90158-0

Sulston, J.E., E. Schierenberg, J.G. White, and J.N. Thomson. 1983. The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev Biol*. 100: pp. 64–119. doi: 10.1016/0012-1606(83)90201-4

Sun, C., M. Cai, A.H. Gunasekera, R.P. Meadows, H. Wang, J. Chen, H. Zhang, W. Wu, N. Xu, S.C. Ng, and S.W. Fesik. 1999. NMR structure and mutagenesis of the inhibitor-of-apoptosis protein XIAP. *Nature*. 401: pp. 818–22.

Sun, H., Z. Nikolovska-Coleska, J. Lu, S. Qiu, C.Y. Yang, W. Gao, J. Meagher, J. Stuckey, and S. Wang. 2006. Design, synthesis, and evaluation of a potent, cell-permeable, conformationally constrained second mitochondria derived activator of caspase (Smac) mimetic. *J Med Chem*. 49: pp. 7916–20. doi: 10.1021/jm061108d

Susin, S.A., N. Zamzami, M. Castedo, T. Hirsch, P. Marchetti, A. Macho, E. Daugas, M. Geuskens, and G. Kroemer. 1996. Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. *J Exp Med*. 184: pp. 1331–41. doi: 10.1084/jem.184.4.1331

Suzuki, M., R.J. Youle, and N. Tjandra. 2000. Structure of Bax: coregulation of dimer formation and intracellular localization. *Cell*. 103: pp. 645–54.

Suzuki, Y., Y. Nakabayashi, and R. Takahashi. 2001. Ubiquitin-protein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase-3 and enhances its anti-apoptotic effect in Fas-induced cell death. *Proc Natl Acad Sci U S A*. 98: pp. 8662–7. doi: 10.1073/pnas.161506698

Szalai, G., R. Krishnamurthy, and G. Hajnoczky. 1999. Apoptosis driven by IP(3)-linked mitochondrial calcium signals. *EMBO J*. 18: pp. 6349–61.

Tait, S.W., and D.R. Green. 2010. Mitochondria and cell death: outer membrane permeabilization and beyond. *Nat Rev Mol Cell Biol*. 11: pp. 621–32. doi: 10.1038/nrm2952

Takahashi, T., M. Tanaka, C.I. Brannan, N.A. Jenkins, N.G. Copeland, T. Suda, and S. Nagata. 1994. Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell*. 76: pp. 969–76. doi: 10.1016/0092-8674(94)90375-1

Tanaka, M., T. Suda, T. Takahashi, and S. Nagata. 1995. Expression of the functional soluble form of human fas ligand in activated lymphocytes. *EMBO J*. 14: pp. 1129–35.

Tanaka, Y., and A.J. Schroit. 1983. Insertion of fluorescent phosphatidylserine into the plasma membrane of red blood cells. Recognition by autologous macrophages. *J Biol Chem*. 258: pp. 11335–43.

Tang, G., Z. Nikolovska-Coleska, S. Qiu, C.Y. Yang, J. Guo, and S. Wang. 2008. Acylpyrogallols as inhibitors of antiapoptotic Bcl-2 proteins. *J Med Chem*. 51: pp. 717–20. doi: 10.1021/jm701358v

Tata, J.R. 1966. Requirement for RNA and protein synthesis for induced regression of the tadpole tail in organ culture. *Dev Biol*. 13: pp. 77–94. doi: 10.1016/0012-1606(66)90050-9

Tawfik, K., B.F. Kimler, M.K. Davis, F. Fan, and O. Tawfik. 2012. Prognostic significance of Bcl-2 in invasive mammary carcinomas: a comparative clinicopathologic study between "triple-negative" and non- "triple-negative" tumors. *Hum Pathol*. 43: pp. 23–30. doi: 10.1016/j.humpath.2011.04.011

Taylor, R.C., S.P. Cullen, and S.J. Martin. 2008. Apoptosis: controlled demolition at the cellular level. *Nat Rev Mol Cell Biol*. 9: pp. 231–41. doi: 10.1038/nrm2312

Thornberry, N.A., H.G. Bull, J.R. Calaycay, K.T. Chapman, A.D. Howard, M.J. Kostura, D.K. Miller, S.M. Molineaux, J.R. Weidner, J. Aunins, and et al. 1992. A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. *Nature*. 356: pp. 768–74.

Thornberry, N.A., and Y. Lazebnik. 1998. Caspases: enemies within. *Science*. 281: pp. 1312–6. doi: 10.1126/science.281.5381.1312

Trautmann, A. 2009. Extracellular ATP in the immune system: more than just a "danger signal." *Sci Signal*. 2: p. e6. doi: 10.1126/scisignal.256pe6

Truman, L.A., C.A. Ford, M. Pasikowska, J.D. Pound, S.J. Wilkinson, I.E. Dumitriu, L. Melville,

L.A. Melrose, C.A. Ogden, R. Nibbs, G. Graham, C. Combadiere, and C.D. Gregory. 2008. CX3CL1/fractalkine is released from apoptotic lymphocytes to stimulate macrophage chemotaxis. *Blood*. 112: pp. 5026–36. doi: 10.1182/blood-2008-06-162404

Tse, C., A.R. Shoemaker, J. Adickes, M.G. Anderson, J. Chen, S. Jin, E.F. Johnson, K.C. Marsh, M.J. Mitten, P. Nimmer, L. Roberts, S.K. Tahir, Y. Xiao, X. Yang, H. Zhang, S. Fesik, S.H. Rosenberg, and S.W. Elmore. 2008. ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor. *Cancer Res*. 68: pp. 3421–8. doi: 10.1158/0008-5472.CAN-07-5836

Tsujimoto, Y., J. Cossman, E. Jaffe, and C.M. Croce. 1985. Involvement of the bcl-2 gene in human follicular lymphoma. *Science*. 228: pp. 1440–3. doi: 10.1126/science.3874430

Vahsen, N., C. Cande, J.J. Briere, P. Benit, N. Joza, N. Larochette, P.G. Mastroberardino, M.O. Pequignot, N. Casares, V. Lazar, O. Feraud, N. Debili, S. Wissing, S. Engelhardt, F. Madeo, M. Piacentini, J.M. Penninger, H. Schagger, P. Rustin, and G. Kroemer. 2004. AIF deficiency compromises oxidative phosphorylation. *EMBO J*. 23: pp. 4679–89. doi: 10.1038/sj.emboj.7600461

van Delft, M.F., A.H. Wei, K.D. Mason, C.J. Vandenberg, L. Chen, P.E. Czabotar, S.N. Willis, C.L. Scott, C.L. Day, S. Cory, J.M. Adams, A.W. Roberts, and D.C. Huang. 2006. The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. *Cancer Cell*. 10: pp. 389–99. doi: 10.1016/j.ccr.2006.08.027

Varfolomeev, E.E., M. Schuchmann, V. Luria, N. Chiannilkulchai, J.S. Beckmann, I.L. Mett, D. Rebrikov, V.M. Brodianski, O.C. Kemper, O. Kollet, T. Lapidot, D. Soffer, T. Sobe, K.B. Avraham, T. Goncharov, H. Holtmann, P. Lonai, and D. Wallach. 1998. Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. *Immunity*. 9: pp. 267–76. doi: 10.1016/S1074-7613(00)80609-3

Vaux, D.L. 2002. Apoptosis timeline. *Cell Death Differ*. 9: pp. 349–54. doi: 10.1038/sj.cdd.4400990

Vaux, D.L. 2011. Apoptogenic factors released from mitochondria. *Biochim Biophys Acta*. 1813: pp. 546–50. doi: 10.1016/j.bbamcr.2010.08.002

Vaux, D.L., S. Cory, and J.M. Adams. 1988. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature*. 335: pp. 440–2. doi: 10.1038/335440a0

Veis, D.J., C.M. Sorenson, J.R. Shutter, and S.J. Korsmeyer. 1993. Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell*. 75: pp. 229–40. doi: 10.1016/0092-8674(93)80065-M

Verhagen, A.M., P.G. Ekert, M. Pakusch, J. Silke, L.M. Connolly, G.E. Reid, R.L. Moritz, R.J. Simpson, and D.L. Vaux. 2000. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell*. 102: pp. 43–53. doi: 10.1016/S0092-8674(00)00009-X

Verhagen, A.M., J. Silke, P.G. Ekert, M. Pakusch, H. Kaufmann, L.M. Connolly, C.L. Day, A. Tikoo, R. Burke, C. Wrobel, R.L. Moritz, R.J. Simpson, and D.L. Vaux. 2002. HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins. *J Biol Chem*. 277: pp. 445–54. doi: 10.1074/jbc.M109891200

Viallanes, H. 1882. Recherches sur l'histologie des insectes et sur les phénomènes histologiques qui accompagnent le développement post-embryonnaire de ces animaux. *Ann Sci Nat Zool*. 14: pp. 1–348.

Villunger, A., E.M. Michalak, L. Coultas, F. Mullauer, G. Bock, M.J. Ausserlechner, J.M. Adams, and A. Strasser. 2003. p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. *Science*. 302: pp. 1036–8. doi: 10.1126/science.1090072

Vogt, C. 1842. Untersuchungen über die Entiwicklungsgeschichte der Geburtshelferkroete (*Alytes obstetricans*). Jent und Gassmann, Solothurn.

Voll, R.E., M. Herrmann, E.A. Roth, C. Stach, J.R. Kalden, and I. Girkontaite. 1997. Immunosuppressive effects of apoptotic cells. *Nature*. 390: pp. 350–1.

Walensky, L.D., A.L. Kung, I. Escher, T.J. Malia, S. Barbuto, R.D. Wright, G. Wagner, G.L. Verdine, and S.J. Korsmeyer. 2004. Activation of apoptosis in vivo by a hydrocarbon-stapled BH3 helix. *Science*. 305: pp. 1466–70. doi: 10.1126/science.1099191

Walensky, L.D., K. Pitter, J. Morash, K.J. Oh, S. Barbuto, J. Fisher, E. Smith, G.L. Verdine, and S.J. Korsmeyer. 2006. A stapled BID BH3 helix directly binds and activates BAX. *Mol Cell*. 24: pp. 199–210. doi: 10.1016/j.molcel.2006.08.020

Walker, N.P., R.V. Talanian, K.D. Brady, L.C. Dang, N.J. Bump, C.R. Ferenz, S. Franklin, T. Ghayur, M.C. Hackett, L.D. Hammill, and et al. 1994. Crystal structure of the cysteine protease interleukin-1 beta-converting enzyme: a (p20/p10)2 homodimer. *Cell*. 78: pp. 343–52.

Wang, J., and M.J. Lenardo. 2000. Roles of caspases in apoptosis, development, and cytokine maturation revealed by homozygous gene deficiencies. *J Cell Sci*. 113(Pt 5): pp. 753–7.

Watanabe-Fukunaga, R., C.I. Brannan, N.G. Copeland, N.A. Jenkins, and S. Nagata. 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature*. 356: pp. 314–7. doi: 10.1038/356314a0

Wei, M.C., T. Lindsten, V.K. Mootha, S. Weiler, A. Gross, M. Ashiya, C.B. Thompson, and S.J. Korsmeyer. 2000. tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. *Genes Dev*. 14: pp. 2060–71.

Weigert, A., S. Cremer, M.V. Schmidt, A. von Knethen, C. Angioni, G. Geisslinger, and B. Brune. 2010. Cleavage of sphingosine kinase 2 by caspase-1 provokes its release from apoptotic cells. *Blood*. 115: pp. 3531–40. doi: 10.1182/blood-2009-10-243444

Weismann, A. 1864. Die Entwicklung der Dipteren. Engelmann, Leipzig. doi: 10.5962/bhl.title.57945

Weissman, A. 1863. Die Entwickelung der Dipteren im Ei nach Beobachtungen an Chironomus spec., Musca vomitoria and Pulex Canis. *Z Wiss Zool*. 14: pp. 187–336.

White, K., M.E. Grether, J.M. Abrams, L. Young, K. Farrell, and H. Steller. 1994. Genetic control of programmed cell death in *Drosophila*. *Science*. 264: pp. 677–83. doi: 10.1126/science.8171319

Whitten, J.M. 1969. Cell death during early morphogenesis: parallels between insect limb and vertebrate limb development. *Science*. 163: pp. 1456–7. doi: 10.1126/science.163.3874.1456

Williams, J.R., J.B. Little, and W.U. Shipley. 1974. Association of mammalian cell death with a specific endonucleolytic degradation of DNA. *Nature*. 252: pp. 754–5. doi: 10.1038/252754a0

Willis, S.N., and J.M. Adams. 2005. Life in the balance: how BH3-only proteins induce apoptosis. *Curr Opin Cell Biol*. 17: pp. 617–25. doi: 10.1016/j.ceb.2005.10.001

Willis, S.N., L. Chen, G. Dewson, A. Wei, E. Naik, J.I. Fletcher, J.M. Adams, and D.C. Huang. 2005. Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes Dev*. 19: pp. 1294–305. doi: 10.1101/gad.1304105

Wilson, K.P., J.A. Black, J.A. Thomson, E.E. Kim, J.P. Griffith, M.A. Navia, M.A. Murcko, S.P. Chambers, R.A. Aldape, S.A. Raybuck, and et al. 1994. Structure and mechanism of interleukin-1 beta converting enzyme. *Nature*. 370: pp. 270–5.

Wolter, K.G., Y.T. Hsu, C.L. Smith, A. Nechushtan, X.G. Xi, and R.J. Youle. 1997. Movement of Bax from the cytosol to mitochondria during apoptosis. *J Cell Biol*. 139: pp. 1281–92. doi: 10.1083/jcb.139.5.1281

Wu, G., J. Chai, T.L. Suber, J.W. Wu, C. Du, X. Wang, and Y. Shi. 2000. Structural basis of IAP recognition by Smac/DIABLO. *Nature*. 408: pp. 1008–12.

Wyllie, A.H. 1980. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature*. 284: pp. 555–6. doi: 10.1038/284555a0

Wyllie, A.H., R.G. Morris, A.L. Smith, and D. Dunlop. 1984. Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. *J Pathol*. 142: pp. 67–77. doi: 10.1002/path.1711420112

Ye, H., C. Cande, N.C. Stephanou, S. Jiang, S. Gurbuxani, N. Larochette, E. Daugas, C. Garrido, G. Kroemer, and H. Wu. 2002. DNA binding is required for the apoptogenic action of apoptosis inducing factor. *Nat Struct Biol*. 9: pp. 680–4. doi: 10.1038/nsb836

Yeh, W.C., A. Itie, A.J. Elia, M. Ng, H.B. Shu, A. Wakeham, C. Mirtsos, N. Suzuki, M. Bonnard, D.V. Goeddel, and T.W. Mak. 2000. Requirement for Casper (c-FLIP) in regulation of death receptor-induced apoptosis and embryonic development. *Immunity*. 12: pp. 633–42. doi: 10.1016/S1074-7613(00)80214-9

Yin, X.M., K. Wang, A. Gross, Y. Zhao, S. Zinkel, B. Klocke, K.A. Roth, and S.J. Korsmeyer. 1999. Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. *Nature*. 400: pp. 886–91.

Yoshida, H., Y.Y. Kong, R. Yoshida, A.J. Elia, A. Hakem, R. Hakem, J.M. Penninger, and T.W. Mak. 1998. Apaf1 is required for mitochondrial pathways of apoptosis and brain development. *Cell*. 94: pp. 739–50. doi: 10.1016/S0092-8674(00)81733-X

Youle, R.J., and A. Strasser. 2008. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol*. 9: pp. 47–59. doi: 10.1038/nrm2308

Yu, J., L. Zhang, P.M. Hwang, K.W. Kinzler, and B. Vogelstein. 2001. PUMA induces the rapid apoptosis of colorectal cancer cells. *Mol Cell*. 7: pp. 673–82. doi: 10.1016/S1097-2765(01)00213-1

Yunis, J.J. 1983. The chromosomal basis of human neoplasia. *Science*. 221: pp. 227–36. doi: 10.1126/science.6336310

Zha, J., H. Harada, E. Yang, J. Jockel, and S.J. Korsmeyer. 1996. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell*. 87: pp. 619–28.

Zhang, J., D. Cado, A. Chen, N.H. Kabra, and A. Winoto. 1998. Fas-mediated apoptosis and activation-induced T-cell proliferation are defective in mice lacking FADD/Mort1. *Nature*. 392: pp. 296–300.

Zhang, J., M. Dong, L. Li, Y. Fan, P. Pathre, J. Dong, D. Lou, J.M. Wells, D. Olivares-Villagomez, L. Van Kaer, X. Wang, and M. Xu. 2003. Endonuclease G is required for early embryogenesis and normal apoptosis in mice. *Proc Natl Acad Sci U S A*. 100: pp. 15782–7. doi: 10.1073/pnas.2636393100

Zhang, N., and Y.W. He. 2005. An essential role for c-FLIP in the efficient development of mature T lymphocytes. *J Exp Med*. 202: pp. 395–404. doi: 10.1084/jem.20050117

Ziegenfuss, J.S., R. Biswas, M.A. Avery, K. Hong, A.E. Sheehan, Y.G. Yeung, E.R. Stanley, and M.R. Freeman. 2008. Draper-dependent glial phagocytic activity is mediated by Src and Syk family kinase signalling. *Nature*. 453: pp. 935–9. doi: 10.1038/nature06901

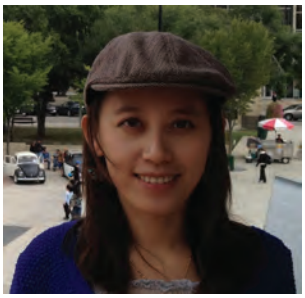
Zobel, K., L. Wang, E. Varfolomeev, M.C. Franklin, L.O. Elliott, H.J. Wallweber, D.C. Okawa, J.A. Flygare, D. Vucic, W.J. Fairbrother, and K. Deshayes. 2006. Design, synthesis, and biological activity of a potent Smac mimetic that sensitizes cancer cells to apoptosis by antagonizing IAPs. *ACS Chem Biol*. 1: pp. 525–33. doi: 10.1021/cb600276q

Zou, H., W.J. Henzel, X. Liu, A. Lutschg, and X. Wang. 1997. Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell*. 90: pp. 405–13. doi: 10.1016/S0092-8674(00)80501-2

Zullig, S., L.J. Neukomm, M. Jovanovic, S.J. Charette, N.N. Lyssenko, M.S. Halleck, C.P. Reutelingsperger, R.A. Schlegel, and M.O. Hengartner. 2007. Aminophospholipid translocase TAT-1 promotes phosphatidylserine exposure during *C. elegans* apoptosis. *Curr Biol*. 17: pp. 994–9. doi: 10.1016/j.cub.2007.05.024

Author Biographies

Ing Swie Goping, PhD, is an Associate Professor of biochemistry and oncology at the University of Alberta. She received her BSc degree in biology from Queen's University, Kingston, Ontario, in 1986, her MSc degree in genetics from the University of Western Ontario, London, Ontario, in 1990, and her PhD degree in biochemistry from McGill University, Montreal, Quebec, in 1995. It was in Dr. Gordon Shore's laboratory at McGill University that she became interested in apoptosis and identified that stress stimulus induced Bax targeting to mitochondria. Dr. Goping went on to study cytotoxic T cell–mediated cell death as a postdoctoral fellow in the laboratory of Dr. Chris Bleackley and elucidated key steps for granzyme B-induced apoptosis. Dr. Goping joined the Faculty of Medicine and Dentistry at the University of Alberta where her group investigates the interplay between apoptosis and cancer cell survival.



Ning Yang received her Master's degree in cell biology from Beijing Normal University (Beijing, China) in 2009. From 2009 to 2011, she studied autophagy using the *C. elegans* model in the National Institute of Biological Sciences (Beijing, China) and focused on anticancer drug screens in BioDuro Company (Beijing, China). Currently, Ning is enrolled in the PhD program in Dr. Goping's laboratory at the University of Alberta.