Hao Wu *Editor*

Cell Death

Mechanism and Disease

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Foreword

The concept of "apoptosis" as a regulated cell death pathway in mammalian cells was first proposed by Andrew Wyllie based on an intriguing observation made during his Ph.D. study that showed dying cancer cells having DNA fragmentation. While Wyllie's work could had been easily dismissed as a "descriptive" study by today's standard and rejected outright, it laid the foundation for the subsequent decades of studies that led to the understanding of the molecular mechanisms of apoptosis. Now the concept of cellular "suicide" is no longer a strange or a novel idea. It has been generally accepted that regulation of cell death is important for development as well as for the maintenance of the normal tissue homeostasis.

The studies on apoptosis led to many conceptual advances that helped us to understand the disease mechanisms. The molecular mechanism that protects mammalian cells from apoptosis were first illustrated by the ground-breaking work from the group of David Vaux and colleagues who showed the ability of Bcl-2 when overexpressed to prolong the survival of cancer cells. Vaux's work provided the second most important principle in oncogenesis, after the uncontrolled and excessive proliferation, that tumor cells must develop mechanisms to evade apoptosis. The two decades of research on Bcl-2 family provided the molecular understanding as how pro- and anti-apoptotic members of Bcl-2 family interact and control the cellular survival. This understanding provided the crucial insights as how such interactions may be manipulated pharmacologically. The exclusive dependence of cancer cell survival on the apoptosis inhibitory effect of Bcl-2 family is now being explored for therapeutic benefits in the treatment of cancers.

The execution of apoptosis in mammalian cells is mediated by the evolutionarily conserved caspase family, a principle first illustrated by the works from my own group. Mammalian caspase family is rather large with at least 12 members which is almost certain due to the complicated tasks that these caspases are involved in. These caspases are arranged into amplification cascades that control either the "intrinsic apoptosis" pathway regulated by the mitochondrial pathway which leads to the activation of "apoptosome" or the "extrinsic apoptosis" pathway which is activated by the ligands of death receptor family. Caspases can be activated to mediate apoptosis by a wide range of physiological and pathological

signaling in different cell types: from DNA damage in cancer cells to tropic factor deprivation in neurons. The consequence of caspase activation, however, is not always lethal: in neurons, limited and localized caspase activation has now been implicated in modulating synaptic plasticity or mediating neurite pruning in fine tuning of neuronal circuitry.

The two decades of research on cell death broke some of the historical dogma such as necrosis can only be passive cell death. It turns out that when apoptosis fails to occur, cells may undergo a form of regulated necrosis, termed "necroptosis" or "programmed necrosis." Necroptosis activated by TNFα is the most understood necrotic cell death pathway. Stimulation of type I TNF receptor by TNFα leads to the transient formation of a membrane complex associated with the intracellular death domain of TNFR1, called "complex I" which includes adaptor protein TRADD, RIP1, and E3 ubiquitin ligases TRAF2 and cIAP1/2. The complex I is then transitioned into one of the two alternative intracellular signaling complexes, "complex IIa" or "complex IIb." The complex IIa, which includes FADD, caspase-8, and RIP1, leads to the activation of caspase-8 and apoptosis. On the other hand, under certain conditions when apoptosis fails to be activated, an alternative complex that includes RIP1 and RIP3 kinases is formed. The kinase activity of RIP1 is required for the formation of complex IIb to mediate necroptosis and sometimes is also involved in mediating the activation of caspases with complex IIa in the absence of cIAP1/2. Interestingly, as shown by Hao Wu's group, the complex IIb formed by RIP1 and RIP3 involves the formation of amyloid-like structure. Given the wide association of amyloid-like structures in neurodegenerative diseases, the formation of amyloid structure in a regulated necrotic cell death pathway is very intriguing. The involvement of RIP1 kinase in mediating acute neurological injuries has already been suggested by the ability of necrostatin-1, a highly specific inhibitor of RIP1 kinase, to protect a wide range of animal models induced by ischemia. It will be interesting to examine the involvement of RIP1–RIP3 amyloid complexes in human neurodegenerative diseases in future.

The history of cell death research argues strongly for the important value of investigators initiated and curiosity-driven research. While there are certainly values for "-omics" approaches where we may get to systematically understand the physical nature of our cells, our body, and our genetic compositions, the mechanisms that control cell death certainly would not had been understood without the hypothesis-driven researches as that had occurred. The researches on cell death mechanisms also provide an excellent example where excellence in basic scientific research not only led to molecular insights to our living world in general but also can be translated into medicines that can treat diseases to improve our lives.

A cell that dies is never in vain.

Boston, MA, USA Junying Yuan

Preface

As editor of this book, I wish to thank several important people, without whose assistance a successful outcome would not have been possible. I was originally approached by Dr. Portia E. Formento, an Editor of Springer Science + Business Media, during the Biophysical Society Meeting in Baltimore, Maryland, in March, 2011, about editing a book on cell death. I hesitated at first. While scientists are always busy, it was an extra busy time for me as my son Alex was preparing to go to college and I was contemplating fresh job opportunities. Portia was convincing enough to persuade me that such a book would be both valuable and timely and that Springer would assist me in accomplishing this at my own pace. I am deeply indebted to her for her support and encouragement.

Although I signed up for the job of putting such a book together then, I did not begin to contact potential contributors until more than a year later, a couple of months before my lab's move from Weill Cornell Medical College to Boston Children's Hospital and Harvard Medical School. Fortunately, the theme of the book, titled "Cell Death: Mechanism and Disease," is exactly as intended and covers the molecular basis of cell death—apoptotic and necrotic—as well as the relevance of cell death mechanisms to understanding, preventing, and treating human disease. I have had the pleasure of working in this highly exciting and competitive field for the past 16 years and have seen its rapid evolution, with one surprising finding after another. The book strives to present a balanced and comprehensive view of the current state of the art.

I am particularly grateful to all the wonderful contributors who truly made the book possible. They are not only amazing scientists but also good friends whom I have come to know over the years. I am especially indebted to David Vaux, a pioneering figure in cell death research, for his commitment to and enthusiasm about the book. Earlier this year, I had the honor of visiting "Davo" (short for Dave Vaux) and his family in Melbourne, where I experienced his hospitality firsthand. I would also like to praise my dear colleague and friend Junying Yuan for writing such a beautiful forward for the book. I am extraordinarily thankful to Daniel L. Dominguez,

the Developmental Editor of the book, who pushed and shoved and made sure that things got done on time. Finally, I take this opportunity to express my appreciation for the boundless love around me, my children Alex and Michael who are my joy and pride, and my current and former lab members who are the heroes behind every research accomplishment from my laboratory.

It is my wish that you will enjoy reading the book as much as I have enjoyed editing it, regardless of whether you are an amateur or an expert in the cell death field.

Boston, MA, USA Hao Wu

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Chapter 1
Historical Perspective: The Seven Ages of Cell Death Research

David L. Vaux

All of science is a stage,
And all the researchers merely players,
They have their exits and entrances,
Each field in its time plays many parts,
Its acts being seven ages.

(with apologies to W. Shakespeare)

Abstract Last year about 20,000 additional papers were listed in PubMed that mentioned the word "apoptosis" or the term "programmed cell death". Searching on the same terms for publications 25 years ago yields only 42 publications. Although it has been recognised for over 150 years that death of some of an organism's cells is a normal physiological process, widespread interest in the field has only come about in recent times, once the mechanisms that implement it were elucidated and abnormalities in the physiological cell death process were linked to human disease. The stages of research into the field of cell death can be likened to the ages of man, except in this case there was an extraordinarily long infancy, and despite the current maturity of the field, it continues to rapidly grow.

Keywords Apoptosis • bcl-2 • Programmed cell death • *C. elegans* • History

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1.1 Infancy 1665–1972

Physiological cell death, namely, death of a cell by a mechanism that has evolved for the purpose of ending the life of one of an organism's cells, has been recognised since Carl Vogt's description of cell death during development of the midwife toad in 1842 [1]. In retrospect, physiological cell death has an even older history, because the first cells to be dubbed "cells" by Hooke in 1665 were from cork—the bark of an oak tree—and these cells fulfil their protective function only after they have died.

As described by Clarke and Clarke [2], following Vogt many histologists recognised that cell death was a normal process that occurred in animals as diverse as insects and vertebrates and was important not only for development but also to balance cell production by mitosis.

Over the years, the descriptions of cell death, and inferences for its role, were remarkably consistent, whether they were referring to cell death in insects or vertebrates. However, the terminology changed, with words such as "chromatolysis" [3, 4] giving way to "programmed cell death" [5], "shrinkage necrosis" [6] and "apoptosis" [7].

Nevertheless, even though interest in physiological cell death stuttered and it was sometimes forgotten only to be rediscovered, its importance was recognised by these pioneers.

1.2 Childhood: Beyond Morphology and Towards Mechanism 1973–1988

Initially, descriptions of cell death were based on the morphology of the dying cells, but this changed as biochemists and geneticists came to the aid of the anatomists, histologists and pathologists.

The first biochemical correlates of physiological cell death were observations of its association with cleavage of the DNA into 180 base pair ladders [8, 9]. Furthermore, as these ladders were not only induced in cells treated with toxins but also in those exposed to hormones such as glucocorticoids [10] and in targets of cytotoxic T lymphocytes [11], such different triggers seemed to activate a common final pathway.

Death of mammalian cells therefore involved activation of pre-existing endonucleases, but there was also evidence from Tata, who was studying regression of tadpoles' tails, that at least in some circumstances production of new proteins was also necessary. Tata showed that the cell deaths that occurred during metamorphosis of tadpoles' tails required production of mRNA and protein, and he speculated that it involved synthesis of a new protease [12].

To identify particular genes involved, invertebrate biologists took a classical genetic approach. While studying cell lineages during development in the nematode *C. elegans*, Sulston noticed that roughly 10 % of the cells died in a process he later referred to as "programmed cell death" because it occurred to the same

cells at the same time of development in each of the embryos [13]. He and Horvitz then induced genetic mutations by feeding the worms a DNA mutagen and searched for worms in which cell death was abnormal: the CED mutants [14–16]. These experiments showed that there was a genetically encoded program for cell death in the worm and, because the mutant worms were otherwise normal, that these genes were specific for cell death and did not play essential roles in other physiological processes.

Despite these seminal discoveries, cell death remained the province of the specialists, whether it was invertebrate biologists such as Lockshin, Sulston and Horvitz, studying programmed cell death in insects and worms, or pathologists, such as Kerr, Wyllie and Currie, studying apoptosis in mammals. I can only speculate that the reason most biologists were reluctant to join the field was a subconscious appreciation of the evolutionary principle of "survival of the fittest". As the product of a billion years of selection for the ability to survive and reproduce, how and why could a cell have evolved a mechanism to kill itself?

Although the total number of papers describing cell death had reached several hundred by the late 1980s, study of cell death remained on the fringe. For example, searches of PubMed in 1988 using the word "chromatolysis" yield 28 papers, the term "programmed cell death" yield 28 papers and the word "apoptosis" yield 49 papers.

In contrast, searching PubMed in 1996 with "programmed cell death" yields 747 publications, with "chromatolysis" yielding 15 and "apoptosis" 4,915. What caused this tremendous growth were three things: (1) molecular identification of the components of the mechanism; (2) finding that abnormalities in cell death caused cancer in humans and (3) the discovery that the mechanisms for programmed cell death in the worm and apoptosis of mammalian cells were one and the same (Fig. 1.1).

1.3 Adolescence—Coming of Age—First Clues to Mechanism, and the Link to Human Disease 1988–1992

The first clue to the molecular mechanism for cell death came from studying Bcl-2 (B-cell leukaemia/lymphoma 2), the gene activated in follicular lymphomas [17]. Tsujimoto and Croce at the Wistar Institute [18], Bakhshi and Korsmeyer at NIH [19] and Cleary and Sklar at Stanford [20] were all interested in identifying the gene at the breakpoint of the t(14;18) chromosomal translocation that was associated with follicular lymphoma, a common non-Hodgkin's lymphoma in humans. In 1986, the full-length cDNA encoding Bcl-2 protein was cloned by Tsujimoto [21] and Cleary [22], but it did not resemble any other gene known at the time, and there were no clues to its function.

To determine whether Bcl-2 could act as an oncogene to transform cells, various groups expressed it in hematopoietic and fibroblast cell lines. While Vaux et al. found that Bcl-2 could promote cell survival but did not act like other oncogenes to

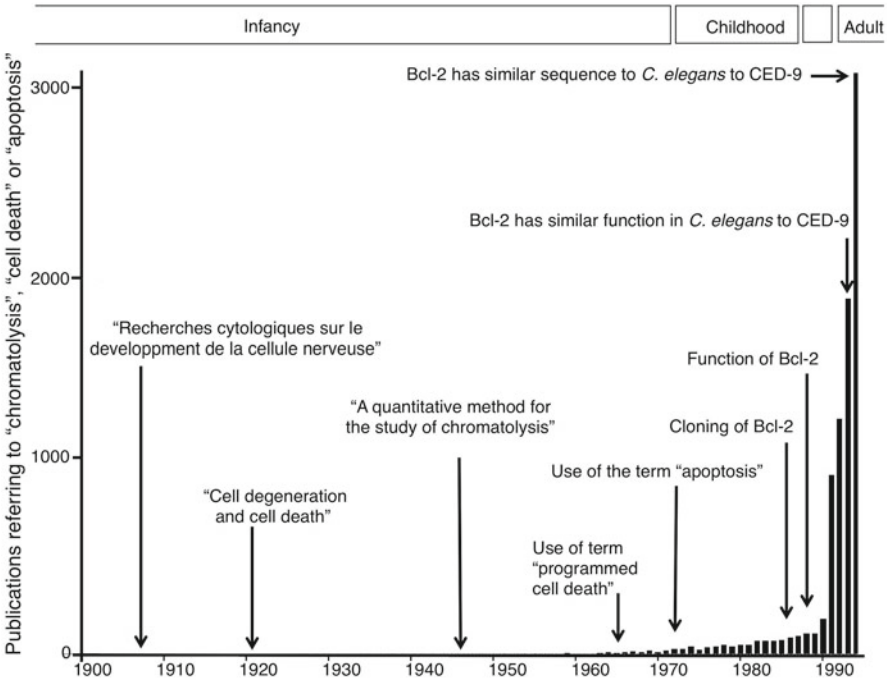

Fig. 1.1 Although cell suicide was recognised to be a physiological process in 1842, few papers referred to the terms "cell death", "chromatolysis" or "apoptosis" until the early 1990s, despite elegant work by early histologists and invertebrate biologists. Interest in the field grew shortly after the bcl-2 gene, which is linked to cancer in humans, was shown to encode an inhibitor of cell death in 1988. Early examples cited above include papers by Collin in 1906 [93], Rossle in 1921 [94] and Campbell and Novick in 1946 [95]. Citation analysis from the Thomson Reuters Web of Science

stimulate cell growth or proliferation [23], Nunez et al. reported that it promoted lymphocyte growth [24]. Reed et al. showed that over-expression of Bcl-2 could transform 3T3 fibroblasts in vivo but not in vitro. However, as the same activity was found for a construct encoding Bcl-2beta, which, in hindsight, we now know to be non-functional, it seems likely that the effects observed were artefactual [25].

The discovery that Bcl-2, a gene implicated in cancer in humans, acted specifically to inhibit cell death, whether due to removal of growth factors [23, 26] or exposure to chemotherapeutic drugs [27], provided the spark that ignited wide and general interest in the cell death field (Fig. 1.2). It became clear that rather than being an obscure topic of minor interest, abnormalities in apoptosis were a cause of cancer in humans. Moreover, the identification of Bcl-2 could provide a handle for identifying the rest of the mechanism.

Until this discovery, it had been easy to dismiss cell death as an accidental event of little significance. Understandably enough, biologists were steeped with Darwinian logic: our genes are the result of billions of years of natural selection for fitness and survival. How could the genes for a mechanism for cell suicide have evolved?

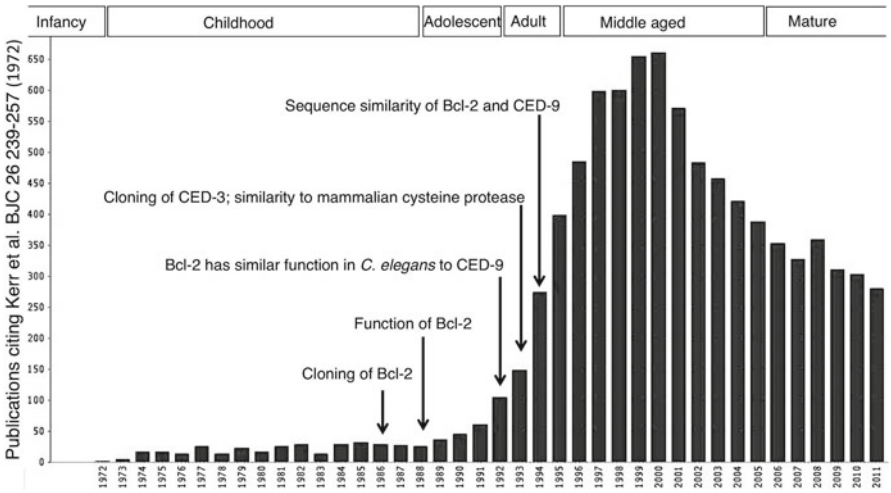

Fig. 1.2 Kerr, Wyllie and Currie adopted the term "apoptosis" for the physiological process of cell suicide in 1972, but for nearly 20 years, their paper received few citations. It was the recognition of the first component of the cell death mechanism, Bcl-2, which linked abnormalities in cell death to cancer in humans, that sparked the explosion of interest in the field and led to a surge in references to the 1972 "apoptosis" paper [7]. The rate of citations accelerated further with the realization that the mechanisms for apoptosis (in mammalian cells) and programmed cell death (of cells in *C. elegans*) were implemented by the same, evolutionarily conserved mechanism in 1992, even before the sequence similarity of Bcl-2 and CED-9 was revealed in 1994. Citation analysis from the Thomson Reuters Web of Science

The discovery that Bcl-2 functioned specifically to regulate cell suicide, and appeared to have no other function, and that by blocking cell death, it was the initial cause of follicular lymphoma meant that apoptosis could no longer be dismissed as an evolutionary oddity.

Furthermore, because over-expression of Bcl-2 could block cell death in response to a wide variety of stimuli but did not protect against apoptosis induced by cytotoxic T lymphocytes, it was clear that there was more than one pathway for cell death, and some of these pathways were independent of the mechanism that could be controlled by Bcl-2 [28].

A short time later, two groups identified a receptor of the surface of cells that could give an apoptotic signal. They had each developed monoclonal antibodies that rapidly induced death of cells of certain lymphocyte lines [29, 30]. Cloning of the antigen recognised by Yonehara's anti-Fas antibody [31] and that recognised by Trauth's anti-APO-1 antibody [32] showed them to be the same molecule, now known as CD95. As CD95 was related to the receptor for tumour necrosis factor (TNF), it provided a big step forward in understanding how certain external signals could trigger a cell to kill itself. For a while, Bcl-2 and CD95 remained isolated components of the cell death mechanism, but their places in an integrated network were established when it became clear that both of them could (indirectly) regulate

the activity of caspases, a family of cysteine proteases. The clue to the role of caspases came not from studying mammalian cells but from experiments involving the worm *C. elegans*.

1.4 Adulthood: More of the Mechanism, Evolutionary Conservation 1992–1994

By 1992, Horvitz's lab had identified about a dozen mutant strains of worm in which cell death was abnormal, but the sequence of the first cloned component, CED-4, was not particularly revealing [33].

What gave an enormous boost to the field was the finding that when human Bcl-2 was expressed in *C. elegans*, it was able to prevent most of the programmed cell deaths that occurred during development [34]. This experiment showed that apoptosis in mammalian cells and programmed cell death in the worm were implemented by the same mechanism, which had been conserved for at least a billion years of evolution. Moreover, it showed that the way to find out how Bcl-2 worked in mammalian cells, and the nature of the cell death mechanism it controlled, was to clone the *C. elegans* cell death genes and identify their mammalian homologues.

The fact that Bcl-2 acted like CED-9, a worm protein that antagonised programmed cell death, suddenly made the worm cell death pathway a topic of major interest in the biomedical community.

When Hengartner in Horvitz's lab cloned the gene for CED-9 [35], it turned out to have similar sequence to Bcl-2. Cloning of the killer gene *ced-3*, which is required for cell death in the worm, showed that it encoded a protease similar to the mammalian cysteine protease responsible for converting pro-interleukin 1 beta into the active form [36]. This turned attention away from endonucleases and to proteases as key effector proteins of apoptosis.

Interestingly, an important role for proteolysis in cell death had been raised before. Back in 1989, Kaufmann showed that treatment of cancer cell lines with a wide variety of chemotherapeutic agents not only induced DNA fragmentation, but it also caused specific cleavage of a number of proteins including PARP, lamin B, topoisomerase I, topoisomerase II, and histone H1 [37]. When the protease responsible was isolated [38], it turned out to be in the same family as CED-3, the cysteine proteases now known as caspases.

When it was found that the substrate preference of caspases, which cleave after aspartic acid residues [39], was similar to that of granzyme B, the key serine protease in the granules of cytotoxic T lymphocytes [40], the picture seemed complete, with activation of latent proteases being the key event in apoptosis [41]. But cell death turned out not to be as simple in mammalian cells as in the worm.

Several groups were trying to figure out how ligation of CD95 caused apoptosis. When proteins associated with ligated CD95 were identified one of them turned out to be a protease, now known as caspase 8 [42]. Using the yeast-two-hybrid system, which showed that CD95 bound to an adaptor (FADD/Mort1) that in turn bound to caspase 8 caspase, Wallach's group independently came to the same conclusion [43, 44].

In an effort to identify the proteases that are activated in cell death, Wang set up assays in cell-free systems [45, 46]. This allowed him to identify several caspases, and also Apaf-1, a caspase-binding adaptor protein that turned out to resemble CED-4 from *C. elegans* [47], which reinforced the notion that the cell death mechanisms in mammals and the worm were similar. However, Wang also discovered that cytochrome c, which normally resides in the mitochondrial inter-membrane space, was critical for activation of Apaf-1 and caspases downstream [48]. The role of cytochrome c, and pro-apoptotic Bcl-2-like proteins Bax and Bak, provided a key point of difference between the pathways identified genetically in *C. elegans* and those that operated in mammalian cells.

Korsmeyer's group had isolated Bax as a protein that was able to bind to Bcl-2 [49]. Bax resembled Bcl-2, but rather than inhibiting cell death, it promoted it. With the cloning of more Bcl-2-like genes and Bcl-2-binding proteins, it turned out that they fell into three subfamilies. Like Bcl-2, Mcl-1, Bcl-x, Bcl-w and A1 were cell death inhibitors; Bax and Bak promoted cell death, and the so-called BH3-only family, which only bears one of the Bcl-2 homology domains, such as Bim, Puma and Bid, promoted cell death by antagonising the anti-apoptotic family members and by activating Bax and Bak [50].

Together, this work from Korsmeyer's and Wang's labs showed how the Bcl-2 family members controlled release of cytochrome c from the mitochondria and hence Apaf-1-mediated activation of caspases. In doing so, they also identified key differences between cell death in mammals and the worm: in mammalian cells activation of Bax or Bak is necessary and sufficient for cell death and also for release of cytochrome c from the mitochondria. When released, cytochrome c can activate Apaf-1 triggering caspase activation, but this is not necessary for cell death. In the worm there is no Bax- or Bak-like protein, and CED-9 directly inhibits CED-4, which in the absence of CED-9 spontaneously activates the caspase CED-3 without the need for cytochrome c. In the worm, the caspase CED-3 is essential for programmed cell death, whereas in most cases, activation of Bax and Bak is the point of no return for death of mammalian cells.

1.5 Middle-Aged Expansion 1994–2005

Not only research on worms but also study of insects have provided important insights into the mechanisms of cell death in mammals. Work on cell death in *Drosophila* in Steller's and Abrams' labs led to the identification of several small pro-apoptotic proteins named reaper, grim and hid [51–54]. The first inhibitor of apoptosis (IAP) genes were found in viruses that infect insect cells [55]. This led to the identification of mammalian IAP homologues [56, 57]. Independently, two of these mammalian IAPs, cIAP1 and cIAP2, were also identified in cell lysates by their ability to form complexes associated with TNF receptor-associated factors (TRAFs) and the cytoplasmic domain of TNF receptor 2 [58]. Two cellular IAP homologues were also identified in *Drosophila*, and their over-expression could suppress cell death triggered by reaper [59].

As IAPs were found in mammalian cells, it seemed likely there would be mammalian equivalents of reaper, grim and hid. By purifying proteins that could be associated with XIAP, Vaux's lab was able to identify Diablo [60]. Independently, Wang's group isolated the same protein, which they termed Smac, because they found that it was released from the mitochondria at the same time as cytochrome c [61].

Together with the work on CD95 and caspase 8, this research showed that there was a pathway to cell death triggered by members of the TNF receptor superfamily that was regulated by IAPs and, in most cell types, operated independently of Bcl-2 family proteins and the mitochondria [62]. Other pathways that could cause cell death independently of Bax, Bak and the mitochondria were also discovered soon afterwards.

The first clues to a mechanism for cell death later termed "necroptosis" or "programmed necrosis" that allowed cells to kill themselves independently not only of Bax, Bak and the mitochondria but also of caspase 8 (and other caspases) came from studying TNF- or CD95 ligand-induced death of L929 fibroblast or Jurkat T lymphocyte cells [63–65]. In these cells, neither over-expression of Bcl-2 nor broad-spectrum caspase inhibitors could prevent cell death. Study of this pathway, which involves the kinases RIPK1 and RIPK3, was boosted with the discovery of an inhibitor of RIPK1, necrostatin [66].

Another mechanism of cell death, sometimes termed "pyroptosis", was discovered that can operate without Bax, Bak and the mitochondria, or caspase 8, but instead involves caspases that had been thought to only be able to process and hence activate cytokines such as interleukin 1. It is now clear that products of infecting micro-organisms can bind to intracellular receptors and trigger activation of the inflammasome, which can activate caspases to cause cell death as well as to release pro-inflammatory cytokines [67–71].

1.6 Maturity: Translation 2005-Present

Researchers have long hoped to harness their knowledge of the cell death process to use therapeutically, either to cause the death of diseased cells, such as cancer cells or immune system cells such as those causing autoimmunity, or to prolong the life of cells that are killing themselves unnecessarily, such as heart muscle cells in heart attacks and neurons in stroke or neurodegenerative diseases.

As the mechanisms of cell death were elucidated, it became clear that empirically discovered conventional cancer treatments, including radiation, chemotherapy and steroids, work, at least in part, by activating the suicide mechanisms in tumour cells. Furthermore, as over-expression of Bcl-2 could increase the resistance of cancer cells to treatment with chemotherapeutic drugs and radiation in vitro, inhibition of anti-apoptotic Bcl-2 family members might enhance the response to therapy.

The finding that anti-apoptotic Bcl-2 family members could be inhibited by a variety of BH3-only proteins suggested that small molecules that bound to the same site might antagonise these Bcl-2-like proteins and cause cancer cells to die.

However, the 3D structure of the interaction [72] was not encouraging, as the binding site did not look "druggable". However, a novel NMR-based structural and medicinal chemistry-based approach developed by Fesik [73] allowed the development of an agent [74] that efficiently binds to and inhibits Bcl-2 and Bcl-x in vivo and is currently undergoing clinical trials.

Another set of pro-apoptotic agents, known as "smac-mimetics" or "IAP-antagonists", have been developed that trigger IAPs to auto-ubiquitylate and be sent for degradation by the proteasome [75–77]. Smac mimetics cause some cancer cell lines to die on their own, but they also increase the sensitivity of a wider range of cancer cells to die in response to TNF-related ligands including CD95L, TRAIL and TNF itself.

Since the cloning of TNF, various death ligands have been tested for their anti-cancer effects. TNF itself causes too many side effects to be used systemically but is still used occasionally in isolated limb perfusions to treat melanoma and soft tissue sarcomas [78, 79]. Antibodies to CD95 proved to cause massive apoptosis in the liver in mice, so they have not been developed for clinical use. Amgen and Genentech have developed candidate therapeutic agents based on the TNF-like cytokine TRAIL that are still being tested.

1.7 Old Age: Senility, or Just Eccentricity?

In no way has the field of cell death research reached its terminal phase, as there is still plenty to be discovered, but at times it does show signs of its age. Just like elderly humans, who start to grow hairs in unwanted places, in the cell death field there have been a number of errant and superfluous outgrowths. As successive tidal waves of data have elevated many candidate proteins as key components of cell death pathways, some have stayed afloat, others have sunk without trace and a few keep re-surfacing, no matter how much counter-data is weighing them down.

For example, rather than defender against death 1 (Dad1) [80] being a cell death inhibitor that acts downstream of Bcl-2 in pathways conserved from plants to animals, it turned out to be a protein needed for transfer of sugars to allow proteins to be trafficked through the ER [81]. In other words, it is not involved in cell suicide; it is required for eukaryotic cell survival.

Survivin, so-named because it bears a baculoviral IAP repeat domain that is characteristic of IAP proteins [82], turned out to have no direct role in regulating cell death but acts together with aurora kinase B, INCENP and Borealin to regulate chromosome segregation during mitosis [83, 84].

Daxx [85] and Dap3 [86, 87] were initially claimed to be adaptor proteins that bound to the death domains of TNF receptor family members, but Daxx turned out to be a histone chaperone [88], and Dap3 turned out to be a component of mitochondrial ribosomes [89].

Apoptosis-inducing factor (AIF) was initially claimed to be a protease released from mitochondria that caused cell death by cleaving DNA [90, 91]. It turned out to

be a protein required for formation of complex I of the mitochondrial electron transport chain that is not a mediator of cell death but is required for mitochondrial function and cell survival [92].

Cell death research has become a major research field that has grown faster than any other. One in every 40 papers published in PubMed in 2011 mentioned "apoptosis", so it will not be forgotten the way it was so many times in the nineteenth and twentieth centuries, especially as this knowledge is translated into new treatments.

Long live cell death!

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Chapter 2
The Intrinsic Apoptotic Pathway

Xuejun Jiang

Abstract The intrinsic apoptotic pathway, also known as the mitochondria-mediated apoptotic pathway, plays important roles in a variety of biological processes and is involved in the pathogenesis of human diseases ranging from immune disorders and neurodegeneration to cancer. Over the last two decades, much has been learned about the molecular basis of this pathway as well as its delicate regulatory mechanisms. The central role of the cellular organelle, the mitochondrion, in this pathway is well appreciated. Importantly, several potential therapeutic agents have been developed to target specific components of this pathway. Further, growing evidence indicates that cytochrome c release from mitochondria, a hallmark of the pathway, does not always lead to irreversible cell death; and the downstream caspase activation can also perform certain non-death biological functions. This chapter reviews our understanding of the intrinsic apoptotic pathway with a focus on the mechanisms and regulation downstream of cytochrome c release. We also discuss unsolved questions and new challenges in this exciting research area. Other important topics, such as the role of the Bcl-2 family in this pathway and the relationship of this pathway with cancer, will be discussed in other chapters of this book and thus will not be elaborated in detail in this chapter.

Keywords Mitochondria-mediated apoptosis • Caspase activation • Mitochondrial outer membrane permeabilization • Cytochrome c • Apaf-1 • Apoptosome • Smac/Diablo • IAP

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2.1 Introduction

The intrinsic apoptotic pathway, also known as the mitochondria-mediated apoptotic pathway, plays important roles in a variety of biological processes and is involved in the pathogenesis of human diseases ranging from immune disorders and neurodegeneration to cancer. Over the last two decades, much has been learned about the molecular basis of this pathway as well as its delicate regulatory mechanisms. The central role of the cellular organelle, the mitochondrion, in this pathway is well appreciated. Importantly, several potential therapeutic agents have been developed to target specific components of this pathway. Further, growing evidence indicates that cytochrome c release from mitochondria, a hallmark of the pathway, does not always lead to irreversible cell death; and the downstream caspase activation can also perform certain non-death biological functions. This chapter reviews our understanding of the intrinsic apoptotic pathway with a focus on the mechanisms and regulation downstream of cytochrome c release. We also discuss unsolved questions and new challenges in this exciting research area. Other important topics, such as the role of the Bcl-2 family in this pathway and the relationship of this pathway with cancer, will be discussed in other chapters of this book and thus will not be elaborated in detail in this chapter.

2.2 The Discovery of the Mitochondrial Pathway, a Historical Overview

The initial discovery of the mammalian mitochondrial apoptotic pathway is full of surprise, excitement, doubt, and debate. In the 1980s and early 1990s, based on the groundbreaking work by Horvitz and colleagues using *Caenorhabditis elegans* genetics [1–3] and subsequent studies in other species, the cysteine proteases known as caspases were firmly established as the conserved molecular executioners of apoptosis. In another line of equally significant research led by Korsmeyer, Cory, and others, the human oncogene Bcl-2 was discovered and was demonstrated to function as an inhibitor of apoptosis [4–6]. The finding that the *C. elegans* apoptotic inhibitor Ced-9 is homologous to Bcl-2 [7] (as well as numerous studies to follow) revealed that the apoptosis pathway is conserved from worms to humans and is important for pathogenesis of diseases such as cancer. However, in mammalian cells, how caspase activation was initiated by triggers such as DNA damage and growth factor deprivation, and how Bcl-2 blocked apoptosis, had not been defined. The underlying mechanisms remained elusive after extensive cellular, molecular, and pharmacological studies. All this set the stage for Xiaodong Wang's landmark discovery of the mitochondrial apoptotic pathway by employing classical biochemistry.

Wang and colleagues started with HeLa cell extracts. In the cell extracts prepared from healthy HeLa cells, caspase-3, the putative molecular marker of apoptosis, is inactive. They simply asked: What does it take to activate caspase-3 in this cell-free

system? Previous evidence indicated that one of the differences between apoptosis and necrosis is that the former process is energy dependent. Therefore a logical albeit simple-minded idea was the following: Is energy in the form of ATP required for caspase-3 activation in this cell-free system? They tested ATP as well as many other nucleotides, supposedly to be negative controls. Curious enough, ATP had a marginal effect, whereas one of the DNA building blocks, deoxy-ATP (dATP), potently activated caspase-3 in the extracts [8]. So in this case, including extra "negative" controls instead of only ADP or AMP clearly paid off. This unexpected observation transformed the puzzle of mechanism into a typical biochemical question that could be addressed by fractionation of the HeLa cell extracts: What components in the crude HeLa cell extracts are necessary and sufficient to support dATP-induced caspase-3 activation?

At first glance, this experimental system appeared to be very artificial with no obvious in vivo relevance. And their biochemical identification of the first needed factor as the life-essential protein cytochrome c [8] further fueled the skepticism, although the in vitro biochemistry was worked out elegantly. Is the whole thing an in vitro artifact, or is cytochrome c really a double-edged sword? The biological nature of cytochrome c did not make the issue easy to resolve. Cytochrome c is an extremely abundant protein in most cells, ruling out the possibility of using overexpression experiments to assess the cellular relevance. The discovery was made in the pre-RNAi era; thus this powerful and nowadays routine technique was not available to them (it would not have been helpful anyhow because knocking down cytochrome c would do more harm to the cell instead of protecting the cell from death). Eventually all the cellular evidence they could gather, such as exquisite association of DNA damage-induced apoptosis with cytochrome c release from the mitochondria to the cytoplasm in the dying cells [8], was merely correlative.

But the doubt inside the lab and the general disbelief from the mainstream apoptosis field did not stop Wang and colleagues. They accomplished identification of all components required for dATP-induced caspase-3 activation and achieved total reconstitution using only purified proteins and dATP [8–10]. Identification of Apaf-1, which shows limited homology with the *C. elegans* apoptotic regulator Ced-4 [9], suggested that the in vitro system might be unveiling part of the truth after all. Both Ced-4 and Apaf-1 contain classical Walker's nucleotide-binding motif, lending an explanation for the mysterious role of dATP. Downstream of Apaf-1, there is the initiator caspase, caspase-9, whose activation is required for eventual caspase-3 activation [10], thus fitting with the standard caspase cascade model: first an initiator caspase (caspase-9 in this case) and then effector caspases (caspase-3 and caspase-7).

Importantly, another study from the Wang Lab [11], and an independent study from collaboration between the Don Newmeyer Lab and the Doug Green Lab [12], demonstrated that overexpression of the anti-apoptotic oncogene product Bcl-2 can prevent apoptosis-associated cytochrome c release. Therefore, if cytochrome c release is indeed the decisive event for downstream caspase activation as suggested by the biochemical studies, this new finding then assigns a biochemical activity for Bcl-2 as an apoptotic inhibitor in mammalian cells. However, in *C. elegans*, the

Bcl-2 homolog Ced-9 appears to directly inhibit the Apaf-1 counterpart Ced-4 without involving cytochrome c [13]. Given that the pathways in mammals and in *C. elegans* are highly conserved, the question remained: Is the biochemical finding concerning the role of cytochrome c in caspase activation relevant at all?

At that period, another series of important developments in the field was mouse genetic studies based on Wang's biochemical discoveries. Multiple groups generated mouse models with genetic deletion of the Apaf-1 or caspase-9 gene [14–17]. The mutant animals showed the same developmental defects as caspase-3-deleted mice [18, 19], including defects in the central nervous system and impaired lymphocyte homeostasis, both of which can be attributed to the blockage of apoptosis. But perhaps the most striking experiment to validate the apoptotic role of cytochrome c in vivo was achieved by combining the results of a detailed biochemical analysis of cytochrome c with genetic replacement (knock-in) in mice. In a systematic mutagenesis study, McLendon and colleagues identified lysine-72 (K72) of cytochrome c to be essential for interaction with Apaf-1 and to mediate Apaf-1-dependent caspase activation in vitro [20]. Importantly, mutation of K72 does not affect the respiration function of cytochrome c [20]. Subsequently, Tak Mak and colleagues, in collaboration with the Wang Lab, created a mouse model with wild-type cytochrome c replaced with the K72A mutant [21]. The mice displayed a rather similar phenotype to those with deletion of the Apaf-1 or caspase-9 genes, and the mutant cells were highly resistant to DNA damage-induced apoptosis. To this point, the pivotal role of cytochrome c in the mitochondria-mediated apoptotic pathway, or intrinsic pathway, has been validated beyond any reasonable doubt.

In retrospect, before Wang's seminal discovery, there had been actually plenty of observations suggesting the potential involvement of mitochondria in apoptosis. For example, Korsmeyer and colleagues had noticed the mitochondrial localization of Bcl-2 previously [5]. Further, in a collaborative work of Don Newmeyer with John Reed, a cell-free Xenopus egg extract system was developed to monitor chromatin condensation and nuclear fragmentation, which are morphological features associated with apoptosis [22]. Using this system, they found that a mitochondria-enriched fraction was required for the onset of these apoptotic features which can be inhibited by Bcl-2 [22]. In light of the mitochondrial apoptotic pathway, all these previous observations make perfect biological sense.

2.3 Mechanism of Cytochrome c-Mediated Caspase Activation

Figure 2.1 depicts a simplified version of the mitochondria-mediated caspase activation pathway. Upon sensing a variety of apoptotic stimuli including DNA damage agents, growth factor deprivation, and many other stressful conditions, mammalian cells respond by releasing cytochrome c from the mitochondria to the cytosol. The released cytochrome c interacts with Apaf-1 and triggers the formation of the

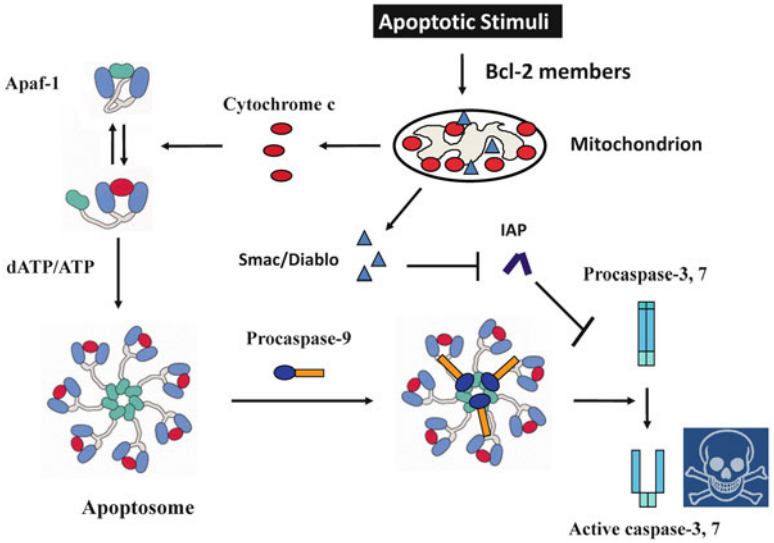

Fig. 2.1 The mitochondria-mediated caspase activation pathway

heptameric protein complex, the apoptosome. The apoptosome then activates the initiator caspase, caspase-9, which in turn cleaves and activates effector caspases, caspase-3 and caspase-7, leading to ultimate apoptotic cell death.

Mechanistically, there are two decisive events in this pathway: (1) mitochondrial outer membrane permeabilization (MOMP) preceding cytochrome c release and (2) downstream cytochrome c-mediated caspase activation. Cytochrome c release is closely regulated by the Bcl-2 family of proteins as well as mitochondrial fusion and fission, a topic discussed in detail in Chap. 4 of this book. Here we focus on the mechanisms acting downstream of cytochrome c release.

2.3.1 Nucleotide, Apaf-1, and Apoptosome

Because Apaf-1 contains Walker's A and B motifs, a relevant question is the exact role of the nucleotide dATP in Apaf-1 function. It was originally believed that hydrolysis of dATP by Apaf-1 was required for apoptosome function [9, 23]. Surprisingly, an in-depth biochemical analysis by Jiang et al. demonstrated that dATP binding but not hydrolysis by Apaf-1 is required for apoptosome function [24]. In fact, Apaf-1 is a rather poor, if active at all, ATPase [24]. Furthermore, they showed that interaction of cytochrome c with Apaf-1 stimulates the nucleotide binding activity of Apaf-1, and the affinity of Apaf-1 to dATP is about five- to tenfold higher than to ATP, likely explaining why dATP is better than ATP in triggering

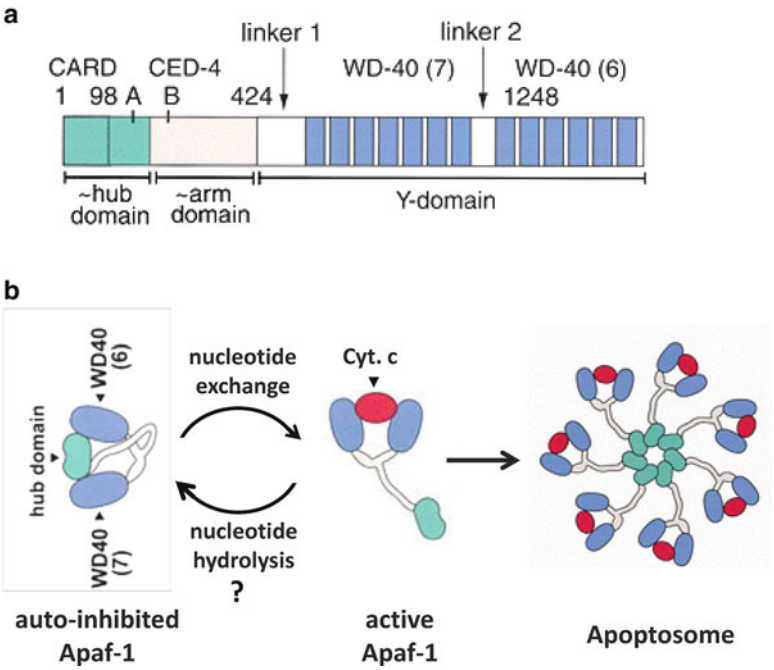

Fig. 2.2 Apoptosome assembly driven by cytochrome c and nucleotide. (**a**) The domain structure of Apaf-1. (**b**) Possible conformational change of Apaf-1 associated with apoptosome formation

caspase activation [24]. Importantly, subsequent studies revealed that even before contacting cytochrome c, Apaf-1 is already in association with nucleotide ADP or dADP [25]. Therefore, precisely speaking, cytochrome c binding activates nucleotide exchange (instead of nucleotide binding) activity of Apaf-1 (Fig. 2.2). Analogous to the regulatory mechanisms for G protein signaling, the (d)ADP-associated Apaf-1 is inactive, and it takes nucleotide exchange to activate its apoptotic function. Therefore, it is likely that Apaf-1 is also regulated by both nucleotide exchange factors (stimulatory) and ATP hydrolysis-activating factors (inhibitory). Indeed, as detailed later in Sect. 2.5.1, Apaf-1 nucleotide exchange can be stimulated by certain apoptotic stimulatory proteins. Intriguingly, as observed in a cell-free reconstituted system, if Apaf-1 binds to cytochrome c in the absence of exogenous dATP/ATP, Apaf-1 will form nonfunctional aggregates and be inactivated irreversibly [25]. This property of Apaf-1 may at least partly explain why apoptosis is an energy-requiring process.

A striking difference between Apaf-1 and its *C. elegans* prototype Ced-4 is that Ced-4 does not require cytochrome c for its activation. Mechanically, the two proteins appear to be operated in opposite modes: by default Apaf-1 is inactive and needs cytochrome c to turn it on, whereas free Ced-4 appears to be active and is kept in check by the anti-apoptotic protein Ced-9. This difference can be explained from a structural perspective. In the case of Apaf-1, cytochrome c binds to its C-terminal

WD40 repeats, which have been shown to have auto-inhibitory activity (i.e., deletion of the WD40 repeats renders Apaf-1 function to be independent of cytochrome c, as assayed in vitro) [26, 27] (Fig. 2.2). In the case of Ced-4, it does not contain WD40 repeats and is thus constitutively active. Applying this logic, the WD40 repeat-containing Drosophila Apaf-1 (dApaf-1) would require cytochrome c for its activation. However, it is still under debate whether dApaf-1 can be stimulated by cytochrome c. In vitro analysis indicates that dApaf-1 can stimulate Drosophila caspase activity without the need for additional protein factors [28]. On the other hand, Steller and colleagues have presented compelling genetic evidence suggesting that during Drosophila sperm maturation, cytochrome c is required for dApaf-1-mediated caspase activation [29, 30]. It is most likely that the WD40 repeat region of dApaf-1 is functionally relevant, but whether the function of this region is fulfilled via dApaf-1 binding to cytochrome c, or to another novel WD40 repeat-binding protein, is unclear. The final verdict may require careful in vitro biochemical reconstitution.

Since cytochrome c is not uniformly required for the activity of Ced-4 and its homologs in all organisms, is the cytochrome c-mediated caspase activation pathway only a new addition to mammals? This is clearly not the case, as the pathway is at least conserved in vertebrates, such as Xenopus and zebrafish [31–33]. More recently, Green and colleagues have presented strong evidence to suggest that this cytochrome c-dependent mechanism is functional even within lower organisms including protostomes and invertebrate deuterostomes [34]. Therefore, it is fair to suggest that cytochrome c-mediated caspase activation is a widespread mechanism not limited to higher organisms. Remarkably, even in *C. elegans*, mitochondria might be somehow involved in apoptosis, because Ced-9 and Ced-4 are localized on the surface of the organelle and translocate to the nuclear membrane upon induction of apoptosis [35].

Although *C. elegans* Ced-4 activity is independent of cytochrome c, apoptosome formation is a unified theme for all tested Ced-4 family proteins. Cryo-electronic microscopy (EM) structures of the mammalian and Drosophila apoptosome were solved by Akey and colleagues in collaboration with the Wang Lab [28, 36–38], and the crystal structure of the *C. elegans* apoptosome was solved by Yigong Shi and colleagues [13, 39]. In all cases, binding of the nucleotide ATP or dATP to Ced-4 family proteins, but not hydrolysis, is essential for apoptosome assembly. As we discuss in more detail later in Sect. 2.5.1, regulation of nucleotide exchange of Apaf-1 is crucial for mammalian apoptosis under multiple biological contexts.

2.3.2 A Unique Mechanism for Caspase-9 Activation

It was generally believed that specific proteolytic processing of a caspase was necessary and sufficient for its activation [40]. However, activation of caspase-9, the effector caspase of the mitochondrial pathway, defies this dogma. The lab of Lazebnik conducted biochemical analysis of caspase-9 activation in cell extracts,

and the results indicated that proteolytic cleavage of caspase-9 is not sufficient for its activation, and caspase-9 needs to remain associated with the apoptosome complex to be active [41]. This conclusion was subsequently confirmed by Wang and colleagues by using purified recombinant proteins [24]. Further, it turns out that proteolytic cleavage of caspase-9 is not only insufficient for its activation, but also it is not even necessary. When all the possible caspase cleavage sites of caspase-9 are mutated, the uncleaved caspase-9 can still be activated, as long as it is associated with the functional apoptosome [24, 36]. Therefore, the apoptosome complex and caspase-9 form a holoenzyme to activate downstream effector caspases.

If caspase-9 cleavage is not required for its activity, why does it undergo autocatalysis, and why is it also processed by the downstream effector caspases? Zou et al. suggested that cleavage by the effector caspases can significantly enhance the enzymatic activity of apoptosome-associated caspase-9, thus acting as a feedforward mechanism [42]. On the other hand, autocatalysis of caspase-9, although having no direct effect on its enzymatic activity, is critical for its regulation by IAP proteins, as described below in Sect. 2.3.3.

2.3.3 IAP Proteins and Their Antagonists

Multiple endogenous proteins, known as inhibitor of apoptosis (IAP) proteins, are able to suppress apoptotic cell death. These proteins possess the same molecular signature, one or several BIR domains. BIR stands for baculovirus IAP repeats, named after the originally discovered IAP proteins from the baculovirus genome by Lois Miller and colleagues [43]. Subsequently, John Reed and colleagues demonstrated that several mammalian IAP proteins can directly inhibit the enzymatic activity of caspases in a BIR domain-dependent manner [44, 45]. It is likely that IAPs have a housekeeping function to prevent accidental caspase activation, which might lead to unwanted cell death. Conversely, if the cell is determined to commit suicide, is there a way to antagonize IAPs so as to ensure effective apoptosis?

Drosophila genetic studies took the lead for the discovery of IAP antagonists. The laboratory of Hermann Steller and that of John Abrams identified pro-apoptotic genes reaper, hid, and grim in fruit flies by genetic screens [46–48]. Biochemically, the proteins encoded by these three genes (RHG genes) exert the same function: suppressing Drosophila IAP1 (dIAP1), as revealed by the laboratories of Steller, Hay, and Miller [49–52]. A detailed mutational and domain analysis indicated that RHG proteins interact with the BIR domain of dIAP1, the functional moiety for caspase inhibition [51]. However, there are no obvious RHG sequence homologs in mammals, making the identification for mammalian functional counterpart(s) of RHG proteins elusive.

Two independent studies led to the discovery of a mammalian equivalent of RHG proteins. Xiaodong Wang and colleagues noticed that there is a second mitochondrial activator of caspases (Smac) in addition to cytochrome c, which they proceeded to identify by biochemical fractionation [53]. David Vaux and colleagues were

inspired by the Drosophila RHG–IAP connection and thus performed affinity purification to search for binding partners of a mammalian IAP protein, XIAP. They identified a novel protein which they named as Diablo [54]. Smac and Diablo turned out to be the same protein, and it functions to antagonize XIAP by binding to its BIR domains.

Only after a series of biochemical analysis and structural studies did it become clear how Smac/Diablo acts equivalently as the RHG proteins. A short N-terminal peptide sequence of Smac, AVPI, located immediately after its mitochondrial targeting sequence, is sufficient for binding and antagonizing the BIR3 domain of XIAP [55]. This short region shares clear homology with the N-termini of the RHG proteins. Crystallography and NMR studies by Yigong Shi's group and Steve Fesik's group, respectively, demonstrated that indeed the first few residues of Smac specifically interact with a groove region of the BIR3 domain of XIAP [56, 57]. This same BIR domain is responsible for inhibition of caspase-9 activity.

Intriguingly, binding of caspase-9 with XIAP follows the same mechanism as binding of Smac with XIAP. Autocatalysis of human caspase-9 at D315 generates a new N-terminal end, ATPF, which is similar to the N-termini of both the RHG proteins and Smac. Indeed, as demonstrated by Alnemri and colleagues, this new N-terminus is essential for caspase-9 interaction with the BIR3 domain of XIAP, and a single A316M mutation of caspase-9 will render the enzyme resistant to XIAP inhibition [58]. Therefore, Smac and cleaved caspase-9 interact with the BIR3 domain of XIAP via the same mechanism, and Smac competitively antagonizes the inhibitory activity of XIAP toward caspase-9 [58]. Further, since XIAP can only interact with cleaved caspase-9, which occurs upon, although is not required for, its activation by the apoptosome, this is obviously a highly economical and effective strategy for XIAP to act as a caspase inhibitor.

Smac can also release XIAP from inhibiting effector caspases. Instead of utilizing the BIR3 domain, or any other BIR domains, XIAP inhibits effector caspases (caspase-3 and caspase-7) through a linker region between its BIR1 domain and BIR2 domain, as revealed by the structural studies from the Shi Lab and Wu Lab independently [59, 60]. This raises the question of how Smac suppresses the inhibitory activity of XIAP toward caspase-3. A detailed biochemical and kinetic analysis by Jiang and colleagues revealed the intricate molecular basis underlying this action [61] (Fig. 2.3): as a homodimer, Smac interacts with the BIR3 domain of XIAP through one of its N-termini, which precedes and facilitates the subsequent low-affinity binding of Smac with the BIR2 domain of XIAP; Smac–BIR2 binding in turn attenuates the caspase-3 inhibitory function of XIAP through preventing binding of XIAP to caspase-3. As such, although the multiple BIR-domain organization of XIAP allows it to simultaneously inhibit caspase-9 and caspase-3, it also makes the protein highly susceptible to inhibition by Smac.

It should be noted that in addition to the BIR domains, most IAP proteins also contain a RING domain and thus possess E3 ubiquitin ligase activity. Work from Steller and others showed that the E3 activity is involved in turning over active caspases; and upon interaction with IAP antagonists, IAPs might also be stimulated to catalyze auto-ubiquitination, leading to their self-destruction [62, 63].

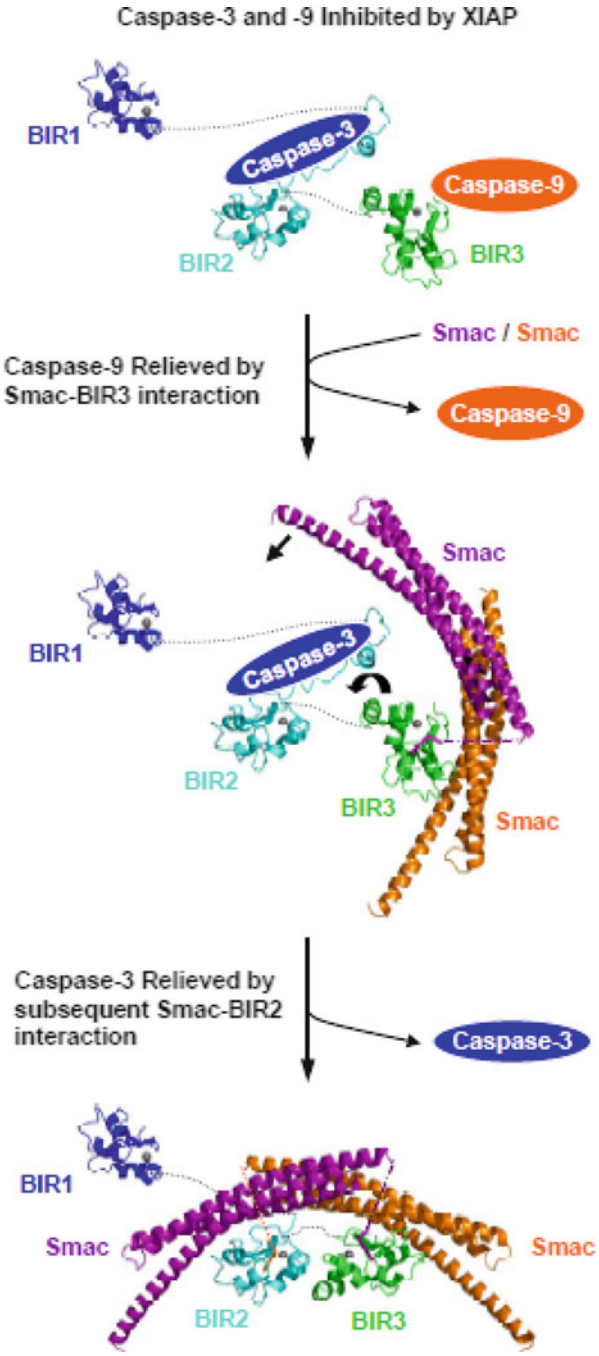

Fig. 2.3 Smac suppresses the inhibitory activity of XIAP toward caspase-9 and caspase-3

Therefore, besides BIR domain-based protein–protein interaction, the E3 enzymatic nature of IAPs is also critical for their apoptotic function.

There are multiple other IAP antagonists in mammalian cells, and they appear to be associated with mitochondria as well. Right after the discovery of Smac/Diablo, several groups identified another IAP antagonizing protein from mitochondria, Omi/HtrA2 [64–69], which appears to work via the similar mechanism of action. Further, Omi/HtrA2 is a serine protease, and in addition to suppressing IAP activity by direct binding, Omi/HtrA2 is also able to antagonize IAPs by proteolytic cleavage.

Interestingly, another putative IAP antagonist, the septin-like protein ARTS/Sept4 discovered by Larisch and colleagues, appears to function by a unique mechanism [70, 71]. Unlike Smac and Omi that are localized inside of mitochondria, ARTS is localized on the surface of the mitochondrial outer membrane, where its interaction with the IAPs can be independent of MOMP [72]. Further, the apoptotic role of ARTS is specific to certain biological contexts and relevant to cancer development. Genetic deletion of ARTS genes in mouse models specifically increased the resistance of stem cells to apoptotic insults and gave rise to higher tendency for tumorigenesis [73]. Consistent with its biochemical role as an IAP antagonist, deletion of the XIAP gene can partially suppress the phenotype of ARTS deletion [73]. Still, an important question is the following: How does ARTS exert such stem cell-selective function? Is it expressed at a higher level in stem cells, or is it regulated in a posttranslational, stem cell-specific manner (localizing outside of mitochondria certainly makes ARTS more susceptible to modulation than Smac or Omi, which reside in a compartment excluded from IAPs)?

2.3.4 Mitochondria as the Apoptosis Headquarters

Study of the mitochondrial apoptotic pathway has clearly established this organelle as the center of regulation for the intrinsic pathway. Many of the key players that control the execution of apoptotic cell death localize inside of mitochondria, including cytochrome c and IAP antagonists, as well as other mitochondria-localized apoptotic factors, such as endonuclease G and AIF, which are released from mitochondria during apoptosis and function together to degrade nuclear DNA [74–76]. But a somewhat philosophical question is the following: Why does this essential organelle for cellular metabolism possess such suicidal capability? There are fascinating theories linking this relationship to the origin of mitochondria in eukaryotic cells and their symbiotic relationship [77]. These theories will not be elaborated in this chapter. Here we discuss mechanistically how the barrier of the mitochondrial membrane is harnessed elegantly for controlling caspase activation.

Both cytochrome c and Smac are encoded by the nuclear genome and thus are synthesized in the cytosol where they suppose to promote caspase activation. However, newly synthesized cytochrome c and Smac are not active as apoptotic factors. Cytochrome c needs to be transported into mitochondria to subsequently be

covalently modified by the prosthetic cofactor heme. Only after such modification can cytochrome c be an active electron transport chain component for ATP synthesis inside of mitochondria and a competent molecule for Apaf-1 activation outside of mitochondria [8]. But now that mature cytochrome c is confined inside the mitochondria, it will only function as a death inducer upon MOMP. Same concept works for Smac, although that detailed mechanism is different. Newly translated Smac has an N-terminal mitochondrial targeting sequence, which is required for its mitochondrial localization but at the same time prevents its interaction with IAPs [53, 56, 57]. Upon mitochondrial import, the mitochondrial targeting sequence is removed by proteolysis, and thus the functional BIR-interacting N-terminus is created. But again, only after MOMP is triggered can the functional Smac be released to interact with cytosolic IAPs.

2.4 Cross Talk of the Mitochondrial Pathway with Other Apoptotic Pathways

2.4.1 Cross Talk with the Death Receptor Pathway

The death receptor-mediated apoptotic pathway, also known as the extrinsic pathway, is triggered by the TNF family of ligands. These ligands activate their corresponding receptors, which through specific adaptor proteins recruit and activate caspase-8 or caspase-10. These caspases are initiator caspases that activate downstream effector caspases. Chapter 12 of this book provides a detailed discussion of this pathway.

The Wang Lab and Junying Yuan Lab independently discovered that caspase-8/10 can also proteolytically activate a pro-death Bcl-2 family protein called BID, thus engaging the mitochondrial pathway as an amplification loop [78, 79]. In this sense, inhibition of the mitochondrial pathway can also decelerate apoptosis triggered by death receptor ligands. Intriguingly, for many mammalian cells (so-called type II cells), inhibition of the mitochondrial pathway by Bcl-2 overexpression can completely block apoptosis induced by extrinsic ligands [80, 81]. A possible mechanism to explain this complete dependency is that there is a high level of IAP expression that inhibits the activity of effector caspases; when MOMP is triggered by caspase-8-activated BID, Smac and Omi are released to antagonize IAPs and thereby liberate effector caspases to execute apoptosis [82, 83].

There is another form of cross talk between the two pathways. In some type II cells, death receptor ligands can induce upregulation of a protein called CAS, which is a stimulator of Apaf-1 [84] (see more detail in the next section). Therefore, the extrinsic pathway can not only turn on the intrinsic pathway through BID cleavage, but it can also enhance apoptosome activity through upregulating CAS.

2.4.2 *Apoptosis Involving Caspase-2 Activation*

Caspase-2 is a very special caspase. Based on its primary structure, it appears to be an initiator caspase. However, it lacks the most characteristic function of a typical initiator caspase: direct activation of effector caspases by proteolytic cleavage. Jiang and colleagues first demonstrated that caspase-2 activates apoptosis by cleaving and activating BID [85]. Therefore, caspase-2-induced apoptosis is dependent on the mitochondrial pathway. Recent studies suggest that stressful conditions such as heat shock and ER stress trigger apoptosis through caspase-2 activation [86, 87].

2.5 Regulation in the Downstream of Cytochrome c Release

2.5.1 *Nucleotide Exchange of Apaf-1*

In addition to the action of the safeguard IAP proteins and their antagonists, there are also other complex mechanisms to regulate apoptosis in downstream of cytochrome c release. One of these mechanisms targets cytochrome c-initiated nucleotide exchange of Apaf-1 (Fig. 2.4). A collaborative effort between Xiaodong Wang's group and Abbott Laboratories identified the chemical compound α-(trichloromethyl)-4-pyridineethanol (PETCM) as a trigger of caspase activation in HeLa cell extracts [88]. Intriguingly, the PETCM effect depends on all the protein components of the cytochrome c pathway but spared the requirement of exogenous ATP/dATP. The residual nucleotide in the cell extracts proved to be sufficient (and essential) for PETCM effect. Further, a series of novel protein factors including the

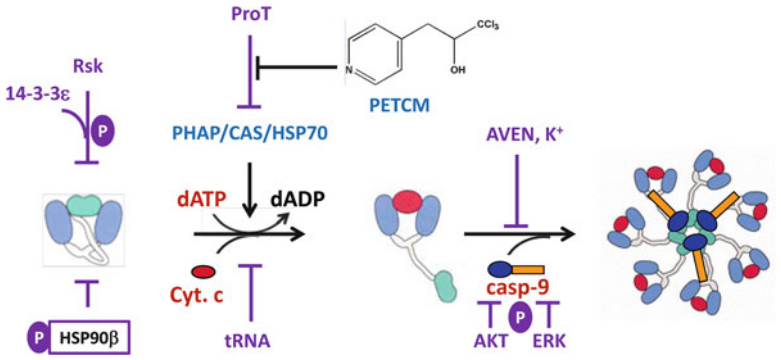

Fig. 2.4 Multiple mechanisms regulate apoptosome function in the downstream of cytochrome c release

negative regulator prothymosin-α (ProT), which is a putative oncogene product, and the positive regulator PHAP proteins, which are reported tumor suppressors, were identified to be involved in PETCM action [88]. Subsequent collaboration between the Wang Lab and Jiang Lab identified two additional protein factors, HSP70 and cellular apoptosis susceptibility (CAS) required for PETCM-induced caspase activation [84]. PHAP, HSP70, and CAS function together to stimulate cytochrome c-dependent Apaf-1 nucleotide exchange; and this stimulatory activity is crucial for caspase activation when both cytochrome c and nucleotide concentrations are low [84].

The crucial role of CAS in promoting Apaf-1 nucleotide exchange and subsequent apoptosome formation has been supported by cellular experiments. Importantly, various apoptotic triggers can upregulate CAS protein levels in cells, and it appears that under these same conditions, CAS is required for effective apoptosis [84]. Therefore, it is possible that CAS upregulation is a commonly used mechanism to ensure apoptosome assembly and eventual apoptosis.

2.5.2 Protein Phosphorylation

Protein phosphorylation has been reported to be another general mechanism regulating the function of the apoptosome-caspase-9 holoenzyme (Fig. 2.4). Reed and colleagues reported that both the AKT pathway and Ras-MAPK signaling can lead to caspase-9 phosphorylation to inhibit cytochrome c-mediated activation of caspase-9 and downstream caspase-3 [89]. This finding is consistent with the proliferating and survival functions of the two signaling pathways. Kornbluth and colleagues revealed that protein phosphorylation can also impact apoptosome assembly through Apaf-1. For example, they found that MAPK signaling promotes Rsk-catalyzed Apaf-1 phosphorylation and subsequent binding of 14-3-3ε, preventing cytochrome c from interacting with Apaf-1 and triggering its oligomerization [90]. They also found that under certain oncogenic conditions, HSP90β becomes hypophosphorylated at certain sites that are normally phosphorylated; and the hypophosphorylated HSP90β inhibits apoptosome activation by sequestering Apaf-1, thus suppressing apoptosis [91]. Therefore, multiple signal transduction pathways communicate with the mitochondrial pathway, particularly its apoptosome components, via modulating protein phosphorylation.

2.5.3 Other Factors Regulating Apoptosome Function

There are many other cellular components that can modulate cytochrome c/Apaf-1-mediated caspase activation by influencing apoptosome function (Fig. 2.4). For example, Hardwick and colleagues reported that a protein factor called Aven can directly interact with Apaf-1 and inhibit its oligomerization [92]. Further, various nonprotein factors have also been shown to regulate apoptosome function. Xiaolu

Wang and colleagues made an interesting observation that tRNA can compete with Apaf-1 for cytoplasmic cytochrome c binding, thus representing a potential protective mechanism [93]. Cellular potassium has also been suggested to control apoptosome function, consistent with the in vitro observation that apoptosome formation is highly sensitive to ion concentration [94, 95]. In conclusion, the apoptosome complex is a major target for regulation of mitochondria-mediated apoptosis.

2.6 Noncanonical Functions of the Mitochondrial Pathway

2.6.1 Settling the Debate of Point of No-Return

It was generally believed that MOMP and cytochrome c release is the point of no-return for cell death. This was considered to be the case even when downstream caspase activation is completely abolished; under such condition, apoptosis may simply succumb to other less understood cell death modes, such as necrosis or mitochondrial catastrophe. However, if this is the case, why does the cell devote substantial resource to regulate downstream caspase activation? It could be argued that those positive regulatory mechanisms will accelerate apoptotic cell death, thus avoiding slower and inflammation-prone necrosis, which could be detrimental to multicellular organisms. But what is the real biological meaning or benefit of these negative regulatory mechanisms that function downstream of the release of cytochrome c?

The model that MOMP is the point of no-return is based on the following observations: (1) cytochrome c release appears to be a rapid, all-or-none event upon many apoptotic triggers [96, 97]; (2) when mitochondrial integrity is severely damaged under certain experimental settings, cells will die either via highly orchestrated apoptosis (when downstream caspase activation is intact) or necrosis (when downstream caspase activation is defective or inhibited) [98]. However, is cytochrome c release always an equivalent to complete loss of mitochondrial integrity, or could there be partial or incomplete MOMP? Under certain physiological conditions, it is formally possible that the initial apoptotic triggers are so delicate that they only induce modest cytochrome c release while maintaining overall mitochondrial integrity. This view is supported by multiple lines of research. For example, genetic deletion of components downstream of cytochrome c release (Apaf-1 or caspase-9) has been shown to prevent developmental cell death of neuronal cells [14–17]; thus, developmental cell death can be halted even after initial cytochrome c release. In addition, under certain conditions, cytochrome c-initiated caspase activation precedes loss of mitochondrial membrane potential, and the downstream caspase activation is required for complete loss of mitochondrial membrane integrity. In this case, activated caspases likely further amplify cytochrome c release by mechanisms such as proteolytic activation of BID or conversion of Bcl-2 from anti-death form to pro-death form [99–104]. Further, as have been argued earlier, the existence of

delicate regulatory mechanisms downstream of MOMP also suggests that cells might adapt fates other than death after cytochrome c release.

Several recent studies further demonstrated that under certain specific biological conditions, MOMP and cytochrome c release are not the point of no-return. By performing single-cell imaging and viability experiments, Green and colleagues found that inhibition of caspase activation downstream of modest cytochrome c release (induced by expression of cleaved BID) allows the same cells to fully recover and proceed into long-term proliferation [105]. A separate study conducted by Morgan Sheng and colleagues revealed that in hippocampal neurons, cytochrome c-mediated caspase activation is required for their long-term depression, which is an essential process for brain development and function [106]. Therefore even caspase activation downstream of cytochrome c release may not necessarily be a point of no-return in some biological contexts.

2.6.2 Non-death Functions of Caspases

Since caspase activation is not always the point of no-return, it is no surprise that caspase activity may be involved in biological processes that are distinct from cell death. There are many such examples of caspase functions in non-death processes, although caspase activity in these contexts is not always controlled by the mitochondrial pathway. It is widely documented that differentiation of various cell types, including sperm and red blood cell differentiation, requires caspase activation [107–109]. Also, neuronal axonal pruning specifically requires caspase-6 activity [110]. These caspase-dependent events share a common feature: while they do not result ultimately in cell death, they all involve significant clearance of cellular components and substantial loss of total cell mass. Thus they can be categorized as "apoptosis-like" or "partial apoptotic" processes. However, some other caspase-dependent, non-death events, such as the aforementioned hippocampal long-term depression [106], do not have such "apoptotic-like" characteristics.

2.6.3 The Unsolved Question of Fate Determination

How does a cell determine whether cytochrome c-mediated caspase activation should execute apoptotic cell death or instead perform a specific non-death biological function? There are two apparent events whose regulation is critical for the determination of cell fate. The first is MOMP or cytochrome c release, and the second is the downstream caspase activation. While the scope of MOMP can be accurately controlled by the Bcl-2 family members, how is caspase activity regulated to realize two extreme cell fates? For example, if caspase activation is triggered to perform a non-death function, how is caspase activity contained presumably

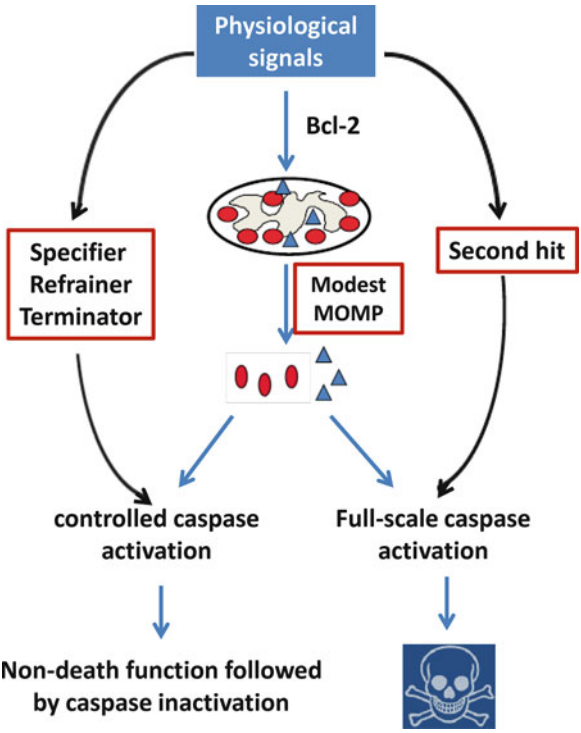

Fig. 2.5 Schemes showing possible mechanisms that determine the apoptotic or non-death functions of the mitochondrial pathway

in a spatially and temporarily limited fashion? How does the activity only act upon the "right" substrates? What instructs the caspase machinery to terminate its function and how? Conversely, if the "purpose" of a modest cytochrome c release under a particular biological condition is for apoptotic cell death, does it take a "second hit" to cooperate with the subtle cytochrome c release to warrant effective apoptosome assembly and caspase activation (Fig. 2.5)? It is most likely that the additional regulatory mechanisms that determine cell fate are instructed by the initial biological cues that trigger the release of cytochrome c.

The current molecular understanding of cytochrome c-mediated caspase activation suggests several possible mechanisms for how caspase activity can be accurately controlled to achieve apoptosis or a non-apoptotic function. For example, to limit or dismantle caspase activity in a non-apoptotic process, the RING domain-containing IAP proteins might be employed to not only inhibit but also degrade active caspases through their E3 ubiquitin ligase activity; apoptosome activity can be terminated by stimulating the dATP/ATP-hydrolysis activity of Apaf-1, thus leading to disassembly of the apoptosome complex. On the other hand, if a modest cytochrome c release is meant to induce rapid apoptosis, a "second hit" such as CAS upregulation could be engaged to stimulate nucleotide exchange of Apaf-1. Further,

additional factors might be involved to direct the activated caspases to act within particular cellular locations and upon specific substrate proteins. To identify, profile, and quantify the cellular substrates of specific caspases, Jim Wells and colleagues have developed an innovative proteomic approach coupling chemical biology and mass spectrometry analysis [111–113]. This will be a powerful tool to facilitate the understanding of the exact role of caspase activity under specific biological conditions.

2.6.4 Noncanonical Functions of the Canonical Components of the Mitochondrial Pathway

Perhaps consistent with the fact that caspase activation does not always activate cell death but can serve other purposes, many components of the mitochondrial pathway have been shown to possess additional functions not related to caspase activation. Mounting evidence indicates that Bcl-2 family members also regulate cellular calcium homeostasis, glucose metabolism, and mitochondrial dynamics [114–118]. Kroemer and colleagues have suggested that Apaf-1 is involved in cell cycle arrest elicited by DNA damage in a caspase activation-independent manner [119]. The canonical caspase inhibitory proteins, IAPs, can also perform other biological functions unrelated to caspases. For example, survivin, a member of the IAP family, is involved in kinetochore function [120, 121]; cIAP1 and cIAP2, also members of the IAP family, are critical regulators of the NF-κB signaling [122].

Interestingly, the synthetic Smac-mimicking compounds have been shown to be able to antagonize the function of cIAP1 and cIAP2 in regulating NF-κB signaling [123–125]. But is there any biological context where Smac regulates NF-κB signaling by antagonizing cIAP1 and cIAP2, but in an apoptosis-independent manner? If so, one would expect that Smac can be released from the mitochondria in the absence of apoptosis. This might be achieved via a modest and reversible MOMP. Alternatively, Smac might be released through a mitochondrial membrane channel specific to Smac but not other mitochondrial proteins such as the initiator of caspase activation, cytochrome c.

The noncanonical functions of members of the mitochondrial pathway have important implications. When we study the physiological and pathological roles of a mitochondrial pathway component, caution needs to be applied when determining whether the observed biological effect can be attributed to mitochondria-mediated apoptosis, or whether it points to a novel non-apoptotic function of this protein.

2.7 Concluding Remarks

After decades of intensive research, the molecular basis and biological functions of the mitochondrial apoptotic pathway have been well established. The role and therapeutic potential of this pathway in various human diseases, especially cancer, have also been explored with several promising therapeutic agents developed. However, many fundamental questions remain to be answered, and various new questions have emerged, due to the recent, unexpected findings indicating that mitochondria-mediated caspase activation can exert other biological functions in addition to cell death and that many canonical components in the pathway are also involved in biological processes not related to apoptosis. These questions should be actively pursued. For example, a fundamental question from the early days is as follows: What is required in order for caspase activation to equate to apoptotic cell death? Is caspase-dependent chromosomal DNA fragmentation or phosphatidyl serine flipping to the outside of the plasma membrane absolutely necessary for apoptosis? Or the other way to ask the same question: What substrate(s) needs to be cleaved by caspases in order to mark the point of no-return? The newly developed cellular substrate profiling techniques will be valuable for tackling this question. Second, what are the biological functions of mitochondria-mediated apoptosis in addition to the developmental and immunological roles identified in mouse gene-targeting experiments? More sophisticated mouse models need to be created for this purpose as conventional knockouts of the pathway components often lead to perinatal lethality. Related to the gene-targeting approach, additional experiments need to be conducted to unambiguously dissect whether the physiological phenotypes caused by a specific gene targeting are due to interference of apoptosis or other non-apoptotic functions of the same gene. Third, since mitochondria-mediated caspase activation has been implicated in non-death biological processes such as those related to brain activity, how can one study such novel function in vivo, especially considering that caspase activation under such contexts is most likely to be modest and transient? Understanding the molecular regulation of caspase activity in these processes (e.g., compartment and substrate specificity, termination mechanisms) will help to guide the generation of reasonable animal models. Lastly, as cytochrome c release or caspase activation is not always the point of no-return, and caspase activation has additional non-death functions, we need to reconsider the value of caspase inhibition therapeutically, as well as other methods to target the pathway (both inhibiting and stimulating it), in treating diseases that are related to the mitochondria-mediated caspase activation pathway but not necessarily its apoptotic function.

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Chapter 3
Molecular Basis of Cell Death Programs in Mature T Cell Homeostasis

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Abstract Overview of Peripheral T Cell Homeostasis
The lymphocyte is a major class of white blood cell that defends the body from attack by infectious agents and confers long-term protection through immunological memory. Lymphocytes are also involved in pathological immune reactions including graft rejection, allergies, and autoimmune disorders. Their remarkable capacity to proliferate up to 5,000-fold in response to antigen must be counterbalanced by a controlled process of removing cells. This is achieved through molecular pathways of programmed cell death, which maintain selective and specific homeostasis of the numbers of lymphocytes and other immune cells. We focus principally on cell death mechanisms in T lymphocytes that control the number of T cells of a given antigen specificity represented in the finite T cell niche. Here, we discuss the central role of caspases in the regulation of the general pathways of cell-extrinsic and cell-intrinsic T cell apoptosis and programmed necrosis with an overview of their importance for human health.

Keywords Lymphocyte • Death receptor • Fas • RICD • Mitochondria • CWID • ALPS • XLP

3.1 Forms of T Cell Death

Although the concept of a programmed form of cell death was envisioned as early as the 1950s, the major supporting data for this process were published in the 1970s and 1980s [1]. These seminal studies were performed in *Caenorhabditis elegans* as

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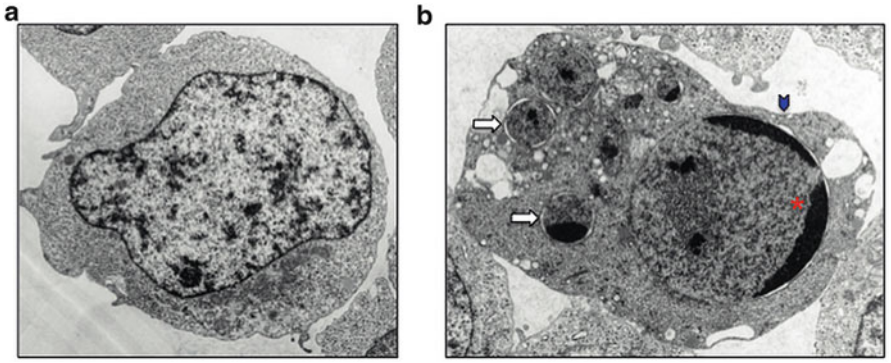

Fig. 3.1 Electron micrographs of healthy (**a**) and apoptotic (**b**) cells. *Red asterisk*: Condensed nucleus; *white arrow*: apoptotic bodies; *blue arrow*: intact membrane. Micrographs courtesy of Dr. Lixin Zheng

part of an effort to trace the ontogeny of the entire worm's embryonic cell lineages. The process of programmed cell death was given the moniker "apoptosis," which is a Greek term referring to leaves falling off trees or petals falling off flowers, by pathologists in 1972 [1]. In recognition of their contributions to understanding apoptosis pathways, Brenner, Horvitz, and Sulston received the 2002 Nobel Prize in Physiology or Medicine [2, 3]. In 1988, independent studies of cell death in mammalian cells by Vaux/Cory/Adams [4] revealed the critical role of BCL-2 (described in detail below) in blocking programmed cell death of tumor cells. These studies were extended by Korsmeyer [5] to demonstrate that BCL-2 also inhibits apoptosis of non-transformed cells. The breadth of studies on cell death has become remarkably vast, and multiple forms of programmed cell death beyond apoptosis have now been identified [6]. The focus of this chapter is on apoptosis in mature T lymphocytes with a brief discussion of a non-apoptotic form of cell death classified as programmed necrosis.

3.1.1 Apoptosis

As elegantly demonstrated in *C. elegans*, apoptosis is a cellular process that results from highly regulated biochemical events terminating in a controlled form of cell death. During *C. elegans* development, 1,090 cells are generated, and 131 of these cells invariably disappear through apoptosis. The genes controlling this process in *C. elegans* are *ced-3*, *ced-4*, *ced-9*, and *egl-1* via a pathway of epistatic relationships in which EGL-1 –| CED-9 –| CED-4 → CED-3 → cell death [7]. Mammalian cells undergoing apoptosis display distinctive hallmarks including cell shrinkage, nuclear condensation, DNA fragmentation, apoptotic bodies, and membrane blebbing (Fig. 3.1) [8].

In late-stage apoptosis, the so-called eat-me signals (e.g., phosphatidylserine) appear on the cell surface and induce phagocytes to rapidly clear dying cells by engulfment [9].

T lymphocytes have great potential for rapid expansion as they carry out their task of orchestrating and executing immunological responses against invading pathogens [10]. As such, apoptosis plays a critical role in homeostatic control of T cell numbers and is important in safeguarding against the autoimmune diathesis caused by rare pathogenic T cells that may expand during a response to infection and overcome peripheral tolerance mechanisms. There are two main pathways discussed in detail below that induce apoptosis: receptor-mediated/extrinsic and mitochondrial/intrinsic. The intrinsic pathway of cell death is an ancient one closely related to the cell death pathway delineated in nematodes. The extrinsic pathway, however, only exists in vertebrates and is initiated by ligation of surface "death" receptors that have major physiological roles in controlling lymphocytes. Interestingly, the emergence of the extrinsic pathway in vertebrates [11] corresponds with the evolution of lymphoid cells, which first appear in basal jawed vertebrates [12], suggesting possible coevolution of death receptor signaling for control of lymphocyte homeostasis.

3.1.2 Non-apoptotic Cell Death

The forms of cell death were originally simply categorized by the dichotomy of either traumatic, injury-induced cell death called necrosis or noninflammatory, programmed cell death via apoptosis. We now appreciate that there are multiple, individually nuanced forms of programmed cell death. Those observed in T cells include apoptosis, necroptosis [13], and autophagic cell death [14]. There are also a variety of mechanisms involving specific molecular pathways that account for pathological cell death due to infectious agents, particularly viral cytopathicity [15]. The importance of non-apoptotic forms of cell death for T cell biology is still being established, but the critical role of apoptosis is irrefutable.

3.2 Central Role of Caspases in Apoptosis

As with most tightly controlled cellular processes, the cascade of events leading to apoptosis involves activation of a series of proteins with several regulatory steps along the way. In the case of apoptosis, the cysteine-dependent, aspartate-directed protease (caspase) enzymes, which are homologous to *C. elegans*' CED-3, are central mediators that function by cleaving substrate proteins after an aspartate residue. Caspases are first synthesized as zymogens that include two enzymatic subunits and an N-terminal pro-domain that, in one class of caspases, contains an 80–100-amino acid protein–protein interaction motif of the death effector domain (DED) or

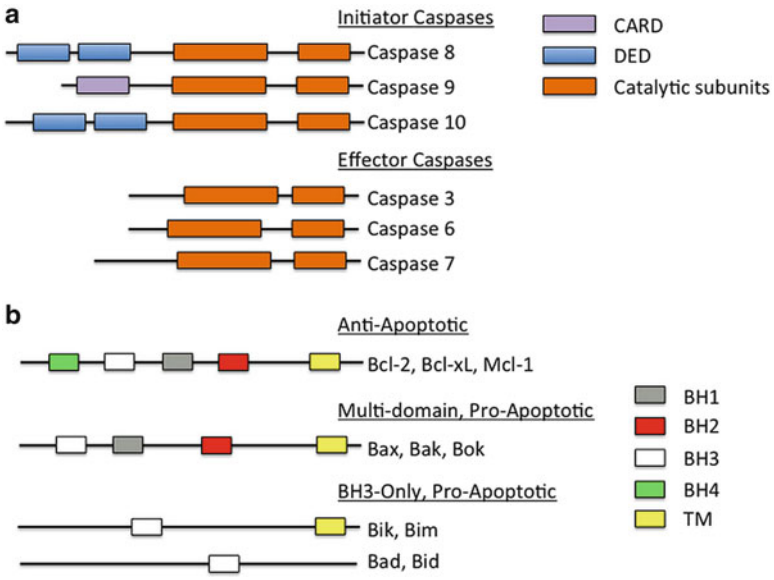

Fig. 3.2 Key protein mediators of extrinsic (**a**) and intrinsic (**b**) cell death. *Colored boxes* denote protein domains as indicated in the legends

caspase recruitment domain (CARD) variety [16]. These domains form a hexahelical bundle of tightly coiled alpha helices known as the death fold that binds through homotypic interactions (i.e., DED–DED or CARD–CARD) to appropriate signaling complexes containing the same death fold [17]. Following processing, the two enzymatic subunits are released and the prodomain is destroyed. Crystal structures of the mature enzyme indicate that two copies each of the enzymatic subunits interact non-covalently to form a heterotetramer [18]. Caspases are involved in apoptosis and inflammation. The inflammatory roles involve processing precursor forms of cytokines and will not be further discussed. Those mediating apoptosis fall into two categories: termed "initiator" and "effector" caspases (Fig. 3.2a). Initiator caspases can be activated by cell-extrinsic signals (caspases 8 and 10) transduced via surface receptors or by cell-intrinsic pathways (caspase 9) initiated by cell stress, such as DNA damage or growth factor withdrawal. Initiator caspases contain either two tandem DED domains (caspases 8 and 10) or a CARD domain (caspase 9), whereas effector caspases (including caspases 3, 6, and 7) lack either recruitment domain. An additional initiator caspase called caspase 2 has more recently been proposed to be important in inducing apoptosis after DNA damage through a signaling complex known as the PIDDosome [19]. Once activated, initiator caspases cleave (and thereby activate) full-length pro-forms of the effector caspases. Effector caspases act by cleaving various protein substrates to inhibit or alter their function and execute the apoptotic program. Cell death substrates that are targeted for cleavage by effector caspases during apoptosis include cytoskeletal proteins (e.g., actin, plectin,

ROCK1, gelsolin), nuclear lamins, and inhibitor of the caspase-activated DNase (ICAD) [20], which upon cleavage lead to cell shrinkage/blebbing, nuclear condensation, and DNA fragmentation, respectively.

Caspase activity is negatively regulated by proteins in the inhibitor of apoptosis (IAP) family [21], which includes XIAP, survivin, c-IAP-1, c-IAP-2, and NAIP. IAP family proteins have multiple functions including ubiquitin ligase activity and inhibition of active caspases. XIAP [22] is the best studied in this family, and it has been demonstrated to bind caspases 3 and 7 with high affinity through its BIR2 domain and caspase 9 with low affinity through its BIR3 domain [23]. This binding profile reveals the role of XIAP as a regulator of the downstream effector caspases that function in both the extrinsic and intrinsic apoptosis pathways discussed below. Once bound to a caspase, XIAP sterically hinders the enzyme active site to prevent execution of the apoptotic program [24]. IAP proteins are, in turn, inhibited by the mitochondrial proteins SMAC/DIABLO [25, 26] and OMI [27] (described further in the discussion of the intrinsic pathway below) to allow apoptosis to proceed. Regulation of caspase 8 is more complex, likely because this protease is unique in its ability to participate in non-apoptotic signaling. Importantly, full-length caspase 8 participates in transducing signals required for T cell activation [28] by augmenting NF-κB activation downstream of the TCR [29]. Moreover, basal caspase 8 activity is required to prevent T cells from undergoing autophagic cell death [14].

3.3 Extrinsic, Death Receptor-Induced Apoptosis and Necroptosis

Cell-extrinsic death pathways are initiated by ligation of death receptors expressed on the surface of lymphoid cells.

3.3.1 TNFR Superfamily Death Receptors

The most important death-inducing receptors on the surface of T cells are members of the TNF receptor (TNFR) superfamily that share structural similarities and conserved cytoplasmic domains. Their signaling depends on trimer formation that is characteristic of TNFR superfamily members and mediated by the pre-ligand-binding assembly domain (PLAD) [30]. The ligands for the DD-containing TNFR proteins are expressed as membrane-bound trimers [31] homologous to TNF-α and can be cleaved from the cell surface by metalloproteinases to generate soluble forms [32]. However, elegant mouse studies have demonstrated that TNFR ligands are most active when in cell-surface, membrane-bound trimer form [31]. The death receptors contain a death domain (DD), which forms the hexahelical death fold described above, that nucleates formation of a protein complex required to

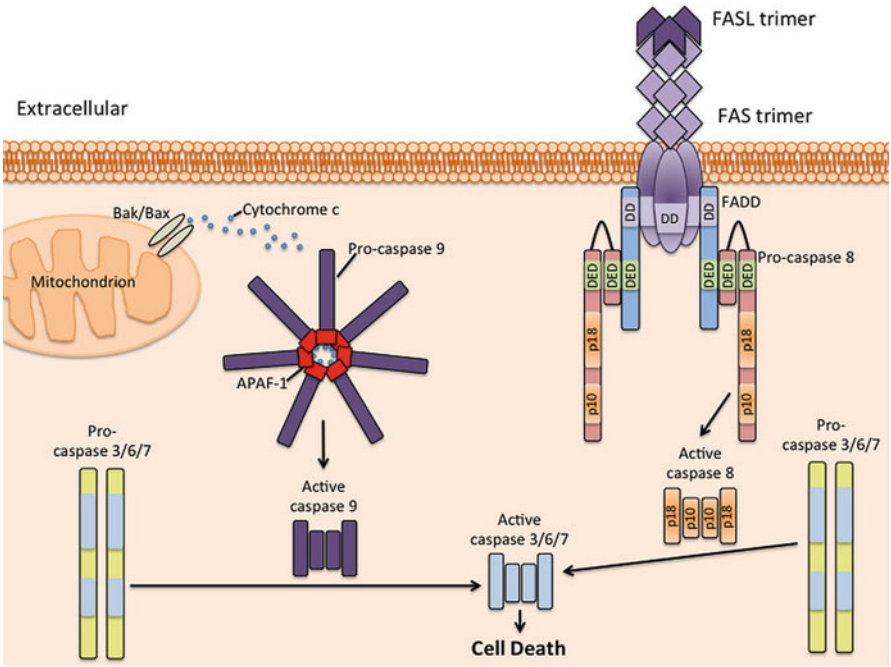

Fig. 3.3 Caspase 8 and 9 activation pathways

transduce the death signal. Receptors included in this family of DD-containing TNFR proteins are FAS, TNFR1, TRAIL-R1, TRAIL-R2, DR3, and DR6 [32]. DR3 and DR6 biology is relatively poorly understood, though ligands have been identified (TL1A [33] for DR3 and APP [34] for DR6). Although they both have some functions in the immune system [35–37], DR3 primarily promotes T cell activation [33], and DR6 functions primarily in the nervous system [34]. Because our current understanding attributes little role in T cell death, DR3 and DR6 will not be discussed further in this chapter.

3.3.1.1 FAS

The prototype for the death receptor family is the FAS receptor (also known as APO-1, CD95, or TNFRSF6), which exists as a multimer, likely a trimer, on the surface of activated T cells due to homotypic interaction of the PLAD [38] domains. Upon binding of membrane-bound, trimeric FAS ligand (FASL), higher order clusters of FAS trimers termed signaling protein oligomerization transduction structures [39] (SPOTS) are formed and permit downstream signaling. The first step in FAS receptor signaling is formation of the death-inducing signaling complex (DISC), which is formed by homotypic interaction of the DD domain of FAS with the DD domain of the adaptor molecule FADD (Fig. 3.3). Structural analysis has revealed

that the stoichiometry of FAS:FADD interactions is 5–7 FAS molecules with 5 FADD molecules [40], consistent with the formation of SPOTS [39]. In addition to a DD domain, FADD also contains a DED domain, which mediates FADD–FADD interactions that stabilize the DISC and recruit the DED-containing pro-caspases 8 and 10. Once pro-caspase 8 and 10 are recruited to the DISC, the pro-caspase zymogens oligomerize and initiate a two-step proteolytic cleavage event that first removes a C-terminal p10 or p12 peptide for caspases 8 and 10, respectively, and then liberates the p18 or the p17 peptide for caspases 8 and 10, respectively, to enable formation of the active caspase heterotetramer consisting of two p10/p12 molecules and two p18/p17 molecules [41]. Active caspases 8 and 10 then cleave the effector caspases to execute the death program.

The susceptibility of a cell to FAS-induced apoptosis is variable, leading to a categorization paradigm in which type 1 cells (e.g., T cells) die efficiently after activation of effector caspases by activated caspases 8 and 10 and type 2 cells (e.g., hepatocytes and pancreatic beta cells) are resistant to death by this pathway unless the caspase cascade is amplified. This amplification occurs when caspase 8 cleaves BID [42–44], generating a pro-apoptotic truncated BID (tBID) protein that amplifies apoptosis signaling by initiating the intrinsic pathway of cell death [45, 46]. XIAP has also been described to be a key reason why type 2 cells are more resistant to FAS death [47].

Negative regulation of FAS signaling is achieved through the activity of c-FLIP, a pro-caspase 8-like protein that is recruited to the DISC via its two tandem, N-terminal DED domains. C-FLIP is a catalytically inactive caspase 8 paralog that decreases the sensitivity of cells to FAS-induced death [48–51] by binding the DISC and inhibiting processing of initiator pro-caspases [52, 53]. Another layer of negative regulation of caspase 8 is provided by TIPE2 [54], a member of the TNF-α-induced protein-8 (TNFAIP8) family that contains a DED domain. TIPE2 functions by binding caspase 8 and inhibiting activation of the transcription factors AP-1 and NF-κB while promoting FAS-induced apoptosis. Mice deficient in TIPE2 succumb to premature death with splenomegaly and multi-organ inflammation [54]. Formation of the DISC can also be impaired by sequestration of FADD by the DED-containing PEA-15 molecule [55].

3.3.1.2 TNFR1

The cytokine TNF-α, which binds TNFR1 and TNFR2, has pleiotropic effects including both cell activation and cell death [56]. TNFR1 but not TNFR2 contains a DD domain important in recruiting adaptor molecules that signal for cell death by apoptosis or necroptosis [32]. Binding of TNF-α to TNFR1 induces consecutive formation of two different signaling complexes [57] on the cell surface (complex I) and then in the cytoplasm (complex II) [58]. Recruitment of the adaptor molecule TNF receptor-associated protein with death domain (TRADD) via its DD domain nucleates complex I and recruits the adaptor TNF receptor-associated factor-2 (TRAF2), a kinase called receptor-interacting protein-1 (RIP1), c-IAP-1, and

c-IAP-2, leading to poly-ubiquitination of RIP1 by the c-IAPs and downstream activation of NF-κB and AP-1 transcription factors [32]. In a second step, the TNFR1 complex is internalized, followed by de-ubiquitination of RIP1 by A20 and CYLD (cylindromatosis), allowing for formation of a cytoplasmic DISC/complex II when TRADD and RIP1 associate with RIP3, FADD, and pro-caspase 8 [58]. Complex II signals the cell to die by either caspase 8-mediated apoptosis [59] or RIP3-mediated necroptosis [60, 61]. RIP1 but not RIP3 contains a DD domain, but both contain a RIP homotypic interaction motif (RHIM) that mediates RIP1:RIP3 interactions [62]. RIP1 and 3 are kinases in the RIP family (including RIP1, RIP2/RICK/CARDIAK, RIP3, RIP4/DIK/PKK, RIP5/SgK288, RIP6/LRRK1, and RIP7/LRRK2) whose substrates and functions are as yet largely unidentified [62].

The disparate cellular outcomes of TNFR1 ligation (i.e., activation, apoptosis, or necroptosis) are determined through complex feedback inhibition that hinges on the ubiquitination status of RIP1. Poly-ubiquitinated RIP1 acts as a prosurvival scaffold molecule that activates NF-κB through complex I, and its kinase activity is not required. NF-κB inhibits cell death through a process involving upregulation of anti-apoptotic molecules including the c-IAP ubiquitin ligases and c-FLIP, which translocates to complex II and inhibits caspase 8 processing [58]. Upon removal of ubiquitin, RIP1 functions as a pro-death kinase as a part of the pro-apoptotic DISC formed by complex II. If caspases are blocked, the RIP kinases can divert death signaling to the pro-necroptotic pathway [57, 58]. An additional regulator of TNFR1 signaling is a DD-containing adaptor called silencer of death domains (SODD), which has been found to associate with TNFR1 and DR3 [63] and negatively regulate their signaling, though the details of its action are still being defined. The importance of TNFR1 in T cell homeostasis is still an open question, as animals deficient in TNFR1 do not accumulate T cells (as would be expected if cell death mechanisms were impaired) but instead exhibit deficient host defense and inflammatory responses [64]. Overall, TNFR1 signaling in T cells appears to lead predominantly to cytokine secretion, pro-inflammatory responses, and cell survival [65] in vivo.

3.3.1.3 TRAIL-R1 and TRAIL-R2

The third major death-promoting TNFR superfamily signaling pathway is induced by a ligand called TNF-related apoptosis-inducing ligand (TRAIL) [66]. TRAIL has five distinct receptors with two, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), containing a functional DD domain and three acting as "decoy" receptors that impair TRAIL-induced signaling by sequestering DR4 and DR5 chains via the PLAD domain [67]. The three "decoy" receptors include the membrane-associated proteins TRAIL-R3/DcR1 and TRAIL-R4/DcR2 and the soluble protein osteoprotegerin/TNFRSF11B [68]. Upon binding of trimeric TRAIL to DR4 or DR5, higher order clusters are formed, resulting in DISC assembly [69]. Similar to the FAS DISC, FADD, pro-caspases 8 and 10, and sometimes c-FLIP are recruited to the DD domain of DR4 or DR5. Unlike the other death receptors, DR4 and DR5 require

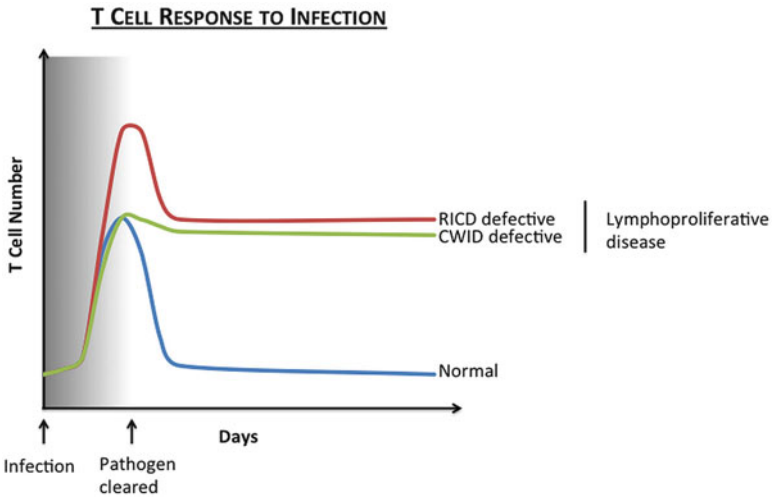

Fig. 3.4 T cell homeostasis in health and disease. *Shading* indicates antigen load from infectious agent. Adapted from Snow et al. Immunol Rev. 2010 Jul;236:68–82

O-glycosylation in order to form the higher order clusters necessary for DISC formation [69]. Moreover, polyubiquitination of pro-caspase 8 after formation of the TRAIL DISC has been found to lead to aggregation of pro-caspase 8 and augmented processing/activation to facilitate cell death [70]. The major roles of DR4 and DR5 signaling seem to be primarily in controlling inflammation and tumor susceptibility [71], but TRAIL can also induce cell death of CD8 T cells that failed to receive cytokine signals from CD4 T cells (i.e., CD4 help) during priming [72].

3.3.2 Restimulation-Induced Cell Death

A major physiological process that employs death receptor-induced apoptosis in T cells is restimulation-induced cell death (RICD). This death mechanism is also called activation-induced cell death (AICD), but we like to distinguish the process of activation from death induction since the latter requires restimulation. RICD functions as a negative feedback mechanism to prevent overexpansion of T cells during an immune response and occurs when T cells that are activated and cycling in the presence of lymphokines (e.g., IL-2) undergo apoptosis in response to a subsequent TCR stimulus [10, 73] (Fig. 3.4). This form of propriocidal regulation of the T cell pool is initiated by signaling through the TCR [74] and depends largely on extrinsic death signaling through FAS [75] and intrinsic death signaling through BIM [76] (discussed below). In appropriate mouse strains with gene deficiencies in FAS (lpr mice) [77] or FASL (gld mice) [75], severe lymphoproliferation and

autoimmunity occur in vivo with defective RICD observed in vitro. Although FAS and FASL are broadly expressed on activated T cells, FAS-dependent RICD is only initiated in cells that receive a TCR stimulus and not in bystander cells of different specificities [74]. The TCR-induced signal that makes a cell competent to die has not been clearly defined, though new protein synthesis is not required [74, 78]. Further studies examining the susceptibility of activated T cells to RICD have identified an important role for cell cycling, as only cells in late G_1 or S phase were found to die by RICD [79]. As discussed in the final section of this chapter, FAS-mediated RICD is a critical process for healthy T cell homeostasis, and a deficiency in any of the required signaling molecules results in severe accumulation of lymphocytes and associated pathologies discussed further below.

3.4 Intrinsic, Mitochondrion-Dependent Apoptosis

In addition to cell-extrinsic death signals propagated by cell surface receptors, a cell-intrinsic pathway of apoptosis has also been well defined. The intrinsic pathway of cell death is triggered by cell stress (e.g., DNA damage or growth factor deprivation) and is dependent on signals propagated from the mitochondria. Mitochondria are organelles within the cell whose major function is the efficient generation of ATP through oxidative phosphorylation in cell metabolism. However, mitochondria also play a critical role in cell death, mainly through release of proteins that induce apoptosis by caspase activation. This release occurs when the outer mitochondrial membrane integrity is disrupted by pore formation, a process tightly controlled by members of the BCL-2 family.

3.4.1 BCL-2 Family Proteins

The BCL-2 family of proteins is a key regulator of mitochondrion-dependent apoptosis, and the family's founding member, BCL-2, is the mammalian homolog of *C. elegans*' CED-9. There are approximately 25 genes in the BCL-2 family, and each contains one or more BCL-2 homology (BH) domains consisting of BH1 through BH4 (Fig. 3.2b). The family is broadly divided into three groups: anti-apoptotic (e.g., BCL-2, BCL-xL, BCL-w, MCL-1, BFL-1), multi-domain pro-apoptotic (e.g., BAX, BAK, BOK), and BH3-only pro-apoptotic (e.g., BIM, BID, BAD, PUMA, NOXA, BMF, BIK, HRK, BLK) [80]. BAX and BAK (and, in certain cell types, BOK) are pore-forming proteins and are termed "activators," while BH3-only proteins are generally considered "sensitizers" since they function to sense various apoptotic stimuli (e.g., growth factor withdrawal sensed by BIM and DNA damage sensed by PUMA). The BH3-only proteins are homologous to *C. elegans*' EGL-1 and promote apoptosis when triggered by appropriate stimuli by binding anti-apoptotic BCL-2 family members and

sequestering them to prevent their association with the multi-domain "activators" of apoptosis. Protein–protein interactions within this family are mediated primarily by the BH3 domain [81]. Regulation of mitochondrion-dependent, intrinsic cell death relies heavily on shuttling of BCL-2 family members between different compartments in the cell. Several of the BCL-2 family members contain a carboxy-terminal transmembrane domain that anchors them into the mitochondrial outer membrane (MOM); however, presence of this transmembrane domain does not dictate constitutive localization to mitochondria. In the case of the major pore-forming, pro-apoptotic proteins, BAK remains inserted in the MOM, while BAX is prevented from accumulating in the MOM by the action of BCL-xL and possibly other anti-apoptotic BCL-2 proteins [81]. The pro-apoptotic, BH3-only protein BID, on the other hand, promotes insertion of BAX in the MOM once it is cleaved by caspase 8 to form tBID [81]. Once BAX and BAK oligomerize, a pore (sometimes called the mitochondrial apoptosis-induced channel, MAC) is formed in the MOM, leading to mitochondrial outer membrane permeabilization (MOMP) and release of mitochondrial proteins that trigger downstream intrinsic apoptotic signaling.

3.4.2 Role of Mitochondrial Intermembrane Proteins

Upon MOMP, soluble mitochondrial proteins including cytochrome *c*, second mitochondria-derived activator of caspase (SMAC, also called DIABLO), OMI, and apoptosis-inducing factor (AIF) are released. Cytochrome *c* is a soluble protein normally sequestered in the intermembrane space of mitochondria and functions as an essential component of the electron transport chain. Upon release into the cytoplasm, cytochrome *c* binds to inositol (1,4,5) triphosphate receptor (IP_3R) present on the membrane of the endoplasmic reticulum (ER) and initiates the release of ER calcium stores [82]. The resulting waves of increased cytosolic calcium induce all mitochondria to release cytochrome *c* as a feed-forward mechanism of amplifying apoptotic signaling [82]. Studies of the BH4 domain of BCL-2 have recently revealed its capacity to bind the IP_3R on ER, blocking release of calcium stores and inhibiting pro-apoptotic calcium signaling [83]. Once cytochrome *c* enters the cytoplasm, it nucleates the formation of a structure termed the apoptosome (described in detail below).

SMAC, OMI, and AIF are also pro-apoptotic mitochondrial intermembrane proteins that are released upon MOMP. SMAC and OMI promote apoptosis by inhibiting the IAP proteins discussed in the caspase section above. SMAC inhibits IAP proteins by physical interaction via its amino-terminal region [84], whereas OMI irreversibly cleaves IAP proteins [85]. AIF mediates caspase-independent death when liberated from mitochondria by calpain-mediated cleavage [86]. Once in the cytoplasm, its nuclear localization signal directs AIF to the nucleus where it condenses chromatin and fragments DNA [87].

3.4.3 APAF-1 Apoptosome

Upon its release from the mitochondria, cytochrome *c* binds a soluble protein called apoptotic protease-activating factor 1 (APAF-1). APAF-1, which is the mammalian homolog of *C. elegans*' CED-4 [88], is present in the cytoplasm and forms a heptameric [89], wheel-shaped signaling platform [90] upon binding cytochrome *c* (Fig. 3.3). APAF-1 is able to form this soluble receptor because it is a tripartite protein containing an N-terminal CARD domain, a nucleotide-binding and oligomerization domain (NOD) with ATPase activity, and C-terminal WD40 repeats [88]. Under steady-state, non-apoptotic conditions, APAF-1 is present in the cytoplasm as an autoinhibited monomer bound to dATP. Once cytochrome *c* binds the WD40 repeats of APAF-1, the NOD ATPase hydrolyzes dATP to dADP to allow for an initial conformational change [91]. However, this is not sufficient for apoptosome assembly, and studies have shown that the dADP must be exchanged for dATP to oligomerize APAF-1 [92]. Once oligomerized, the CARD domain of APAF-1 becomes accessible and enables recruitment of pro-caspase 9 through CARD:CARD interactions. As described for the extrinsic pathway above, oligomerization of the pro-caspase zymogen enables proteolytic processing that produces active caspase 9 [93]. The effector caspases 3, 6, and/or 7 are subsequently activated through caspase 9-mediated cleavage to proteolyze cell death substrates.

3.4.4 Cytokine Withdrawal-Induced Death

For T cell biology, the most appreciated inducer of intrinsic apoptosis is cytokine withdrawal. At the conclusion of an immune response to infection, the expanded T cell populations must contract to maintain homeostasis of T cell numbers. This vital contraction is mediated largely by cytokine withdrawal-induced death (CWID) [10, 94]. CWID is primarily understood in the context of IL-2 deprivation but can also occur when other common gamma chain cytokines (e.g., IL-4, IL-7) are abruptly removed from the environment [95]. The pro-apoptotic, BH3-only protein BIM is a key mediator of CWID [96, 97] whose expression is tightly controlled by signals downstream of cytokine receptors. When key cytokines are withdrawn, T cells sense this as growth factor deprivation, resulting in upregulation and stabilization of *bim* mRNA as well as reduced turnover of the BIM protein [98, 99]. Moreover, the pro-apoptotic protein PUMA is also upregulated upon cytokine withdrawal and functions synergistically with BIM, though it can mediate BIM-independent CWID under certain conditions [100]. Finally, degradation of the anti-apoptotic MCL-1 protein also contributes to cell death induced by withdrawal of growth cytokines [101]. These events collectively result in a shift in the balance between anti- and pro-apoptotic BCL-2 family members, tilting in favor of cell death through MOMP and apoptosome formation. In patients with defective CWID, abnormally high numbers of lymphocytes accumulate as a result of impaired T cell contraction (Fig. 3.4) [102].

3.5 Diseases of Failed Lymphocyte Apoptosis

The importance of homeostatic control of lymphocyte cell numbers in health and disease has become evident through identification and characterization of patients with failed lymphocyte apoptosis (Fig. 3.4).

3.5.1 Autoimmune Lymphoproliferative Syndrome

Autoimmune lymphoproliferative syndrome (ALPS) is a clinical condition in which RICD fails to occur due to mutations in genes critical for homeostatic control of T lymphocytes through FAS receptor signaling. An enigmatic population of mature double-negative ($CD4^{-}CD8^{-}$) T (DNT) cells accumulates in ALPS patients and is used as a diagnostic indicator [103], though the nature of the DNT cells is still far from completely understood. Massive accumulation of T cells in the lymph nodes and spleen occurs due to disruption of the canonical FAS pathway [104] and results in autoimmune cytopenias. Mutations in FAS, FASL, and caspase 10 are responsible for the large majority of ALPS disease cases [103]. Mutations in the FAS receptor have been found that can be classified as homozygous, heterozygous, or somatic, where heterozygous, dominant-interfering mutations are most common followed by heterozygous somatic (acquired instead of inherited) mutations [103]. Notably, in somatic ALPS, mutations in the FAS gene are found only in the DNT cells, emphasizing their connection with disease pathogenesis. Dominant interference of FAS signaling by the presence of mutated FAS within a pool of wild-type FAS is caused by poisoning of the trimeric receptor complex with a signaling-incompetent chain [38]. The small subset of patients with an ALPS-type presentation who have been screened for but lack mutations in known disease genes are categorized as ALPS-unknown patients. There are also family members of affected ALPS patients who carry a mutation in the disease-causing gene and have in vitro defects in RICD but do not display clinical manifestations of ALPS. These individuals are referred to as healthy mutation-positive relatives (HMPR), and their resistance to disease highlights the importance of genetic modifiers and background genes in disease penetration [105].

Other unique diseases related to ALPS have also been discovered. Patients with mutated caspase 8 have a distinct disease termed caspase eight deficiency state (CEDS), which is characterized by immunodeficiency, lymphoaccumulation, and autoimmunity [28] due to the role of caspase 8 in non-apoptotic signaling pathways. Another subcategory of ALPS-like disease, RAS-associated ALPS-like disease (RALD), is caused by somatic, activating mutations in NRAS [102] or KRAS [106, 107] in hematopoietic cells. RALD patients exhibit defective CWID [102] and RICD [76] due to reduced levels of the pro-apoptotic BIM protein caused by exuberant protein degradation and mRNA instability caused by RAS signaling. Interestingly, the same genes mutated in RALD are also mutated in a subpopulation

of pediatric patients with the severe myelodysplastic/myeloproliferative disease juvenile myelomonocytic leukemia (JMML) and a subpopulation (approximately 35 %) of adult patients with chronic myelomonocytic leukemia (CMML) [108]. However, the expanded monocyte population in these cancers is monoclonal, while in RALD the accumulating cells carrying the mutated NRAS or KRAS are of multiple different hematopoietic lineages and, therefore, likely occurred in an early progenitor cell that has not yet undergone malignant transformation [108].

3.5.2 X-Linked Lymphoproliferative Disease-1

A more rare lymphoproliferative disease of failed lymphocyte apoptosis is X-linked lymphoproliferative disease-1 (XLP1), which is caused by deficiency of the SH2-containing adapter protein (SH2D1A/SAP) [109]. XLP1 patients are immunodeficient and exhibit spontaneous lymphoproliferation and fulminant, often fatal, infectious mononucleosis after infection by the B cell-tropic Epstein–Barr virus (EBV). EBV infection in XLP1 patients is also often associated with secondary hemophagocytic lymphohistiocytosis (HLH) in which a pro-inflammatory state induces macrophage expansion and phagocytosis of other blood cells. The SAP adaptor binds to SLAM family members as well as TCR signaling components [110], making it an important player in the adhesion and signaling events required for robust T cell activation [111]. These defects have recently been found to be the cause for lethal CD8 T cell accumulation in response to EBV infection since T cells from SAP-deficient XLP1 patients fail to form robust T cell:B cell conjugates or transduce a strong enough TCR signal to pass the threshold required for RICD [112].

3.6 Concluding Remarks

Programmed cell death is a critical regulator of T cell homeostasis that is required to prevent pathological accumulation of lymphocytes. The study of genetic immunological diseases has yielded fascinating insight into the basic biology of programmed cell death. Moreover, the study of human pathways has revealed clear differences and additional complexity compared with *C. elegans* or even other mammals. The continued exploration of lymphocyte homeostasis through the lens of human genetics promises to yield additional medically important insights.

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Chapter 4
Bcl-2 Family and Their Therapeutic Potential

Qian Liu, Xiaoke Chi, Brian Leber, and David W. Andrews

Abstract Bcl-2 family proteins play a pivotal role in regulating programmed cell death that is essential for cell homeostasis in multicellular organisms. Tumors develop when cells bypass regulated cell death and grow faster than they die, while in degenerative diseases there is too much cell death. Thus understanding how Bcl-2 proteins modulate cell survival will provide novel important insight into therapeutic targets, as evidenced by recent successes specifically targeting and killing cancer cells. Here we describe the current models for regulation of apoptosis by Bcl-2 family proteins with a specific emphasis on the predicted therapeutic targets. Limited success of current efforts in targeting Bcl-2 family proteins including antisense oligonucleotides and small-molecule inhibitors suggests that emerging techniques and strategies will be required to identify a new generation of drugs targeting Bcl-2 family proteins. We review some of the more promising therapeutic opportunities for treating diseases that have been revealed by our current mechanistic understanding of these proteins.

Keywords Bcl-2 family • Apoptosis • Cancer • Mitochondria • ER • Small-molecule inhibitors • Membrane

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4.1 Introduction and Overview

Bcl-2 family proteins interact with each other to determine cellular fate through programmed cell death. They do this by regulating mechanical permeabilization of the outer mitochondrial membrane. Because the process depends on direct physical interactions and mechanical changes its regulation is significantly different than other signal transduction or metabolic pathways. As defects in the regulation of cell death have been associated with serious diseases, the Bcl-2 family constitutes an important therapeutic target, especially for cancer. In this chapter we discuss how Bcl-2 family proteins regulate cell death as these mechanistic details provide the rationale for new drug development for the treatment of cancer and other diseases. Current progress in the development and testing of such agents and novel methods to guide screening for the next generation of pharmaceuticals that target the Bcl-2 family are also described. The implications of our current understanding for future drug discovery in diseases other than cancer are also discussed.

4.2 Bcl-2 Family, Cell Death, and Diseases

4.2.1 Apoptosis and Cancer

In multicellular organisms, cell number is regulated by a highly integrated network of control mechanisms. Aside from adding new cells when required during development and repair, the appropriate eradication of dysfunctional or redundant cells is also essential, as these may pose a risk to the collective. Bcl-2 family proteins play a key role in this "self-removal" by regulating several complex processes including autophagy, necrosis, and apoptosis [1]. Of these the functions of Bcl-2 family proteins in apoptosis have been the most extensively studied.

Excessive apoptosis leads to neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's diseases and immunologic disorders like AIDS, while insufficient apoptosis allows cells to survive inappropriately and results in cancer and autoimmune disorders [2]. Cancer remains a global threat to health and longevity as even with the currently available treatments including surgery, chemotherapy, radiation therapy, immunotherapy, and monoclonal antibody therapy there were an estimated 12.7 million cancer cases and 7.6 million cancer deaths in 2008 [3]. Furthermore, even curative therapy can be quite toxic. As cancer cells divide and grow in an unregulated fashion, determining how they bypass cell death might suggest new ways to treat cancer. Understanding the steps in apoptosis and how they are regulated is a key first step to this goal.

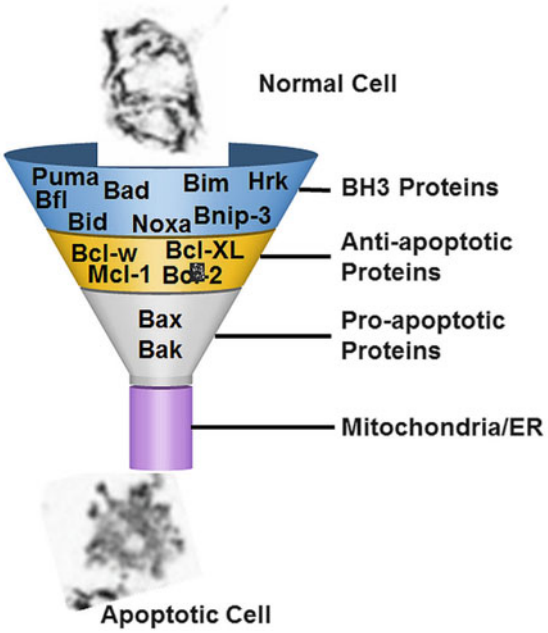

Fig. 4.1 Bcl-2 proteins regulate programmed cell death

4.2.2 Bcl-2 Family in Apoptosis

In broad terms apoptosis can be triggered by either of the two signaling pathways: an extrinsic pathway that responds to extracellular ligands of death receptors located on the plasma membrane and an intrinsic pathway. This latter pathway is elicited by a variety of stressors including growth factor withdrawal [4], DNA damage [5], and unfolded protein stress [6]. Detachment of cells from the extracellular matrix induces a form of cell death called anoikis that is similar to the intrinsic pathway but has several unique features not discussed here [7]. Bcl-2 family proteins regulate apoptosis through all of these pathways but is crucial for the intrinsic pathway, which is also referred to as the mitochondrial pathway. As a key step in this process Bcl-2 family proteins promote or prevent in a hierarchical fashion the mitochondrial outer membrane permeabilization (MOMP) that commits a cell to die. In cells undergoing apoptosis, MOMP mediates the release of factors such as cytochrome *c* and SMAC from the inter-membrane space to the cytoplasm [8, 9], where they activate or prevent the inhibition of a series of proteases called caspases that ultimately cleave various substrates in the cell leading to death (Fig. 4.1). It is at this step of activation of the final "executioner" caspases that the intrinsic and extrinsic

pathways converge [10] with MOMP amplifying cell death signaling by the extrinsic pathway. For most cell types MOMP can be considered the point of no return in cell death. Thus, understanding how Bcl-2 family proteins regulate MOMP is crucial for finding new therapeutic targets for the treatment of cancer and other diseases.

4.2.3 Bcl-2 Family and Cancer

The importance of the dysregulation of apoptosis and the role of Bcl-2 family proteins was initially recognized two decades ago with the realization that they are involved in the developing stage of many cancers [11]. This led to the observation that deregulation of Bcl-2 family proteins is a critical feature that makes cancer cells distinguishable from normal cells [12]. More recent analyses investigating copy number variations across many cancer genomes (including both cell lines and primary clinical samples) confirmed this result as amplified expression of anti-apoptotic members such as Bcl-2 and Mcl-1 were among the most common abnormalities noted [13]. Aside from the role in generating cancer, anti-apoptotic Bcl-2 members are a major contributor to the acquisition of resistance to cancer treatment including chemotherapy and radiotherapy [14]. Recently, it has been proposed that some cancer cells are "addicted" to the presence of high levels of anti-apoptotic Bcl-2 proteins for tumor maintenance and survival independent of treatment [15, 16], a phenomenon that is not observed in normal cells. For example, chronic lymphocytic leukemia (CLL) cells are addicted to expression of Bcl-2 that is largely bound to the activator Bim whose function is discussed below [17]. These findings suggest that by targeting Bcl-2 family member interactions it may be possible to selectively kill cancer cells either as a monotherapy or synergistically by combination with standard chemotherapy.

4.3 Bcl-2 Family Portrait

The gene *bcl-2* was discovered as a translocation partner into the immunoglobulin heavy-chain gene locus in the t(14;18) chromosomal translocations found in B cell non-Hodgkin lymphoma in 1985 [18]. As an unusual oncogene that did not directly promote cell growth the oncogenic potential of *bcl-2* was only demonstrated in 1988 [19] and then identified as an anti-apoptotic activity shortly thereafter [20]. The expansion of the Bcl-2 family to include both pro- and anti-apoptotic relatives started in the early 1990s [21]. Since then a large and growing number of Bcl-2 family members have been identified by biochemical, genetic, and molecular techniques, and while their individual functions have been characterized there is still much to learn about how they function in concert.

The Bcl-2 family is defined by amino acid sequences known as Bcl-2 homology (BH) regions [22, 23]. According to the protein functions and the number of regions

contained, relatives of Bcl-2 are classified into three groups (Fig. 4.1): The anti-apoptotic proteins (Bcl-2, Bcl-w, Bcl-XL, Mcl-1, and A1) share up to four BH regions named BH1–4 and prevent cells from entering apoptosis. The pro-apoptotic effectors (Bax and Bak) contain three regions, BH1–3, and promote cell death by oligomerization and forming pores in membranes that mediate MOMP. The third group of BH3-only proteins shares only one region (BH3) and includes Bid, Bim, Bad, Bmf, Bik, Puma, Noxa, Hrk, Blk, Nip3, bNip3, Mule, and likely other proteins. Many of these proteins appear to be largely unstructured. However, it should be noted that at least Bid may be an outlier in the group as it has structural features found in both the multidomain pro- and anti-apoptotic members.

In response to a variety of different cellular stress signals, BH3-only proteins become "activated" through different mechanisms and in turn directly or indirectly activate Bax/Bak by inducing conformational changes and oligomerization leading to MOMP [24, 25]. It is also clear that anti-apoptotic proteins can bind to either BH3-only proteins, or Bax/Bak, to prevent cell death. Thus the functional convergence point for the family is the activation of Bax/Bak that mediates MOMP and commits the cells to apoptosis [26]. Together the three groups of Bcl-2 family proteins form a complex network that regulates cell death by controlling the integrity of the mitochondrial outer membrane (MOM) [27].

The multidomain Bcl-2 family members, both pro- and anti-apoptotic, share a "signature" fold composed of an alpha helical bundle formed by seven or eight helices surrounding a central helical hairpin. The different sizes of a surface-accessible hydrophobic groove formed by helix α2 to α5, α7 and α8, partially explain the relationship between structure and function of Bcl-2 proteins. In Bcl-XL [28], Mcl-1 [29], and Bcl-2 [30] the grooves act as "ligand receptors" for the BH3 region of other family members as determined by NMR spectroscopy or X-ray crystallography experiments demonstrating that the BH3-peptides derived from pro-apoptotic proteins bind to this hydrophobic groove [31–34]. Mutagenesis studies indicate that this binding is required for anti-apoptotic activity. In at least Bcl-w [35, 36] and Bax [37], the groove is blocked by an extra C-terminal helix, which needs to be released for activation to occur. For Bid [38, 39] and Bak [40], the groove is much shallower and narrower compared to other Bcl-2 members. As a result cytoplasmic proteins and fragments of Bid and Bak do not stably bind ligands. However these putative binding surfaces are likely to be dynamically regulated in certain circumstances as discussed below. It is the exact structure of the groove that is believed to be the major contributor to the selectivity of protein–protein interactions that regulate MOMP.

The main cellular localizations of Bcl-2 members include the cytoplasm as well as the cytoplasmic face of mitochondria and endoplasmic reticulum (ER). It has become evident that dynamic interactions with specific intracellular membranes are crucial for the function of these proteins. The anti-apoptotic proteins and multi-region pro-apoptotic proteins Bax and Bak contain a hydrophobic membrane-binding sequence at the carboxyl-terminus. Because of this similarity and direct biochemical evidence they are classified as belonging to a large group of tail-anchored membrane proteins. The membrane-binding region of tail-anchored

proteins typically spans the membrane and therefore is usually referred to as the transmembrane (TM) region of the protein. The TM region not only helps the protein to anchor into either the MOM or the ER by spanning the membrane in a α-helical conformation [41] but also plays a major role in directing the protein into its specific cellular destination [42, 43]. Within this scheme there are notable variations found for Bcl-2 family proteins: Similar to conventional tail-anchored proteins Bak is found exclusively membrane anchored in MOM and, to a lesser extent, the ER membrane. In contrast, Bax, Bcl-XL, and perhaps Mcl-1 are normally found in the cytoplasm as soluble proteins in equilibrium with a peripherally membrane-bound form. These proteins only insert into mitochondria or ER when in response to a signal during apoptosis they undergo a conformational change that exposes the TM region [44]. Similarly, some of the BH3-only proteins, including Bim, Noxa, Bik, and Hrk (and possibly Puma), contain tail-anchored regions at their C-termini, which appear to function in regulated targeting to membranes. Normally these proteins are either not expressed or sequestered elsewhere in healthy cells and require transcriptional or posttranslational events in order to migrate to their membrane destination [45–51].

The amount and activity of individual Bcl-2 family proteins is regulated at multiple levels. The transcription of genes encoding anti-apoptotic proteins is increased by a variety of cellular and extracellular factors such as STAT, AP-1, interleukins, interferon [52, 53], and other stress stimuli [54]. By contrast, the tumor-suppressor p53 acts as a transcriptional activator of many pro-apoptotic members [45, 46, 55–57]. At the posttranscriptional level, alternative splicing of a primary transcript is involved in regulating almost every Bcl-2 family member. Splicing regulates the gene products in different cell types by determining both the presence and absence of a TM region that affects subcellular localization and by varying the combinations of the BH regions which are usually encoded in separate exons [45, 58–68]. Posttranslationally, the Bcl-2 family proteins are regulated through phosphorylation [69–73], proteolytic cleavage [74–76], and ubiquitin-dependent degradation [77, 78] and other modifications. The targets of these modifications are located in several critical regions of the proteins including the unstructured loop region (ULR, between the first and second helices), BH3 region, TM region, and other member-specific regions. The combination and integration of all these regulatory mechanisms allow either large or minor modulations of protein levels and localizations in different cell contexts, thus mediating physiological activities.

Knockout studies have demonstrated that Bcl-2 members have specific physiological roles in vivo. Bcl-XL regulates the survival of erythroid progenitors and neuronal cells. Its deficiency in mice leads to embryonic lethality and excessive cell death of immature lymphocytes and neurons [79], while over-expression correlates with resistance to chemotherapeutic agents [80]. Bcl-2 is necessary for the survival of mature T and B lymphocytes. Its loss results in growth retardation, loss of hair pigmentation, renal failure, and apoptosis of lymphocytes [81], and its over-expression correlates with poor survival of animals with tumors [82]. Mcl-1 not only plays an important role in the implantation of the developing embryo [83] but also is essential for both the development and maintenance of lymphocytes [84] and the hematopoietic system [85]. Over-expression of Mcl-1 is frequently seen in

hematopoietic and lymphoid cancers such as multiple myeloma and chronic lymphocytic leukemia [86, 87]. Bcl-w regulates spermatogenesis, and its ablation leads to male infertility [88]. It has also been reported that Bcl-w may play an important protective role in neurons in the diseased brain [89] and that overexpression promotes the growth of non-small-cell lung cancers [90]. A1 is involved in inhibition of certain types of neutrophil apoptosis [91], and high-level expression in mice perturbs late-stage B lymphocyte function [92].

The multi-region pro-apoptotic proteins Bax and Bak also display critical functions. The absence of Bax leads to inappropriate survival of neuronal cells during development [93], altered homeostasis of lymphoid and reproductive organs [94], and tumor promotion [95–97]. Its expression level is increased by stresses such as DNA damage and ischemia–reperfusion injury [98]. The phenotypic abnormalities caused by the depletion of Bak are subtle as *bak*$^{-/-}$ mice develop normally without any age-related disorders. However, bax$^{-/-}$bak$^{-/-}$ animals displayed multiple developmental defects in several tissues beyond those seen in the absence of Bax alone. These cells show profound resistance to multiple apoptotic stimuli including staurosporine, ultraviolet radiation, growth factor deprivation, and etoposide [26, 99], suggesting that these two proteins have functionally overlapping but not entirely redundant roles.

Most studies of the physiological functions of BH3-only proteins have focused on a single member. Bid, which serves as a bridge between the intrinsic and extrinsic apoptotic pathways, is necessary for caspase-2-induced apoptosis [100]. At the enzymatic level Bid has been reported to facilitate lipid transfer activity between mitochondria membranes [101]. At the organ level its presence is required for myeloid homeostasis and tumor suppression [102]. The protein also mediates the demise of nerve cells in cerebral ischemia [103]. In the nucleus Bid is involved in the DNA damage response [104].

While there have been fewer studies of other BH3 proteins, accumulating evidence suggests a wide range of functions for these proteins. Bim has been reported to have essential functions in embryogenesis, the control of hematopoietic cell death, and the fate of anergic B cells and also acts as a barrier against autoimmunity [105, 106]. Its ablation in mice results in increased numbers of lymphocytes, plasma cells, and myeloid cells and facilitates the development of fatal autoimmune glomerulonephritis [107]. Puma functions as a tumor suppressor and is required for hematopoietic cell death triggered by ionizing radiation (IR), deregulated c-Myc expression, and cytokine withdrawal [108, 109]. It is also critical in cytokine deprivation-induced apoptosis of mast cells [109] and necessary and sufficient for neuronal apoptosis induced by DNA damage in sympathetic neurons [110]. Together with another p53 transcriptionally activated BH3 protein, Noxa [111, 112], Puma is also involved with regulating neural precursor cell death. Finally, depletion of Bmf impairs B cell homeostasis and accelerates gamma irradiation-induced thymic lymphoma development [113].

Taken together these studies indicate that the various Bcl-2 family proteins are assigned specific physiologic as well as cell-specific responsibilities. Many (but not all) of these functions have been revealed because alteration of individual family members results in dysregulated “baseline” apoptosis. However, understanding how

Bcl-2 family proteins fulfill their duties as a goal to improve therapeutics requires an understanding of their molecular mechanism of action and the rules governing how the various family members interact.

4.4 Regulation of Apoptosis by Bcl-2 Family Proteins

The observation that Bcl-2 blocks the activation of caspases by interacting with Bax led to the original "rheostat" model of Bcl-2 regulation in which anti- and pro-apoptotic proteins bind to each other to neutralize Bax-mediated potentiation of apoptosis. According to this model the relative ratio of pro- and anti-apoptotic proteins determines the cell fate [21, 114]. This original model provided the foundation of our current understanding of apoptotic regulation but fails to explain the binding specificity of the subsequently described Bcl-2 family members. Accumulating evidence pointed to hierarchical roles of BH3-only proteins in initiating apoptosis and the requirement of Bax/Bak for MOMP (Fig. 4.1).

To account for the observation that individual cells often express multiple Bcl-2 family proteins ascribed similar functions, two competing schemes were proposed: the derepression model and the direct-activation model (Table 4.1).

4.4.1 Derepression Model

The derepression model assumes that in both healthy and diseased cells Bax and Bak are constitutively active and sufficient to mediate MOMP unless bound to and inactivated by anti-apoptotic Bcl-2 family members. Therefore it was postulated that the pro-apoptotic function of BH3-only proteins occurs indirectly as they function solely by releasing Bax/Bak from their anti-apoptotic partners. This occurs through the BH3-only proteins competitively binding to the pocket of the anti-apoptotic proteins and thus without direct interactions between the BH3-only proteins and Bax/Bak. Once sufficient Bax/Bak are released, apoptosis occurs [115–117].

This model is based on two sets of observations: the selective interactions between the anti-apoptotic proteins and Bax/Bak and the differential binding affinities between anti-apoptotic proteins and BH3 peptides derived from BH3-only proteins. The former observation was based on the initial identification of Bax as a Bcl-2-binding protein. Binding specificity was inferred from the fact that Bak was found sequestered by Bcl-XL and Mcl-1 but not by Bcl-2 [21, 116, 118]. However, these binding interactions were observed by immune- and co-precipitation of cell lysates in the presence of detergents such as Triton X-100 and CHAPS. Relatively quickly it was revealed that nonionic detergents such as Triton X-100 artifactually promote interactions between Bcl-2 family proteins. Only much later was it discovered that CHAPS disrupts authentic interactions between Bcl-2 members [119].

Table 4.1 Comparison between regulatory models by Bcl-2 family proteins

	Models			
MOMP players	De-repression model	Direct-activation model	Embedded together model	Unified model
Bax/bak	Constitutively active	Activated by BH3-only activators	Activated by BH3-only and other activators	Activated by BH3-only activators
Anti-apoptotic proteins	Sequestering active bax/bak	Sequestering BH3-only proteins	Mutual sequestering of anti-apoptotic proteins with BH3-only proteins and bax/bak	Sequestering both BH3-only activator (Mode-1) and activated bax/bak (Mode-2)
BH3-only proteins	Inhibiting anti-apoptotic proteins	Activators: activating bax/bak	Activators: activating bax/bak	Activators: activating bax/bak
		Sensitizers: displacing activators from anti-apoptotic proteins	Sensitizers: inhibiting anti-apoptotic proteins by mutual sequestration	Sensitizers: derepress the inhibition from anti-apoptotic proteins more effectively in Mode-1 than Mode-2
Membrane	Passive recipient of Bcl-2 members	Passive recipient of Bcl-2 members	Interaction induces conformational and functional changes in Bcl-2 family proteins.	Not emphasized
Other important features			The interactions are reversible equilibria determined by local concentrations of proteins	Links mitochondrial dynamics and apoptotic regulation

It was also argued that these specific interactions are not constitutive in cells as only a small fraction of Bax/Bak can be co-immunoprecipitated with anti-apoptotic proteins [120, 121]. Thus it was suggested that in cells binding of anti-apoptotic members to Bax/Bak might require other factors.

The combinatorial specificity of BH3-only proteins for specific anti-apoptotic proteins is an important observation that underpins this model and is well substantiated by observations showing that BH3 peptides derived from BH3-only proteins have different binding affinities for anti-apoptotic members, varying from nanomolar affinity to undetectable. Bid, Bim, and Puma potently engage all the anti-apoptotic members; Bad only binds Bcl-2, Bcl-XL, and Bcl-w, but not A1 and Mcl-1; and NOXA is a specific antagonist of Mcl-1 and A1 [115]. However, there is a question of whether experiments with BH3 peptides and in vitro binding experiments appropriately substitute for the full-length BH3-only proteins in live cells. This has physiologic relevance as in vivo the anti-apoptotic members and activated BH3-only proteins functionally interact in a membrane environment but have rarely been shown to interact with each other in solution [122].

4.4.2 Direct-Activation Model

The direct-activation model postulates that BH3-only proteins can directly bind and activate Bax/Bak and that the role of anti-apoptotic proteins is solely to sequester the BH3-only members to prevent this activation. Since only Bim, tBid (the truncated active form of Bid), and possibly Puma have been reported to directly activate Bax/Bak [123–126], they are called "activators," whereas the remainder of the BH3-only members are classified as "sensitizers." In this model, sensitizers work indirectly by competing with "activators" for binding to anti-apoptotic proteins, thereby releasing the activators to activate Bax/Bak and induce apoptosis [15].

The direct-activation model introduced two sets of competitive interactions. One is the competition for "activators" between anti-apoptotic proteins and Bax/Bak, and the other is between the "activators" and "sensitizers" that compete for anti-apoptotic proteins. Thus this model broke the conventional concept of "neutralization." However the model requires direct interaction of Bax/Bak with "activators," an interaction that proved notoriously difficult to demonstrate unambiguously. However, experiments with Bak-deficient mitochondria and blocking antibodies first demonstrated that tBid could bind Bak to trigger MOMP and release cytochrome *c* [124]. Furthermore by using liposomes as a model membrane, it was shown that only Bid and Bim (using either the BH3-only proteins or BH3 peptides) could directly induce the activating conformational change of Bax and Bak, indicating that the division of BH3-only proteins into those that work via Bax/Bak (activators) and those that work by inhibiting anti-apoptotic Bcl-2 proteins (sensitizers) was valid [119, 123, 126, 127]. In addition to this functional evidence, structural support for a BH3-only protein binding to Bax was contributed by the observation that a stapled Bid/Bim BH3 peptide (stapling is a chemical modification that stabilizes the peptide in a helical state) bound directly to Bax in solution. Unexpectedly

this binding occurred through a novel site composed of helix α1, α6, and the loop between α1 and α2 [128, 129]. However, as with previous experiments it remains unclear whether stapled peptides faithfully recapitulate binding by the full-length protein, and whether this interaction occurs in cells remains unknown.

Less evidence for direct binding of Bak to BH3-only proteins has accumulated because Bak is inserted constitutively into the MOM which makes it hard to purify and study. Thus the activation of Bak might involve different mechanisms from those proposed for Bax. At a minimum regulation of Bak does not require migration of the protein from cytoplasm to mitochondria. On the other hand both Bax and Bak have been observed to undergo autoactivation [130, 131] and cross-activation [132, 133], i.e., activated Bax can then activate Bak and vice versa. According to the direct activation model, this should only occur when all the relevant anti-apoptotic proteins have been occupied by BH3-only activators or sensitizers. However, it was not supported by experiments showing that Bcl-2 can directly inhibit autoactivation of Bax/Bak [130, 131].

4.4.3 Embedded Together Model

Neither the derepression model nor the direct-activation model fit all the relevant observations. And in different conditions (including cell types, cell stages, stimuli, signaling) one model explains findings better than the other [15, 117, 134, 135]. Using a simplified in vitro system containing full-length proteins and liposomal membranes further data was generated suggesting the need for an alternative model. The recognition that the membrane was not just a passive recipient of Bcl-2 family proteins but induces structural alterations with functional consequences led to the elaboration of a third model called embedded together (Table 4.1).

In this model, each Bcl-2 member has different conformations when on- and off-membranes and the interactions with membranes and between Bcl-2 family proteins are tightly regulated by multiple parallel equilibria. The BH3 activators can recruit not only anti-apoptotic proteins but also Bax/Bak to the membrane and activate them by changing their conformations. Regulation by mutual sequestration relegates the terms activator and inhibitor to semantics. Once in the membrane, the anti-apoptotic proteins sequester BH3-only activators to prevent them from activating Bax/Bak; meanwhile, they compete with already activated Bax/Bak to prevent its further oligomerization. Sensitizer BH3-only proteins may also displace both activators and activated Bax/Bak from anti-apoptotic proteins to promote apoptosis. In this model, the activation of Bax/Bak at the membrane is the rate-limiting step in MOMP [136–138].

The embedded together model integrates the previously existing two models and extends them to include multiple reversible equilibria and a dynamic role for the membrane. It reinforces that many of the functional interactions between the Bcl-2 proteins only occur in membranes upon unique conformational changes. For example, independent interactions between tBid and either Bcl-XL or Bax were detected only when membranes were present [119]. Thus it is important to measure the rates

and extent of these interactions in the right context. Second, it proposes that anti-apoptotic proteins can also be "activated" in the sense that their function changes upon recruitment to membranes by Bcl-2 family proteins including themselves. Upon the direct binding of BH3 activators, Bcl-2 inserts at least part of α-helices 5 and 6 into membranes, a conformation change critical for it to inhibit Bax oligomerization [139, 140]. Also the BH3 sensitizers such as Bad can recruit anti-apoptotic proteins like Bcl-XL into membranes whereupon mutual sequestration inhibits the function of both proteins determined by the binding equilibrium between them [141]. Third, it specifies how anti-apoptotic proteins inhibit Bax/Bak-mediated MOMP. By observing that Bcl-XL has multiple mechanisms of inhibiting Bax including by competitively inhibiting the tBid:Bax interaction in a simplified liposome system and mitochondria assays, we first proposed that one mechanism by which Bcl-XL inhibits Bax oligomerization is by keeping Bax off of the MOM [142]. This observation was later verified in live cells using FLIP–FRAP [143]. Further Bcl-XL acts as a dominant-negative Bax in the membrane permeabilization process through competing with Bax for binding to tBid, competing with cytoplasmic Bax for membrane-bound active Bax, and preventing a membrane-induced reversible Bax conformational change [142]. Finally, the embedded together model describes a series of ordered events for how tBid triggers Bax activation: tBid binds to membranes rapidly, which allows it to interact with Bax; based on this direct binding Bax undergoes reversible conformational changes and inserts into membranes; there Bax oligomerizes and permeabilizes membranes [119]. Overall, the embedded together model fits most of the data in the field and anticipates many new components in the mechanisms by which Bcl-2 family proteins regulate apoptosis.

4.4.4 Unified Model

When the concept that anti-apoptotic proteins prevent apoptosis by inhibiting both BH3-only activators and Bax/Bak became more widely accepted, additional efforts were made to distinguish and partition these two types of inhibition. By defining the sequestration of activators by the anti-apoptotic proteins as Mode1 and the inhibition of Bax/Bak by anti-apoptotic proteins as Mode2, either mode could be invoked alone to prevent MOMP. This led to a proposal for a unified model in which Mode1 appears to be less effective compared to Mode2 (Table 4.1). In addition, Mode2 prevents mitochondrial fusion, promotes mitochondrial fragmentation, and affects the kinetics of MOMP. The two modes display distinct physiological consequences regarding the mitochondria morphology. In cancer cells addicted Bcl-2 family proteins or sensitized to stress by the effects of oncogenic transformation on pro-apoptotic Bcl-2 proteins (coined "primed cells" by the Letai group) may affect the predominance of one mode over the other. It is also possible that predominance of modes can be greatly affected by cell types and different treatments [144]. This model for the first time formalized the relationship between mitochondrial dynamics and apoptotic regulation.

4.4.5 *Unresolved Issues in the Regulation of Apoptosis*

One critical aspect of apoptosis regulation that is almost universally assumed (with rare dissenting voices [145, 146]) is the importance of Bax/Bak oligomerization in mediating MOMP. Thus detailed understanding of the mechanism of this process is of paramount importance, and recently suggestive data points to several alternative mechanisms.

It has been proposed that Bax/Bak oligomerization requires the formation of symmetric homodimers and occurs in the following sequence: A widely acknowledged conformation change in the N-terminus of Bax starts the process (and results in exposure of an epitope recognized by the 6A7 monoclonal antibody in Bax). Presumably a similar process occurs for Bak. This is rapidly followed by the exposure of the BH3 region that is then buried in the hydrophobic groove of a neighboring Bax or Bak monomer. The resulting homodimer is linked by reciprocal BH3:groove interactions and can then bind to other cognate homodimers through an α6:α6 interface. This new interface allows oligomerization of homodimers (presumably always in multiples of two) generating a structure that can permeabilize the MOM. The same BH3:groove hetero-dimer was observed for Bax:Bak and suggested that Bax/Bak cross-activation occurs through a similar mechanism [147–149].

This model was recently supported by observations made by two other groups. Functional evidence comes from knock-in studies using specific Bax mutants. The results indicated that activation of endogenous Bax in HCT116 cells is impeded by mutations in the α6:α6 homo-oligomerization sites [150]. Structural evidence comes from crystal studies that provide snapshots of the different stages of Bax homodimerization. This data suggested that binding of the Bid-BH3 peptide causes Bax to release a "latch" (α6, α7, and α8) from its core helical structure (α2α5) through BH3:groove interaction. This latch is further displaced by another activated Bax and results in the formation of a symmetric Bax homodimer with BH3:groove swapping [151]. As the definition of latch/core is not consistent with the original model that Bax inserts into MOM by its α5 and α6 hairpin [152], it is not clear whether this crystal structure-based model reflects what happens in cells. It seems most likely that it results from a stage in the unfolding process prior to insertion into the lipid bilayer that in the absence of a membrane generates a dead-end complex. In this context other domain swapping (α5 and α6)-derived homodimers previously reported for anti-apoptotic proteins such as Bcl-XL [153–155] and Bcl-2 [156] are now considered crystallization artifacts [155].

Structural methods including NMR spectroscopy were used to explore the process of Bax activation. The stapled Bid/Bim BH3 peptide has improved helicity so that it can directly bind Bax in solution, which involves a different interface in Bax from that usually proposed. This novel interface comprises helices α1, α6, and the loop between α1 and α2 [128, 129]. After BH3 binding, Bax undergoes a series of conformational changes in which the unstructured loop region between the first two helices is displaced, the TM region is mobilized for membrane translocation,

and the BH3 region is exposed to propagate the death signal. Again whether the stapled peptide accurately models the full-length protein in vivo remains unclear. Nevertheless, understanding how this complex transitions into the membrane is critical for future therapeutic exploitation with small molecules.

Comparing these two models, both agree that an activated Bax or Bak molecule must bind with two interfaces to oligomerize: we term the BH3 and the groove side the "front" and the α6 side the "rear". Thus the difference between the models is whether Bax oligomerizes by binding "front:rear:front:rear" or "rear:front:front:rear" etc. Both require further elucidation of the conformation of activated Bax, which requires using full-length proteins and membranes. Solution studies particularly those using a misleading membrane mimic detergent like CHAPS are unlikely to be helpful for reasons mentioned above. Furthermore, because of the different locations of "unactivated" Bax and Bak, it is still formally possible that different mechanisms are used to propagate oligomers of the two proteins. Resolution of these issues will provide important insights for discovering small-molecule agonists or antagonists of Bax/Bak activation. Efficient agonists of Bax/Bak activation are critical for testing a prevailing theory that activation of pro-apoptotic proteins will be better tolerated by normal cells than cancer cells due to an unused anti-apoptotic reserve in normal cells.

4.5 Targeting Bcl-2 Family for Cancer Therapy

The tremendous progress in understanding how the Bcl-2 family controls apoptosis reviewed above has helped delineate effective strategies to target Bcl-2 family in cancer cells. For example the regulatory mechanisms indicate potential therapeutic benefit in targeting the anti-apoptotic proteins including Bcl-XL, Bcl-2, and Mcl-1 that has now been realized. Structural information directed the design of the drugs that mimic a BH3 region from a BH3 sensitizer (e.g., ABT-737 and ABT = 263). Meanwhile understanding the global regulation of apoptosis by the Bcl-2 family proteins in cancer cells did not only shed light on why targeting the Bcl-2 family may selectively kill cancer cells. Importantly and yet to enter large clinical trials is testing chemical modulators of Bcl-2 family proteins as drugs to selectively enhance the toxicity of conventional chemotherapy.

The concept of the "addiction" of certain types of malignancies to specific anti-apoptotic proteins [17, 157] helped pinpoint what should be attacked—i.e., initiating stalled MOMP in cancer cells requires removal of the "blocks" in apoptosis [158]. Using the embedded together model as a guide, there are several possible ways that MOMP can be blocked [159]: In the case of relative insufficiency of the BH3-only proteins, the anti-apoptotic proteins sequester all the available BH3 activators so that Bax/Bak are not activated and no MOMP ensues. The use of BH3 mimetics of sensitizers here will displace the BH3 activator bound by anti-apoptotic members, effectively increasing the amount of free activator capable of

turning on Bax/Bak. In cells with high levels of Bax/Bak but low levels of functional activator proteins a Bax activator may provide selectivity. However if the levels of Bax/Bak are very low with respect to the anti-apoptotic proteins (or as in Jurkat cells absent due to gene mutation), BH3 mimetics will be ineffective. By contrast in cases with anti-apoptotic proteins highly over-expressed (e.g., due to chromosomal translocation or genetic deletions involving negative regulatory miRNAs) such that all BH3 activators and activated Bax/Bak are sequestered, BH3 mimetics derived from either sensitizers or activators are predicted to selectively kill cancer cells with little harm to the normal cells in which all the pro-apoptotic proteins are not sequestered.

A very promising method of personalizing treatment arises from the differences in the selectivity of binding of different anti-apoptotic proteins to BH3 proteins. By using BH3 peptides to determine functionally the dependence of specific cancer cells on particular Bcl-2 family proteins ("BH3 profiling") it may be possible to tailor treatment. In BH3 profiling mitochondria isolated from cancer cell lines or patient samples are treated with either Bad or Noxa BH3 peptide (that only target Bcl-XL/Bcl-2 or Mcl-1, respectively); those peptides that elicit MOMP in vitro can predict the cells' responses to BH3 mimetics or chemotherapy [16, 160–162].

4.5.1 Targeting Anti-apoptotic Proteins

There is a strong rationale for interfering with endogenous apoptosis regulators in cancer cells. One strategy to accomplish this is down-regulating the anti-apoptotic Bcl-2 proteins. There are numerous natural and synthetic chemicals, such as flavopiridol and sorafenib that down-regulate the expression of pro-survival Bcl-2 members; however, these usually work by indirect mechanisms such that other cellular effects are inevitable [163]. However, by combining this approach with BH3 profiling it may be possible to optimize these off-target effects. As an alternate strategy, oblimersen sodium was designed as a Bcl-2-specific antisense phosphorothioate oligodeoxynucleotide that is complementary to the *bcl2* gene. This reagent should decrease the cellular level of functional Bcl-2 transcript [164]. Unfortunately it has only modest clinical potency in several solid tumors and hematologic malignancies probably due to its poor penetration into cells and extremely short half-life in the blood resulting in low concentrations that are exposed to the cancer [165–167].

Consequently, more attention was focused on inhibiting anti-apoptotic proteins using small-molecule inhibitors. This strategy is based on the structures of anti-apoptotic proteins complexed with BH3 peptide derived from pro-apoptotic proteins, where the hydrophobic groove of the anti-apoptotic proteins is occupied by the α-helix of the BH3 peptide. The expectation was that the inhibitors would release the sequestered pro-apoptotic Bcl-2 members and thereby induce Bax/Bak-dependent apoptosis in cancer cells. Below we overview the most developed small molecules that target Bcl-2 family proteins.

4.5.1.1 Pan-Bcl-2 Inhibitors

There are two inhibitors that are expected to target multiple anti-apoptotic proteins that are the subject of ongoing clinical development: gossypol and obatoclax. Because of their spectrum of activity they are deemed pan-Bcl-2 inhibitors. Gossypol and its analogs have modest binding affinities to Bcl-2, Bcl-XL, and Mcl-1, ranging from hundreds of nanomolar to tens of micromolar. They are effective at inducing cytochrome *c* release in a variety of tumor cell lines [168–171]. They are currently in Phase II clinical trials both as a single agent and in combination with conventional chemotherapy [172]. However nonselective toxicity may be a problem. Gossypol has been reported to induce cell death even in the absence of the apoptotic executors Bax and Bak [173]. To address this problem, a newer derivative BI-97C1 (sabutoclax) has been developed that shows minimal off-target effects with little cytotoxicity in cells without Bax/Bak [174]. At the cellular level it induces apoptosis in a B-cell lymphoma cell line and inhibits cell growth in prostate and lung cancer cell lines. Moreover in combination with other treatments the Phase I clinical trial results indicated significant positive responses in advanced solid tumors [175, 176]. Thus, sabutoclax will likely be tested as monotherapy in phase III clinical trials.

Obatoclax (GX15-070) was discovered by screening natural compounds that disrupt Bcl-2 family protein:protein interactions [177]. In a fluorescence polarization assay, obatoclax displays IC_{50} values from 1 to 7 μM for all anti-apoptotic Bcl-2 members. Preclinically it showed activity in a number of cancer types such as non-small-cell lung cancer, mantle cell lymphoma, and multiple myeloma [178]. However the mechanism(s) by which obatoclax works is still controversial. Although it was initially demonstrated to disrupt the interaction of Mcl-1:Bak on MOM [179] and can up-regulate the BH3-only Mcl-1-specific sensitizer Noxa [180], other investigators reported that it induces cell death in a Bax/Bak-independent fashion by nonselective toxicity [173, 181]. In Phase I clinical trial it displayed single-agent anticancer activity with reasonable tolerance [182], and currently it is under Phase II clinical trial where it has shown modest clinical activity [183].

4.5.1.2 Bcl-2 Selective Inhibitors

The pan inhibitors display broad spectrum of activity by interacting with multiple anti-apoptotic proteins making them potentially effective in a wide range of cell lines. However the low specificity may lead to off-target toxicity, thereby limiting their application in anticancer treatment. Furthermore, clinical data with more specific inhibitors suggests that highly effective pan inhibitors may be too toxic to normal cells. In discussing agents that selectively target Bcl-2 proteins, we will divide the anti-apoptotic proteins into two groups with different interacting partners: Bcl-XL/Bcl-2 that Bad prefers binding and Mcl-1 that binds to Noxa.

ABT-737/263/199: Targeting Bcl-XL/Bcl-2

ABT-737 and ABT-263 developed by Abbott Laboratories are inhibitors of both Bcl-XL and Bcl-2. Designed using SAR-by-NMR [184] to mimic the BH3-only protein Bad, ABT-737 displays nanomolar affinities for soluble fragments of Bcl-XL and Bcl-2 [185] that are at least 100-fold higher than the pan inhibitors discussed above. Mechanistic studies suggested that ABT-737 induces cell death by displacing BH3-only proteins from the binding pocket of Bcl-XL in a Bax/Bak-dependent manner [186]. In vitro studies demonstrated that ABT-737 has significant cytotoxicity in many different cancer cell lines both as monotherapy and in combination with other chemotherapy reagents [187–189].

However, the specificity and selectivity of ABT-737 for binding to full-length proteins in live cells or mitochondria-like environments were assumed to mimic those seen in the in vitro screen. We have recently demonstrated using fluorescence lifetime imaging microscopy-based Forster resonance energy transfer (FLIM-FRET) in live MCF-7 cells that ABT-737 can displace Bad and tBid but not Bim from Bcl-XL and Bcl-2 [190].

The closely related derivative ABT-263 was developed to achieve increased oral bioavailability, lower nonspecific binding to serum proteins, and higher solubility. It has similar binding profile to anti-apoptotic proteins and potent activity against cancer cell lines [191]. In our test system using MCF-7 cells, ABT-263 has comparable activity to ABT-737 in disrupting the complexes between Bcl-XL/Bcl-2 and Bad/tBid and displays the same inhibitory pattern, i.e., ABT-263 does not prevent the binding of Bim to Bcl-XL/Bcl-2 [192]. Whether this effect extends to other cell lines is not yet known. However, taken together our results suggest that it may be difficult to predict which tumors will respond best to ABT-263 without a biomarker. In clinical trials, ABT-263 showed notable benefits in lymphoid malignancies as monotherapy or in combination with chemotherapy [193, 194] but to date only modest response in small-cell lung cancer when used as a single agent [195, 196].

The most common adverse event after ABT-737 and ABT-263 treatments is thrombocytopenia, which is actually an on-target toxicity effect due to the critical dependence of platelet development and survival on the function of Bcl-XL [197, 198]. Platelets are anucleate cells that develop from megakaryocytes in the bone marrow, and the megakaryocyte-specific gene deletion of Bcl-XL gene results in abnormal morphology and a drastically decreased life-span of nascent platelets, with severe thrombocytopenia in mice [199]. It is therefore expected that a Bcl-XL inhibitor leads to thrombocytopenia. Interestingly, double knockout of Bax and Bak protects the megakaryocytes from the effects induced by Bcl-XL deletion or ABT-737 treatment, further indicating that the intrinsic pathway needs to be restrained for normal platelet production [199, 200]. In contrast, Bim and Bid seem to be dispensable in this apoptotic pathway as deficiency of Bid, Bim, or both did not reduce thrombocytopenia in Bcl-XL knockout mice [200]. This probably reflects the importance of Puma and/or Bad in the platelet survival, as both of these are bound by Bcl-XL. This significant unwanted on-target effect of ABT 737 (and ABT 263) has restricted the use in clinical trials of acute leukemia. However, it is intriguing to

note that this problem has been circumvented by the development of a new derivative ABT-199 that selectively targets Bcl-2, but not Bcl-XL [201], benefiting from this selectivity as Bcl-2 deletion does not cause thrombocytopenia. Thus, ABT-199 can be used in higher doses to achieve more complete Bcl-2 inhibition. Currently it is being evaluated in Phase I trials for patients with relapsed or refractory chronic lymphocytic leukemia. Another potential approach that may permit the use of ABT-263 for solid tumors would be to protect platelets by inhibiting Bax and Bak.

Specifically Targeting Mcl-1

Mcl-1 is an anti-apoptotic Bcl-2 protein that is over-expressed in many cancers [13] and tightly regulates the development, maintenance, and chemotherapeutic resistance of malignancies [202–205]. Cell death can be induced in cancer cells by genetic deletion of Mcl-1 even in the presence of other anti-apoptotic proteins, indicating a special role for Mcl-1 in cancer cells [204, 205]. Consistent with this, Mcl-1 has several unique structural and functional features compared to the other anti-apoptotic proteins. Mcl-1 has a longer amino-terminus that is intrinsically unstructured. This may account for interactions that not only provide the targeting sites for ubiquitin-dependent [77, 78, 206, 207] and ubiquitin-independent [208] degradation that result in the short half-life of Mcl-1 [209] but also target Mcl-1 to mitochondria. At this site some Mcl-1 undergoes a further cleavage with subsequent relocalization to the mitochondrial matrix which is critical for Mcl-1 to fulfill its non-apoptotic mitochondrial function, regulating fission/fusion [210]. Therefore it appears that it is primarily the MOM-targeted Mcl-1 that performs its anti-apoptotic function where it binds to specific pro-apoptotic proteins. Together with its role regulating fission/fusion from the mitochondrial matrix Mcl-1 is a crucial survival factor for many different cell types, including all early hematopoietic lineages and progenitor populations [85, 211]. Thus Mcl-1 is an ideal target for therapy especially in those cancers addicted to Mcl-1 such as acute myeloid leukemia and Myc-induced lymphomas [205, 212].

It is understandable therefore that there is considerable interest in developing specific Mcl-1 inhibitors. As a proof of principle, Mcl-1 antisense oligonucleotides showed synergistic activities in promoting apoptosis when combined with chemotherapeutic drugs [213, 214]. However, reflecting the problem with oblimersen, there has been no movement into clinical trials due to the issues of stability and delivery. Peptides or modified peptide based on selected BH3 regions have also been reported to selectively bind the hydrophobic groove of Mcl-1: Bim_S2A [215] and SAHB-Mcl1-BH3 [216] inhibit Mcl-1 and promote Bak-dependent cell death through a Noxa-like mechanism. A small-molecule inhibitor has recently been developed using the same strategy used in the discovery of ABT-737 [217]. This molecule selectively binds Mcl-1 with a dissociation constant less than 100 nM, and providing that it is functional in cells it is anticipated to show clinical benefits. However the preliminary results suggest potent activities only when combined with

chemotherapy, which raises a question whether the extra mitochondrial role and the unusual organellar localization pose a barrier to drug activity [211].

A Novel Path to Screen Selective and Active Bcl-2 Inhibitors

All the currently available selective inhibitors for Bcl-2 family were developed using truncated proteins or peptides in solution. This might affect the efficacy of the drugs to function in cells due to the significant effects of membrane binding on the conformation and the relative binding affinities for different partner proteins. For example a full-length Bim mutant called Bim_S2A binds as tightly to Bcl-XL/Bcl-2 as the wild-type Bim protein on mitochondria in MCF-7 cells [190]. In contrast the BH3 peptide derived from Bim_S2A binds only truncated Mcl-1 in solution [215]. Another indication is that both ABT-737 and ABT-263 fail to displace full-length Bim from mitochondrial Bcl-XL and Bcl-2 in live MCF-7 cells [190, 192], a feature that could be critical for inducing Bax/Bak-dependent apoptosis in some cells. Taken together, there is a need to screen Bcl-2 family inhibitors using full-length proteins in membranes, membrane-like environments, and live cells.

We have made significant progress in filling this gap. By measuring the inhibition of the direct interactions between full-length recombinant proteins in a liposome system that recapitulates MOMP [119, 142], a few active compounds have been found after screening. These molecules are more potent than ABT-737 in inhibiting the membrane-bound form of Bcl-XL [unpublished data]. Taking the system even closer to the physiologic environment of cancer cells, we have also developed a system in MCF-7 breast cancer cells with the stable or the transient expression of fluorescent proteins fused to Bcl-2 family members. The direct protein:protein interactions between Bcl-2 proteins on mitochondria can be quantified by FLIM-FRET. Furthermore, we can measure the effect of mutagenesis or drugs on these interactions [192]. Using this system we observed that in MCF-7 cells the interactions between Bcl-XL/Bcl-2 and Bim are not sensitive to mutations of the conserved BH3 region of Bim compared to the effects of similar mutations in Bad and tBid. ABT-737 and ABT-263 selectively inhibited binding of Bcl-XL/Bcl-2 to Bad and tBid but not to the major isoforms of Bim. Significantly, one of the hits in our screen released Bim from Bcl-XL in preliminary experiments [unpublished data], demonstrating the power of employing full-length proteins in a physiologic context during screening.

4.5.2 Targeting the Pore-Forming Pro-apoptotic Proteins

By extending the concept of cancer cells being “addicted” to anti-apoptotic proteins one can envision that with an appropriate stimulus these cells are “primed” to die, i.e., there is a lower threshold for triggering apoptosis in certain malignant cells compared to normal cells [218]. This suggests another avenue to target the Bcl-2

family in cancer cells as directly activating restrained Bax/Bak may selectively kill cancer cells. This opens the possibility of using direct Bax/Bak activators.

The first compound with such activity was a peptide that includes part of the Bax pore-forming domain which targets to mitochondria and induces cytochrome *c* release, thereby triggering caspase-dependent apoptosis. The peptide caused tumor regression after direct intratumoral injection in a nude mouse xenograft model [219]. However as this peptide is an active version of Bax, this clinically inconvenient administration route may be required to kill cancer but not normal cells.

Computational screening was used to identify a Bax activator BAM7 that directly and selectively activates Bax. BAM7 engages the novel "rear pocket" Bax activation site (helices α6, α1, and α2), promotes Bax oligomerization, and induces cell death in a Bax-dependent fashion. BAM7 does not interact with the hydrophobic groove of anti-apoptotic proteins or pro-apoptotic Bak, demonstrating its selectivity in binding and activating Bax [220].

4.6 The Clinical Implications of Bcl-2 Localization at the Endoplasmic Reticulum

Other than mitochondria, the Bcl-2 family proteins are also found at the endoplasmic reticulum (ER). At this location Bcl-2 regulates the cellular consequences of protein misfolding and release of calcium from storage. With increasing levels of misfolded mutated proteins, cancer cells rely on the unfolded protein response (UPR) for survival more so than many normal cells [221]. Activated UPR has three effectors, one of which is IRE1. This is a transmembrane ER-located protein that will oligomerize once released from ER chaperones after the accumulation of unfolded proteins [222]. IRE1 binds to the activation site of Bax/Bak and prevents their activation and oligomerization. Bax/Bak activators under development may disrupt this interaction and induce cell death in cancers with highly active UPR [223].

Autophagy is another pathway of cell death/survival that is mediated by events that are initiated at the ER. It maintains cell survival following a variety of extracellular and intracellular stimuli by degrading misfolded or aggregated proteins and damaged organelles allowing recycling of the amino acids [224]. Beclin1 is an essential trigger for the new membrane formation that is critical for autophagy to proceed and has been identified as a BH3-only protein that binds specifically ER-localized Bcl-2 [225, 226]. This interaction can be disrupted by phosphorylation mediated by Jun kinase (JNK) of several serine residues in the unstructured loop region of Bcl-2, thereby freeing Beclin to mediate new membrane formation [227]. It was also reported that Bad can release Beclin1 from the hydrophobic groove of Bcl-2 [228]. Therefore, it would be expected that BH3 mimetics such as ABT-737 can induce autophagy in a similar way, an effect also shared by the pan inhibitors gossypol and obatoclax [229, 230] and the BH3 mimetic S1 [231].

Because of the dual nature of autophagy to promote either cell survival or death in particular circumstances how this pathway will modulate the overall effect of Bcl-2 inhibitors in cancer cells remains to be determined.

4.7 Targeting Bcl-2 Family in Other Diseases

Other than cancer, dysregulation of the Bcl-2 family is observed in several other serious diseases such as diabetes and traumatic brain injury (TBI). However in these cases, excessive rather than inhibited cell death is at fault.

Diabetes is characterized by hyperglycemia due to insulin deficiency. It is either the result of autoimmunity against the pancreatic β-cell that produces insulin (type 1) or due to reduced insulin secretion (type 2) [232]. In both cases, apoptosis ensues after the mitochondria pathway being activated by cell death signals, contributing to the loss of β-cell mass/function.

In type 1 diabetes, the death stimuli arise from a combination of pro-inflammatory cytokines (e.g., IL-1 β/TNF-α + IFN-γ) [233, 234]. These signals trigger a JNK/c-Jun phosphorylation pathway that causes the up-regulation of sensitizer BH3-only proteins DP5 and Bad, thus releasing BH3-only activator protein Puma from anti-apoptotic proteins [234–236]. Furthermore, activated JNK phosphorylates Mcl-1 making it susceptible to ubiquitin-mediated degradation [237, 238]. The formation of nitric oxide induced by cytokines also leads to ER stress, further aggravating the situation [239, 240]. Interestingly, unlike in immune and epithelial cells, apoptosis induced by ER stress in β-cells seems to occur via up-regulation of Puma rather than Bim, suggesting a cell-specific response [241, 242]. Immune cells such as CD8+ T cells also release granzyme B that activates Bid by cleavage inducing apoptosis [243]. Collectively these effects result in MOMP due to Bax/Bak oligomerization [236].

In type 2 diabetes, glucolipotoxic insults induce apoptosis mainly through the ER stress pathway. This response involves Puma up-regulation by transcription factor FoxO3 as well as DP5 up-regulation through JNK pathway and down-regulation of Bcl-2 and Bcl-XL [237, 244].

TBI is a heterogeneous disorder that is a major health issue worldwide. Research into the pathophysiology reveals that TBI occurs after a primary insult from biomechanical forces and involves ATP depletion and ion-pump failure. There is also a secondary insult that has a pivotal role in the continued neuron cell death after the TBI. In the secondary insult, an abnormally high level of calcium is reached in mitochondria resulting in membrane depolarization and opening of calcium channels. This overload of calcium in the cytoplasm is believed to trigger cell death [245]. Several studies have confirmed the critical role of mitochondria and Bcl-2 family members in neuron cell survival [246, 247]. Bcl-2, Bcl-XL, Bcl-w, and Mcl-1 all are found on the MOM in normal healthy neuron cells, inhibiting MOMP [248].

Thus diabetes and TBI represent two diseases among others in which undesired cell death can be triggered by diverse death signals, emphasizing the difficulty of preventing apoptosis by interfering with upstream activation. In this respect,

preventing the activation of the executioners Bax and Bak as the final common pathway emerges as a plausible target which is currently being pursued by several groups. If cells are prevented from dying by inhibition of Bax and Bak the question remains as to how beneficial these "undead" cells will be to the organism and whether or not they can repair or at least function long enough to be replaced.

4.8 Epilogue

Understanding the regulation apoptosis by the Bcl-2 family has led to important insights into cell biology and the development of the first generation of exciting new compounds with excellent therapeutic potential in cancer. Further refinement of screens, and the search for compounds that disrupt interactions other than sensitizer: anti-apoptotic protein binding, promises exciting times ahead.

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Chapter 5
IAP Proteins and Their Therapeutic Potential

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Abstract Precise regulation of cell death and survival is essential for proper maintenance of organismal homeostasis, development, and immune system. Deregulated cell death can lead to developmental defects, neuropathies, infections, and cancer. Inhibitor of apoptosis (IAP) proteins are evolutionarily conserved regulators of cell death with impact on numerous cellular processes. Initially identified as inhibitors of cell death during viral infection, IAP proteins block cell death by direct inhibition of apoptotic proteases (X chromosome-linked IAP) or by preventing caspase activation following death receptor engagement (cellular IAPs). Recently, the role of IAP proteins as ubiquitin ligases has emerged as a critical feature for their ability to modulate various signaling pathways (e.g., NF-κB, MAPK, NOD) and to influence cellular fate. In addition, IAP proteins are often overexpressed in human malignancies presenting them as attractive targets for anticancer therapy. Among the different strategies employed to target IAP proteins, IAP antagonists—small-molecule mimetics of the natural IAP antagonist SMAC—have garnered most attention. IAP antagonists have successfully passed initial safety scrutiny in clinical trials and are about to enter a critical phase in their development in which their antitumor efficacy will be tested in the hopes of providing novel treatments for cancer patients.

Keywords IAP • Inhibitor of apoptosis • BIR domain • TNF • SMAC • XIAP • c-IAP • RING domain • Apoptosis • NF-kB • Cancer • IAP antagonist • SMAC mimetic • Necroptosis

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5.1 Inhibitor of Apoptosis Protein Family

The evolutionarily conserved family of inhibitor of apoptosis (IAP) proteins encompasses structurally related regulators of many critical cellular processes [1]. IAPs were originally identified in baculoviruses because of their ability to inhibit virus-induced apoptosis and allow viral amplification [2, 3]. Subsequently, through bioinformatics efforts and functional screens, IAP genes and proteins were identified in all metazoan organisms including several Drosophila and eight human IAP proteins [4]. Among the human IAP proteins, X chromosome-linked IAP (XIAP) and cellular IAP1 (c-IAP1) are probably the most studied, although other IAP proteins (NAIP, c-IAP2, ML-IAP, survivin, ILP2, and Apollon; Fig. 5.1) also play important roles in cell survival, cell cycle, inflammation, and overall homeostasis. In Drosophila, IAPs are critical for fly development and survival, and genetic deletion of Drosophila DIAP1 causes massive apoptosis and death of fly embryos [5].

IAP proteins contain a signature baculovirus IAP repeat (BIR), and several IAPs also have additional protein domains [1]. BIR domains are conserved 70–80 amino acid zinc-coordinating domains that are present in one to three copies and regulate protein–protein interactions that are necessary for IAP function [6]. Several IAP proteins (such as XIAP, c-IAP1, c-IAP2, and ML-IAP) contain a carboxy-terminal really interesting new gene (RING) domain, which imparts them with ubiquitin ligase activity [7]. A ubiquitin-associated (UBA) domain in cellular IAPs (c-IAPs), XIAP, and ILP2 allows their association with monoubiquitin and polyubiquitin

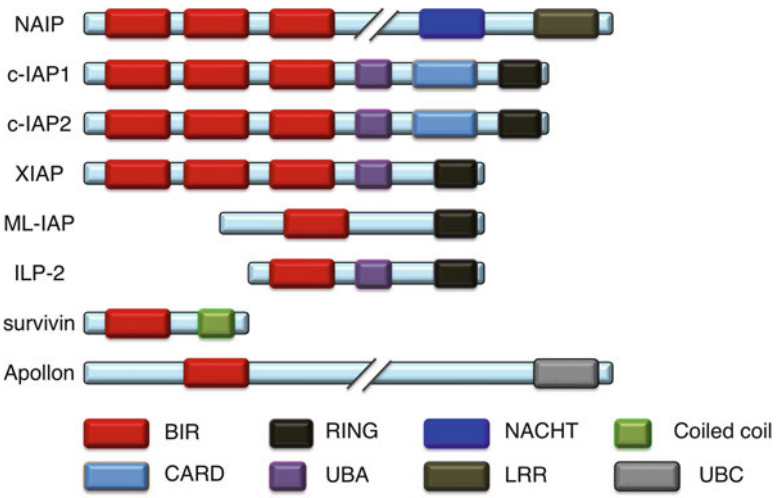

Fig. 5.1 Schematic representation of human IAP proteins. *BIR* baculovirus IAP repeat, *CARD* caspase recruitment domain, *LLR* leucine-rich repeat, *NACHT* NAIP, CIITA, HET-E, and TP1, *RING* really interesting gene, *UBA* ubiquitin-associated domain, *UBC* ubiquitin-conjugating domain

chains [8, 9], while the carboxy-terminal ubiquitin-conjugating (UBC) domain of BRUCE promotes ubiquitin attachment [10, 11]. The cellular IAPs also possess a caspase-recruitment domain (CARD) [12]. Survivin contains a coiled-coil region, which mediates interaction with chromosomal passenger complex (CPC) proteins and ensures proper subcellular localization [13]. The leucine-rich repeat (LRR) and nucleotide-binding and oligomerization domain (NOD) of NAIP are required for its role in innate immunity [14, 15]. This chapter focuses primarily on RING-containing IAP proteins (XIAP, c-IAP1, c-IAP2, ML-IAP) and their role in cell survival and signaling pathways with an emphasis on potential new therapeutic treatments.

5.2 Ubiquitin Ligase Activity of IAP Proteins

Ubiquitination involves covalent modification of target proteins with the 76-amino acid protein ubiquitin and requires the enzymatic activity of a ubiquitin-activating enzyme (E1), a UBC enzyme (E2), and a ubiquitin ligase (E3) [16] (Fig. 5.2). Monoubiquitination occurs when a single-ubiquitin molecule is attached to a lysine residue of the substrate protein [16]. However, ubiquitin contains seven lysine residues and a free amino-terminus, thus allowing the synthesis of polyubiquitin chains through eight different isopeptide linkages [17]. The combinatorial complexity of ubiquitination is enabled by tens of different E2 enzymes and, especially, by hundreds of ubiquitin ligases that link the substrate proteins to the ubiquitination machinery [18]. Ubiquitin ligases can act as single proteins or exist as multisubunit complexes, and most of them contain either a RING domain or a HECT domain [19]. The assembly of ubiquitin chains can be reversed by deubiquitinating enzymes (DUBs), which permits the fine-tuning of the ubiquitination signaling [20]. Finally, various ubiquitin moieties are recognized by a diverse group of ubiquitin-binding domains that interpret this information and generate numerous biological outcomes [21]. The lysine 48- and 11-linked ubiquitin chains predominantly target proteins for proteasomal degradation, while lysine 63-, amino-terminal methionine-, and, in some case, lysine11-linked chains provide a scaffolding platform for the assembly of signaling complexes [16, 22].

IAP proteins are RING domain ubiquitin ligases that promote the assembly of a variety of polyubiquitin chains on themselves and their substrates in collaboration with E2 enzymes of the UbcH5 family [22, 23] (Fig. 5.2). The substrates of E3 activity of IAP proteins are as diverse as the signaling pathways that they regulate. XIAP and c-IAP proteins can promote ubiquitination of several apoptotic proteins including effector proteases—caspases and the endogenous IAP antagonist, second mitochondrial activator of caspases (SMAC) [24–26]. But the best-known substrates for the E3 ligase activity of c-IAP proteins are kinases receptor-interacting protein 1 (RIP1) and NF-κB-inducing kinase (NIK) [27–29]. Ubiquitination of RIP1 and NIK is crucial for the regulation of cell death and signaling processes and is discussed later in this chapter. Other substrates involve signaling adaptors tumor necrosis factor receptor-associated factors 2 and 3, TRAF2 and TRAF3, as well as

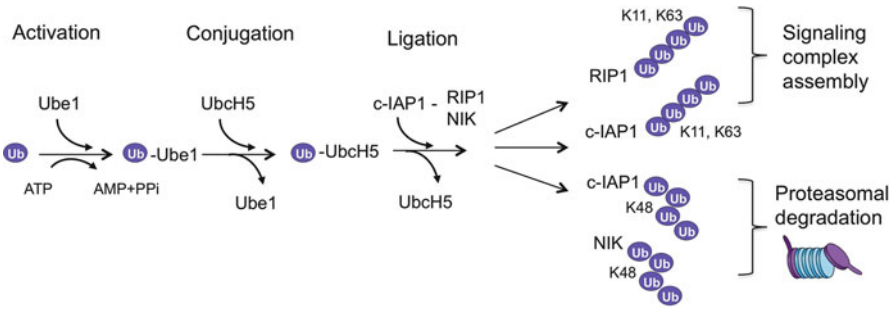

Fig. 5.2 The enzymes and reactions of the IAP-mediated ubiquitination. Activation reaction involves transfer of ubiquitin to an E1 enzyme (Ube1) in an ATP-dependent fashion and leads to transfer of activated ubiquitin to an E2 enzyme (UbcH5) in the conjugation reaction. The E2 with ubiquitin binds E3 ubiquitin ligase (c-IAP1), which can also bind a substrate—commonly via a different protein interaction domain—and thus allows the ubiquitin ligation to occur. When polyubiquitin chains are assembled this process will be repeated with a lysine (K) residue of the ubiquitin molecule itself serving as a substrate. The assembly of K63- or K11-linked polyubiquitin chains on RIP1 or c-IAP1 itself promotes the formation of signaling complexes, while K48-linked ubiquitination of NIK or c-IAP1 targets them for proteasomal degradation

several regulators of innate immunity [30–32]. IAP-mediated ubiquitination and regulation of the stability of their substrates are discussed in the context of various pathways that rely on the E3 ligase activity of IAP proteins for the proper spatial-temporal execution of signaling directives.

5.3 Regulation of Cell Death Pathways by IAP Proteins

5.3.1 Role of IAP Proteins in Apoptotic Pathways

Cell death can be carried out by three major pathways: apoptosis, necrosis/necroptosis, and authophagy. Apoptosis is a carefully orchestrated cell death program that relies on cysteine-dependent aspartyl-specific proteases—caspases, while necroptosis occurs when caspase activity is blocked [5, 33, 34]. Caspase-dependent apoptotic cell death can be initiated by extrinsic or intrinsic stimuli. Intrinsic cell death is activated by cellular stress, developmental cues, or growth factor withdrawal that lead to disruption of internal cellular integrity including damaging mitochondria [35, 36] (Fig. 5.3). The major regulators of the intrinsic, mitochondrial cell death pathway are Bcl-2 family of proteins where the pro-apoptotic initiators such as Bim or Bid and effectors like Bak and Bax counteract the inhibitory action of anti-apoptotic proteins Bcl-2, Bcl-x_L, and Mcl-1 [37]. Tipping of the balance in favor of pro-apoptotic Bcl-2 proteins leads to cytochrome c release, formation of the apoptosome complex around Apaf-1, and activation of caspase-9. Activated caspase-9 subsequently activates caspases 3 and 7 leading to cell death [38].

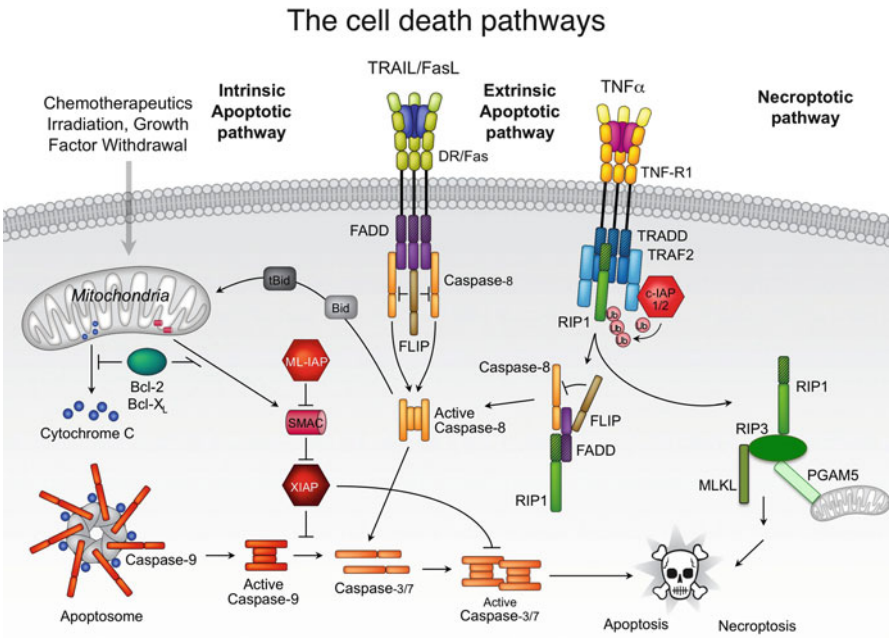

Fig. 5.3 Apoptotic and necroptotic signaling pathways. Programmed cell death is executed through apoptotic (caspase-dependent) and necroptotic (caspase-independent) signaling pathways in response to various stimuli including environmental stress or growth factor deprivation (intrinsic apoptotic pathway) and activation of TNF family receptors (extrinsic apoptotic or necroptotic pathway). *Bcl-2* B-cell lymphoma 2, *Bcl-x*$_L$ B-cell lymphoma-extra large, *BH3* Bcl-2 homology 3, *BID* BH3-interacting domain death agonist, *c-IAP* cellular IAP, *Caspase* cysteine-aspartic protease, *DR* death receptor, *FADD* fas-associated death domain, *FLIP* FLICE inhibitory protein, *ML-IAP* melanoma IAP, *RIP* receptor-interacting protein, *SMAC* Second mitochondrial activator of caspases, *tBID* truncated BID, *TRADD* TNFR-associated death domain, *TRAF* TNF receptor-associated factor, *TNFα* tumor necrosis factor α, *TNFR1* TNF receptor 1, *Ub* ubiquitin, *XIAP* X-chromosome-linked IAP

The extrinsic cell death pathway is initiated by binding of tumor necrosis factor (TNF) family death ligands to death domain (DD) containing TNF receptors causing their aggregation and recruitment of the receptor-associated death-inducing signaling complex (DISC) [39] (Fig. 5.3). Death receptors 4 and 5 (DR4 and DR5) and Fas recruit adaptor protein Fas-associated death domain (FADD) and caspase-8, which triggers caspase-8 activation and consequent activation of effector caspases 3 and 7 [40]. TNFR1 and DR3 engage additional proteins in their receptor complexes including TNFR-associated death domain (TRADD), receptor-interacting protein RIP1, TRAF2, and c-IAP1 and 2 [41, 42]. Cellular IAP 1 and 2 ubiquitinate RIP1 with a variety of linkages, most prominently K63- and K11-linked polyubiquitin chains, within the TNFR1 receptor-associated complex [23]. This prevents the formation of the cytosolic pro-apoptotic complex (complex II) that is deprived of TNFR1 but engages FADD and caspase-8 and consequently blocks caspase-8

activation [41]. Removal of c-IAP proteins or ubiquitin moieties from RIP1 by deubiquitinating enzymes A20 or CYLD allows the formation of a fully functional complex II, caspase-8 activation, and cell death [22, 29]. An additional brake on this pathway is achieved by the inhibitory caspase-8-like molecule FLICE inhibitor protein (FLIP), which counters activation of the death receptor apoptotic pathway by competing with caspase-8 for recruitment into the death receptor complex with FADD [41, 43]. These two apoptotic pathways are not insulated from each other but rather interact through caspase-mediated amplification of death signal and via proteolytic cleavage of Bid, which can further enhance extrinsic death signaling through stimulation of the mitochondrial pathway [44].

Both apoptotic pathways converge at the activation of effector caspases 3 and 7, and only one true endogenous inhibitor of caspases in mammals, XIAP, can block cell death at this point [45]. Active caspases operate as dimers that consist of a pair of interacting large and small catalytic subunits [46]. The BIR3 domain of XIAP binds the conserved four amino-terminal amino acids of the p12 small subunit of processed caspase-9 [47, 48]. This small peptide constitutes the IAP-binding motif (IBM) that is present in several caspases and IAP-antagonistic proteins. Binding of XIAP BIR3 prevents caspase-9 dimerization, thus blocking its activation. Direct inhibition of caspases 3 and 7 involves a different mechanism. In this case, the linker region between the BIR1 and BIR2 domains of XIAP binds the substrate-binding groove of activated caspases with BIR2 further stabilizing these interactions [49–51].

As nature always ensures multiple ways for regulation of critical cellular processes, even inhibitors have their own inhibitors, and in the case of IAP proteins it is SMAC [52, 53]. Apoptotic stimuli cause the release of processed, mature SMAC from mitochondria into the cytosol where it can bind BIR2 and BIR3 domains of XIAP via its IBM and preclude XIAP interaction with caspases [54, 55]. Other IAP proteins do not bind or inhibit caspases at physiologically relevant levels. However, ML-IAP, ILP2, and c-IAP proteins (predominantly through their BIR3 domains) bind SMAC with high affinities and block SMAC-mediated inhibition of XIAP [56–58]. In addition, IAP proteins and SMAC regulate each other's protein stability via ubquitination. The RING domain-containing IAP proteins can ubiquitinate and destabilize SMAC, while SMAC binding stimulates autoubiquitination of IAP proteins leading to their degradation [25, 59–61]. In addition to blocking caspase activation and activity, IAP proteins can promote caspase ubiquitination and proteasomal degradation [24, 26]. Finally, ubiquitin ligase activity of IAP proteins regulates their own stability as c-IAP1-mediated ubiquitination of XIAP and c-IAP2 promotes their proteasomal degradation [62, 63].

5.3.2 Role of IAP Proteins in Necroptosis

In instances when TNFR1-mediated cell death signaling is halted by caspase inhibition, an alternative pathway can be engaged—necroptosis. Necroptosis is a regulated form of necrotic cell death initiated by TNF signaling and mediated by kinases RIP1

and the related protein RIP3 [34] (Fig. 5.3). Although TNF-stimulated pathways are best studied, pathogen recognition receptors (PRRs), Toll-like receptor, NOD-like receptors, and RNA helicases can induce necroptosis as well. Caspase inhibition and the presence of RIP3 (whose expression is very restricted in human tissues and cell lines) following TNF stimulation enable RIP1–RIP3 interaction via the RIP homologous interaction motif (RHIM) [64]. Interestingly, while the kinase activity of RIP1 is not required for induction of NF-κB signaling or apoptosis, it is critical for necroptotic cell death and RIP1–RIP3 association [65, 66]. c-IAP proteins are negative regulators of necrotic cell death due to their E3 ligase activity that promotes RIP1 ubiquitination and thereby blocks RIP1 recruitment into cytoplasmic cell death-stimulating protein complexes [67]. Again, and similar to TNF-stimulated apoptotic signaling, the absence of c-IAP proteins or deubiquitination of RIP1 allows RIP1 to enter the cytoplasmic complexes. However, in contrast to the apoptotic pathway, absence of the adaptor protein FADD or inhibition of caspase-8 activity is required for RIP1–RIP3 interaction and cross-phosphorylation to form a functional necrosome complex [34, 68]. Therefore, the E3 ligase activity of c-IAP1 and 2 represents an important checkpoint for necrotic cell death.

Mediators and effectors of necrotic cell death appear to reside in the mitochondrial cellular compartment, although, undoubtedly, more will be identified. Mixed lineage kinase domain-like (MLKL) binds phosphorylated RIP3 and appears to be required for necrotic cell death [69, 70]. Another recently identified RIP3-binding partner is a phosphatase, PGAM5, that provides a mitochondrial link for the RIP1–RIP3–MLKL complex to ensure efficient execution of necrosis [71]. Future studies involving genetic ablation of these mediators of necrotic death signaling will address their physiological relevance for this highly specialized form of cell death.

5.4 IAP Proteins as Critical Modulators of Signaling Pathways

5.4.1 Regulation of Canonical NF-κB Signaling by IAP Proteins

Regulation of cell survival by IAP proteins is not limited to cell death pathways. A number of studies conducted in recent years have established IAP proteins as important regulators of MAPK, NOD, and in particular NF-κB signaling pathways (Fig. 5.4). The NF-κB family of transcription factors regulates transcription of a vast number of genes involved in cell survival, inflammation, and immunity [72]. Five NF-κB family transcription factors, NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB, and c-Rel, act as homodimers or heterodimers, and their activation is regulated by phosphorylation and ubiquitination [73]. In canonical NF-κB signaling, the inhibitor of κB (IκB) binds NF-κB proteins RelA and p50 in the cytoplasm to prevent them from entering the nucleus in unstimulated cells [73]. When TNF binds TNFR1 it triggers the recruitment of the proximal receptor-associated complex

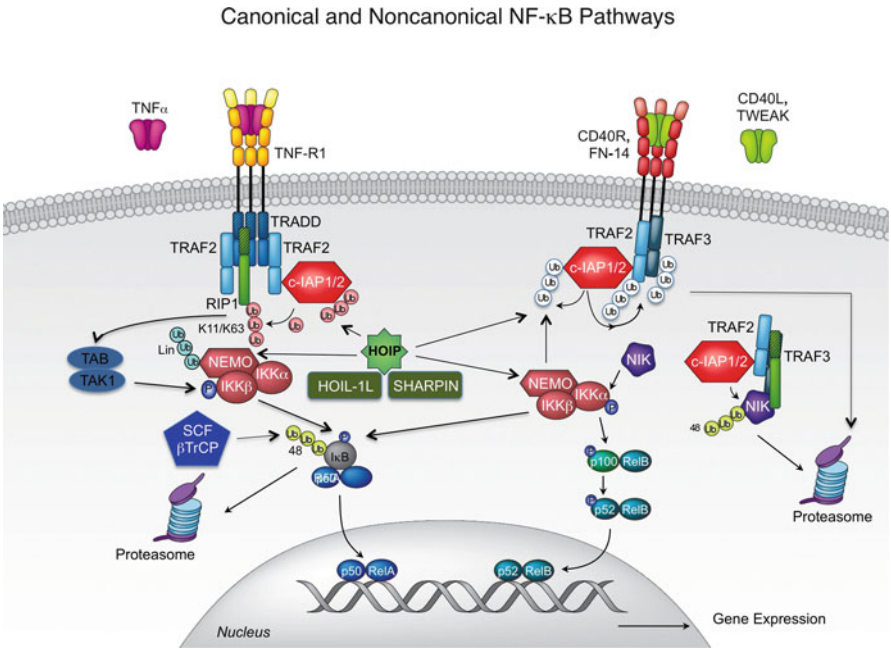

Fig. 5.4 Canonical and noncanonical NF-κB signaling pathways. Cellular IAP proteins function as positive regulators of canonical and negative regulators of noncanonical NF-κB signaling pathways. *c-IAPs* cellular IAPs, *FN14* fibroblast growth factor-inducible 14, *HOIL-1* heme-oxidized IRP2 ubiquitin ligase-1, *HOIP* HOIL-1L-interacting protein, *IKK* IκB kinase, *NEMO* NF-κB essential modulator, *NIK* NF-κB-inducing kinase, *RIP* receptor-interacting protein, *TAB* TAK1-binding protein, *TAK1* TGF-β-activated kinase 1, *TRADD* TNFR-associated death domain, *TRAF* TNF receptor-associated factor, *TNFα* tumor necrosis factor α, *TNFR1* TNF receptor 1, *TWEAK* TNF-related weak inducer of apoptosis, *Ub* ubiquitin

starting with TRADD and followed by RIP1, TRAF2, and TRAF2-associated c-IAP1 and c-IAP2 [31, 41]. Aggregation at the receptor complex leads to c-IAP-mediated ubiquitination of RIP1, TRAF2, and themselves to enable the recruitment of IκB kinase, the IKK complex, the transforming growth factor β-activating kinase 1, TAK1–TAK1-binding protein 2/3 (TAB2/3) complex, and the linear ubiquitin chain assembly complex, LUBAC [74].

TAB2/3 from the TAK1–TAB2/3 complex and IKKγ or NF-κB essential modifier (NEMO) from the IKK complex bind polyubiquitin chains on RIP1 to bring kinase TAK1 in proximity of IKKβ, thereby allowing IKKβ phosphorylation and subsequent IκB phosphorylation by IKKβ. Phosphorylation of IκB serves as a recognition signal for the E3 ligase complex SCFβ–TRCP, which promotes IκB ubiquitination and degradation. Autoubiquitination of c-IAP1/2 proteins allows the recruitment of LUBAC and assembly of linear polyubiqutin chains on NEMO and RIP1 [74, 75]. LUBAC comprises two regulatory components: SHANK-associated RH domain interactor (SHARPIN) and heme-oxidized IRP2 ubiquitin ligase 1 homolog (HOIL-1L), and the E3 ligase HOIL-1-interacting protein (HOIP) [76–78].

Linear polyubiquitin chains are believed to stabilize TNFR-associated signaling complexes, and elimination or reduction of LUBAC levels diminished NF-κB signaling [79]. In addition to the TNFR1-associated complex, c-IAP1 and 2 and their ubiquitin ligase activity are critical for the assembly of a number of TNF family receptors where they provide an important link and a platform for the assembly of distal kinase and E3 ligase complexes IKK, TAK1/TAB2/3, and LUBAC [31]. This has been shown for DR3, FN14, LT-βR, CD40, and CD30, and it is likely true for all TNF family receptors that rely on the adaptor protein TRAF2 and E3 ligases c-IAP1/2 for the stimulation of canonical NF-κB signaling [70, 80].

5.4.2 *Regulation of Noncanonical NF-κB Signaling by IAP Proteins*

Contrary to their positive regulatory role in the activation of the canonical NF-κB pathway, c-IAP proteins are key negative regulators of the noncanonical NF-κB signaling (Fig. 5.4) [81]. The noncanonical signaling pathway is initiated by NIK, which phosphorylates IKKα leading to phosphorylation and subsequent partial proteasomal degradation of p100 to p52 [82]. However, in unstimulated cells NIK is kept at extremely low levels due to constitutive ubiquitination by c-IAP1 and 2 proteins, which target it for proteasomal degradation [27]. The cytoplasmic complex comprising adaptor proteins TRAF2 and TRAF3 links the E3 ligases c-IAP1/2 with their substrate, NIK, to repress the activation of this signaling pathway [31, 83]. Activation of a number of TNF family receptors (including FN14, LT-βR, CD40) by their respective ligands or agonistic antibodies results in the recruitment of TRAF2, TRAF3, and c-IAP1/2 at the receptor complex [31, 83]. This membrane-associated aggregation leads to dimerization of c-IAP proteins and stimulation of their E3 ligase activity, which promotes c-IAP-dependent TRAF2, TRAF3, and c-IAP ubiquitination and subsequent degradation of these proteins [31, 84]. Absence of TRAF2, TRAF3, and c-IAP proteins liberates NIK, which accumulates in cells to activate noncanonical NF-κB signaling. Therefore, c-IAP proteins are instrumental for keeping this signaling pathway suppressed and thus preventing unwanted induction of cytokine expression and inflammation.

5.4.3 *Regulation of Other Signaling Pathways by IAP Proteins*

Similar to their positive regulatory role in the canonical NF-κB pathway, c-IAP proteins are also critical for MAPK activation following the stimulation by TNF family ligands. In the absence of c-IAPs, TNFα-, TL1A-, TWEAK-, LIGHT-, or CD40-induced activation of JNK and p38 signaling is drastically reduced [31, 85]. Furthermore, c-IAP1 and 2 are required for the induction of gene expression by TNF family ligands/receptors [31, 80]. Therefore, c-IAP proteins are critical regulators of the NF-κB and MAPK signaling pathways downstream of the TNFR

superfamily. However, IAP proteins are also important for several other signaling pathways.

Defense against external pathogens is frequently carried out by the innate immunity systems and cells, which employ Toll-like receptors (TLRs), NOD-like receptors (NLRs), and DNA- and RNA-sensing receptors. Bacterial peptidoglycans activate NOD receptors leading to their oligomerization and recruitment of RIP2, TRAF2, and c-IAP proteins [32]. RIP2 is then ubiquitinated by c-IAP1 and 2, which brings IKK and TAK1/TAB2/3 complexes into proximity and promotes activation of NF-κB and MAPK pathways [86]. In addition to c-IAP proteins, XIAP also promotes RIP2 ubiquitination, thus enabling LUBAC recruitment into NOD2-associated complexes and more sustained signaling [87]. The NOD signaling pathway is a rare example where both XIAP and c-IAP proteins can promote ubiquitination of the same substrate, RIP2, and in doing so possibly ensure a stronger pro-inflammatory response.

The functional importance of IAP proteins extends into caspase-mediated regulation of the pro-inflammatory cytokine IL-1β. In response to cell damage or pathogens, caspase-1 gets activated to cleave IL-1β to its active form [88]. However, the absence of IAP proteins allows enhanced processing of IL-1β in TLR-primed macrophages by both caspase-1 and caspase-8 [89]. Interestingly, IAP proteins are believed to block RIP3 activation to prevent NLRP3-stimulated IL-1β cleavage [89]. Thus, in addition to regulating cell death pathways through inhibition of RIP proteins, IAP proteins also modulate pathogen-stimulated IL-1β production and NLRP signaling.

5.5 Relevance of IAP Proteins for Human Malignancies

IAP proteins have been implicated in a number of human pathologies, particularly so in cancer. Elevated levels of IAP mRNA and protein levels have been documented in various tumor types, sometimes with a striking expression preference in cancerous versus normal tissues [90]. For example, survivin is normally not expressed in the majority of adult tissues—with the exception of cells with high renewal potential like placenta and bone marrow stem cells—but can be detected in majority of examined tumor types [91]. Survivin expression in tumor cells is mostly driven by genomic amplification, MAPK signaling pathways, or increased promoter activity [91]. Another IAP protein, ML-IAP, is also scarcely expressed in adult tissues but present at relatively high levels in melanomas, bladder, kidney, and lung cancers [90]. Interestingly, recent study suggests that ML-IAP is highly expressed in human and rodent eyes, although the functional relevance of this selective expression is not clear apparent yet [92]. ML-IAP potentially contributes to resistance to standard-of-care chemotherapeutic agents, and in many tumors its expression has been linked to poor outcome prognosis [90]. In melanomas, ML-IAP expression has been linked to the pro-survival oncogene microphthalmia-associated transcription factor (MITF), and MITF may exert some of its anti-apoptotic activity through the regulation of ML-IAP expression [93].

Expression of XIAP, c-IAP1, and c-IAP2 in human cancers is associated with poor prognosis in several tumor types [90]. Furthermore, downregulation of IAP expression is clearly correlated with the activation of apoptosis in cancer cells as knockdown of XIAP, c-IAP1, and c-IAP2 sensitizes numerous cells to death receptor-, chemotherapeutic agent-, or γ-irradiation-induced cell death. Cellular IAP proteins are also implicated as potential oncogenes, thus further supporting their importance for human cancers. The 11q21–q23 chromosomal region, which harbors *c-IAP1* and *c-IAP2* genes, is amplified in various types of tumors including esophageal carcinoma, hepatocellular carcinoma, cervical cancer, liver cancer, pancreatic cancer, non-small-cell lung cancer (NSCLC), glioblastomas, and meduloblastoma [90, 94, 95]. In addition, the syntenic genomic region encoding c-IAP genes is amplified in murine tumors as well, and c-IAP1 overexpression promotes tumor growth and hepatoma formation [96]. Besides amplification, other genetic evidence comes from the t(11;18)(q21;q21) translocation, which fuses the BIR domains of c-IAP2 with the mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) in MALT lymphomas [97–99]. Approximately half of the surveyed cases of MALT lymphomas harbor this translocation resulting in a c-IAP2–MALT1 fusion protein that constitutively activates NF-κB signaling [100]. The c-IAP2–MALT1 fusion protein-stimulated pro-survival and pro-inflammatory signaling favors cancer progression and enhances resistance to anticancer therapies.

Besides promoting tumor progression, IAP proteins have also been implicated in tumor cell mobility, invasion, and metastasis. XIAP and survivin cooperate to promote metastasis via the activation of cell motility kinases Src and FAK [101]. At the same time, antagonism of IAP proteins can block tumor cell migration and invasion [101]. Finally, ubiquitin ligase activity of IAP proteins seems to be instrumental for their ability to modulate several different pathways (in addition to NF-κB and cell death signaling) involved in tumor regulation. It was reported that c-IAP1 can promote ubiquitination of the tumor-suppressor protein MAD1 leading to its degradation and enhancement of Myc-dependent tumorigenesis [102]. On the other hand, XIAP, c-IAPs, and ML-IAP modulate kinase C-RAF stability and consequently MAPK signaling and cell motility [103, 104]. Furthermore, XIAP and c-IAP1 can also ubiquitinate Rho GTPase Rac1, and the regulation of Rac1 stability provides another modality for the modulation of cell migration [105]. Overall, elevated expression in numerous tumor types combined with their established functional importance for the regulation of cell death, survival, and signaling pathways as well as resistance to anticancer therapies makes IAP proteins attractive targets for therapeutic intervention.

5.6 Targeting IAP Proteins for Therapeutic Intervention

Several strategies have been attempted for targeting IAP proteins. Among these, the most advanced and attractive approaches involve SMAC-mimicking small-molecule antagonists and antisense oligonucleotides [90, 106]. Antisense approach has been focused on targeting XIAP and survivin and has yielded oligonucleotides AEG35156

and LY2181308, respectively (for further information on targeting survivin with LY2181308 please see [91, 107, 108]). AEG35156 efficiently decreased XIAP levels even at low nanomolar concentrations and showed antitumor activity in vivo in xenograft models of several cancer types [109]. The correlation of XIAP downregulation and tumor-inhibiting activity in combination with chemotherapeutic agents, death receptor agonists, or radiation therapy prompted the advancement of AEG35156 to clinical studies [109, 110]. AEG35156 was well tolerated in patients and showed expected pharmacokinetic properties and promising hints of antitumor activity [111]. Phase I/II non-randomized study of AEG35156 further confirmed its tolerability in patients, dose-dependent knockdown of XIAP, and some induction of apoptosis [112]. However, randomized Phase II trial in patients with refractory AML showed that AEG35156 did not provide antitumor benefit in the re-induction chemotherapy protocol [113]. Unfortunately, pharmacodynamic studies were not performed in this trial leaving the question of whether efficient knockdown of XIAP was achieved unanswered.

SMAC-derived amino-terminal peptides mimic the function of mature active SMAC. Such peptides have been extensively used to validate targeting of IAP proteins and to generate high-affinity binders for select BIR domains of IAP proteins [6]. It became apparent very early in these efforts that alanine was the preferred residue at the amino-terminal end and that high-throughput screening would not necessarily identify high-affinity ligands for the rather shallow and surface-exposed IAP peptide-binding site [114, 115]. Those early validation studies demonstrated the ability of SMAC-derived peptides to promote cell death in cancer cells and in in vivo models in combination with chemotherapeutics or the death receptor ligand TRAIL/Apo2L [116–119]. The following years witnessed independent development of several types of SMAC-mimicking IAP antagonists, which possessed higher affinities for BIR domains, better pharmacological properties, and single-agent activity in cell-based assays and in vivo tumor models [6]. Additionally, two major classes of IAP antagonists emerged with distinct size and potency but still relying on very similar mechanistic principles: monovalent IAP antagonists that emulate a single SMAC AVPI motif and bivalent antagonists that consist of two AVPI motifs connected by a chemical linker [6] (Fig. 5.5). The major difference between monovalent and bivalent IAP antagonists (apart from size) lies in the ability of bivalent antagonists to simultaneously bind the BIR2 and BIR3 domains of XIAP leading to enhanced activation of caspases [27, 120–123].

5.6.1 Mechanism of Action of IAP Antagonist

Mechanistic exploration of IAP antagonism has led researches to several unexpected discoveries. First, treatment of cells with IAP antagonists leads to rapid proteasomal degradation of c-IAP1 and c-IAP2 proteins [27, 124]. The molecular origin of this event, which was not expected when IAP antagonists were first designed, resides in the conformational change that binding of IAP antagonists promotes in the c-IAP1 protein [125]. Binding of IAP antagonists allows opening of the

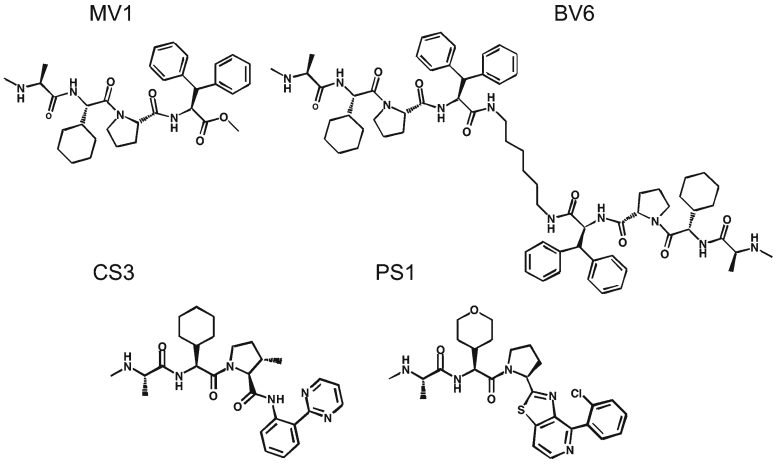

Fig. 5.5 Examples of monovalent (MV1 and PS1), bivalent (BV6), and c-IAP selective (CS3) IAP antagonists

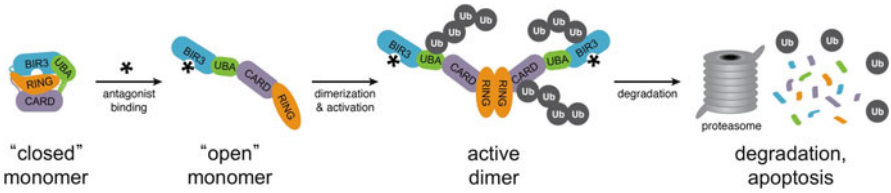

Fig. 5.6 IAP antagonists trigger a conformational change in c-IAP1 leading to RING dimerization and activation of E3 ligase activity

c-IAP1 structure and promotes RING domain dimerization [125]. Since RING dimerization is a prerequisite for the E3 ligase activity [126, 127], antagonist binding to c-IAP1 boosts E3 ligase activity resulting in autoubiquitination and subsequent proteasomal degradation (Fig. 5.6). Elevated ubiquitin ligase activity of c-IAP proteins also leads to TNF-independent RIP1 ubiquitination and activation of the canonical NF-κB pathway [27, 29, 124, 128]. However, due to the rapid degradation of c-IAP proteins, this boost of E3 ligase activity is short-lived but sufficient for the activation of noncanonical NF-κB signaling [27, 124]. As long as c-IAP proteins are present NIK is ubiquitinated and degraded. But with the loss of c-IAP proteins NIK accumulates and activates the noncanonical NF-κB pathway [105, 115]. Both of the pathways stimulate expression of a number of NF-κB responsive genes, among them TNFα, which can activate TNFR1 signaling in an autocrine or a paracrine fashion [29, 105, 115, 128]. Normally, this would cause activation of proliferative NF-κB and MAPK signaling. However, in the absence of c-IAP proteins RIP1 cannot be ubiquitinated [23, 28, 29, 31]. Instead, RIP1 joins FADD and caspase-8 to form the cytosolic pro-apoptotic complex and promote cell death [29, 128]. Thus, without TNFα production IAP antagonists cannot induce cell death as single agents. Indeed, most of the cell types and tumors that are sensitive to IAP antagonist

treatment have elevated levels of TNFα. Although IAP antagonists predominantly induce apoptotic cell death, in the absence of FADD or caspases-8, or when caspase activity is inhibited, TNFα and IAP antagonists can activate necrotic cell death [34]. Necroptosis is limited to cells that also express RIP3, a critical mediator of this form of cell death [68]. But regardless, this implies that IAP antagonists can promote cell death even when caspase activity is blocked and expands the eventual applicability of these agents.

5.6.2 c-IAP and XIAP Selective Antagonists

Although the majority of IAP antagonists targets the number of IAP proteins (XIAP, c-IAPs, ML-IAP) with comparable affinities, several antagonists selective for a particular IAP or a group of IAP proteins have been described as well [6]. Structural information on the particular BIR domains of IAP proteins and increased understanding of IAP biology and antagonism have enabled development of selective antagonists. CS3, c-IAP selective 3, antagonist is over 2,000-fold more selective for c-IAP1 over XIAP [129]. This compound activates cell death in sensitive cell lines, promotes c-IAP1 and c-IAP2 degradation, and stimulates canonical and noncanonical NF-κB signaling [129]. However, cell death induction by CS3 was much weaker in comparison to chemically related pan-IAP antagonist PS1 [129] (Fig. 5.5). Thus, antagonism of c-IAP proteins is required and sufficient for induction of cell death and stimulation of NF-κB pathways and cytokine production. Nevertheless, antagonism of both XIAP and c-IAP proteins is needed for the efficient activation of cell death.

Development of XIAP selective compounds has been oriented on preventing XIAP from binding to and inhibiting caspase-3. A high-throughput screening for such a molecule identified a compound TWX-024, which can disrupt XIAP–caspase-3 interaction and sensitize otherwise resistant cancer cell to TRAIL treatment [130]. An alternative screen of combinatorial chemistry library identified a polyphenylurea class molecule, TPI 1396-34 [131]. This agent also prevented XIAP-mediated inhibition of caspase-3 and showed antitumor activity in vivo. Yet another approach using rational design yielded a series of tripeptides with selectivity for XIAP BIR2 domain and ability to sensitize cancer cells to TRAIL [132]. In spite of initial progress and the need for such reagent, neither of these compounds has been further optimized to produce a highly selective XIAP antagonist. The perfect XIAP-targeting compound should allow simultaneous antagonism of the BIR2 and BIR3 domains, thus enabling broad activation of caspases 3, 7, and 9.

5.6.3 Pro-apoptotic Combinations of IAP Antagonists

IAP antagonists possess single-agent pro-apoptotic activity in sensitive cancer cell lines, but they also combine well with other cytotoxic agents including chemotherapeutics, death receptor agonists, oncogenic kinase inhibitors, and radiation therapy [90].

The ability to combine with the other anticancer agents greatly expands the number of malignancies where IAP antagonists could be used. The list of cancer types where these combinations were demonstrated in cell culture and in in vivo xenograft tumor models includes but is not limited to pancreatic, lung, colon, prostate, breast, ovarian, and skin cancers as well as chronic and acute leukemia [90]. Probably the most efficacious combination involves IAP antagonists and death receptor agonists. In many cancer cell lines that are otherwise resistant to TRAIL or death receptor agonistic antibody treatments, IAP antagonists can lower the apoptotic threshold and promote synergistic killing of tumor cells [122, 133–135]. Although c-IAP proteins play a role in this combination, antagonism of XIAP is instrumental for bypassing the need for mitochondrial amplification of death receptor-stimulated apoptosis [122, 135, 136]. The synergistic combination of IAP antagonists and death receptor agonists also does not require TNF signaling, because it relies on the abrogation of XIAP-mediated inhibition of caspases [109, 123]. Thus, even in those cells and tissues where IAP antagonists do not stimulate NF-κB activation and TNF production, antagonism of XIAP will allow efficient combination with death receptor agonists in large number of malignancies.

5.6.4 Clinical Development and Future Perspectives of Targeting IAP Proteins

At least five different IAP-targeting small molecules have entered clinical trials to test their applicability for anticancer treatments. Genentech, Novartis, Aegera Therapeutics/Human Genome Sciences, Tetralogic Pharmaceuticals, and Ascenta Therapeutics have concluded, or are about to conclude, the Phase I clinical trials with their monovalent and bivalent IAP antagonists [90]. These antagonists stimulated similar responses, and neither of them seemed to invoke serious safety concerns (at least based on the data available at the moment). Monovalent compound GDC-0152 from Genentech was the first IAP antagonist to enter human clinical trials [137]. Administration of GDC-0152 demonstrated linear pharmacokinetics over a wide range of doses with no signs of significant toxicity [137]. LCL161, an orally available monovalent IAP antagonist from Novartis, was well tolerated in cancer patients, and no dose-limiting toxicities were reported. Treatment with LCL161 showed target antagonism as evidenced by c-IAP1 degradation and upregulation of cytokines monocyte chemoattractant protein 1, MCP1, and IL-8 [90]. Similarly, bivalent antagonists HGS1029 and TL32711 from Aegera Therapeutics/Human Genome Sciences and Tetralogic Pharmaceuticals, respectively, demonstrated proportional pharmacokinetics across their dose ranges and were well tolerated although transient lymphopenia and neutrophilia were reported in some patients [90]. At higher dose these agents promoted downregulation of c-IAP1, elevation of MCP1, and processing of caspases 3 and 7.

Future clinical trials should test the efficacy of IAP antagonists alone and in combination with death receptor agonists, chemotherapeutics, or other targeted agents such as kinase inhibitors. However, to develop IAP antagonists as therapeutic agents it will be critical to identify predictive markers and diagnostic tools that will

allow selection of the optimal patient population for this approach. Since c-IAP1 degradation occurs in tumor and non-tumor cells, as well as in responsive and non-responsive cancer cells, loss of c-IAP1 could be a potential indicator of IAP antagonist activity but not predictive of treatment outcome. Evidence of cell death, manifested as caspase activation in some cases but not always due to the possible necroptosis induction, would thus serve as a more definitive indicator of efficacy. But above all, the presence of TNF—a crucial mediator of IAP antagonist-stimulated cell death pathways—could provide a very meaningful gauge of the sensitivity of cancer cells and tissues for IAP antagonist treatment. Nevertheless, TNF is relatively unstable, and developing reliable tools for its accurate detection in patient samples presents challenges that still need to be resolved.

The controlled regulation of cell death and survival is essential for proper maintenance of organismal homeostasis, and deregulated cell death can lead to developmental defects, infections, and cancer. IAP proteins are evolutionarily conserved ubiquitin ligases that regulate cell death and various signaling pathways (e.g., NF-κB, MAPK, NOD) to control cellular fate. Elevated expression in many cancer types, potent anti-cell death activity, and contribution to survival signaling pathways make IAP proteins promising targets for therapeutic intervention in human malignancies. However, our understanding of IAP proteins and cellular processes they regulate is far from complete, and future studies, both in basic research and in clinical setting, should focus on fully unraveling the biological roles of IAP proteins and thereby open up additional therapeutic strategies.

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Chapter 6
Cell Death and Cancer

David L. Vaux

Abstract Research into the mechanisms and regulation of cell death has not only given insights into how cancers arise but also provided targets for the development of novel treatments. A cancer is a clonally related population of cells that persists, in part, because cells with altered DNA have been able to survive. It follows that genetic mutations that prevent a cell from killing itself are likely to promote the development of cancer. On the other hand, a therapy for cancer will be effective if it causes cancer cells to die at a greater rate than the rate at which new cancer cells are produced, provided that the treatment does not cause damage to normal cells that are incompatible with survival of the patient. As most conventional chemotherapies provoke cell suicide in addition to having direct toxic effects, cancer cells that express high levels of cell death inhibitors will be resistant to treatment but might be susceptible to a combination of conventional chemotherapy combined with drugs that antagonise cell death inhibitory proteins, such as members of the Bcl-2 and IAP protein families.

Keywords Bcl-2 • IAPs • Cancer • Apoptosis • Navitoclax • BH3-only • Smac mimetic

6.1 Evading Apoptosis Is a Hallmark of Cancer

In their influential review, Hanahan and Weinberg listed six alterations that are essential for malignant growth, the "Hallmarks of Cancer" [1]. This list included oncogenic changes such as unlimited ability to divide and the ability to invade

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tissues. One of the hallmarks was the ability to evade programmed cell death (apoptosis). Nevertheless, as pointed out by John Kerr in 1972, apoptotic cells can still be found in every cancer [2].

These two superficially contradictory observations can be reconciled if we consider that a tumour will grow as long as the rate of cell division exceeds the rate of cell death. Therefore, reduced expression of genes that are required for apoptosis, or increased expression of genes that inhibit apoptosis, is likely to promote neoplasia, even if some cancer cells still die by apoptosis.

Indeed, Kerr's observation that apoptotic cells can still be found offers a degree of hope: if cancer cells still carry the effector machinery to kill themselves, all we need to do is to find a drug that increases the rate at which it is triggered so that the rate of cell death exceeds the rate of cell division, and the cancer will be cured. Cell death mechanisms are not only involved in causing cancer, but they also might provide an Achilles' heel.

The first evidence implicating failure of apoptosis as a contributing factor in the development of neoplastic disease came with the realisation that the function of Bcl-2, the product of the gene translocated in follicular lymphoma, was to inhibit apoptosis, rather than to promote cell division, like other oncogenes known at the time [3]. Since then there has been an abundance of experimental evidence in animal models showing that failure of apoptosis can promote cancer as well as correlative data from human studies of associations between cancer and abnormal expression of genes involved in cell death (Table 6.1).

6.1.1 Bcl-2 Is the Archetype of a Novel Class of Oncogenes: Inhibitors of Cell Death

Cancers begin with acquisition of an inheritable (genetic or epigenetic) change in a single cell. The first oncogenic mutations to be discovered were those that activated genes encoding growth-promoting proteins [4, 5] or those that inactivated genes that normally prevent cell proliferation [6, 7]. As translocations that cause over-expression of Bcl-2 are strongly associated with follicular lymphoma, the finding that the Bcl-2 protein does not regulate cell proliferation but inhibits physiological cell death [3] identified inhibitors of cell death as a novel class of oncogenes.

Formal in vivo proof that inhibition of cell death by Bcl-2 could cause cancer came through creation of transgenic mice that over-express Bcl-2. While transgenic mice expressing Bcl-2 alone develop a disease characterised by accumulation of non-malignant cells, rather than cancer [8], when Bcl-2 transgenics were crossed with Eμ-myc transgenic mice to make Bcl-2-Eμ-myc double transgenics, they developed leukaemia very quickly [9]. Together with the in vitro results [3], these experiments showed that inhibition of cell death per se is not sufficient to transform a normal cell to malignancy but that inhibition of cell death can contribute to malignancy by stopping elimination of cells with damaged DNA or cells over-expressing growth-stimulating oncogenes such as c-myc.

Table 6.1 Cell death genes implicated in cancer

Gene	Function	Evidence	Reference
Bcl-2	Inhibits cell death mediated by Bax and Bak	Association: Translocated in follicular lymphoma. Experimental: Accelerated leukaemia when crossed with c-myc transgenic mice	[3, 9, 58]
p53	Promotes apoptosis by inducing Puma (but has other functions as well)	Association: Li–Fraumeni syndrome in humans, loss-of-function mutations in >50 % human tumours Experimental: Spontaneous tumours in p53 KO mice	[15, 59]
MDM2	Ubiquitylates p53, causing it to be degraded	Amplified in tumours	[60, 61]
Bax	Promotes apoptosis	Mutated in some tumours	[62]
XIAP	Inhibits caspases	Elevated expression variety of tumours	[63, 64]
ML-IAP	Not known	Amplified in melanomas	[32]
cIAP1 and cIAP2	Keep levels of NIK low, allow TNF to efficiently activate p65/RelA, ubiquitylate RIPK1	Amplifications in many tumour types cIAP2 translocations in MALT lymphoma	[31, 36, 65]

Mutation or misexpression of practically any gene can cause cell stress, provoking an apoptotic response. This has resulted in many genes that do not have a primary role in regulating cell death being assumed to be cell death genes. For example, c-myc, survivin

In a similar way, transgenic mice expressing other anti-apoptotic Bcl-2 family members Mcl-1 and Bcl-x [10, 11] or mice with deletions to pro-apoptotic genes such as Puma or BIM [12, 13] show perturbed cell populations, develop malignancies or accelerate the development of neoplasia in cancer-prone strains of mice.

In follicular lymphoma the increased level of Bcl-2 is due to t14:18 translocation that puts the Bcl-2 gene under control of the immunoglobulin regulatory regions. Genetic analysis of different types of cancers has shown that there are other mechanisms that allow altered expression of Bcl-2 family members. For example, the microRNAs miR-19, 25, 32 and 92 have been reported to decrease the expression of the pro-apoptotic Bcl-2 family member BIM, miR-125b has been reported to decrease the levels of Bak and miR-483-3p has been reported to decrease the levels of Puma (reviewed in [14]).

Because expression of genes for anti-apoptotic proteins such as Bcl-2 and Bcl-x, or reduced expression of genes for proteins required for apoptosis, such as Bax and Bak, would reduce the likelihood of a cancer cell killing itself, such cells would be at an advantage in the Darwinian competition among tumour cells for survival. While some of the genetic mutations mentioned above, such as translocations involving Bcl-2 genes, appear to occur early in the course of disease, cells with other changes, such as epigenetic alterations or loss of heterozygosity, could be selected for during all stages of the disease, including during the course of treatment with conventional chemotherapeutic agents.

6.1.2 The Tumour-Suppressor p53 Promotes Apoptosis

The tumour-suppressor gene p53 is the most commonly mutated gene in human cancer [15]. Humans heterozygous for mutations in p53 (Li–Fraumeni syndrome) and mice with loss of one or both copies of p53 spontaneously develop tumours of multiple types. The discovery that p53 is required for thymocytes to undergo apoptosis due to radiation-induced DNA damage [16], together with the discovery that p53 is a direct transcriptional inducer of the pro-apoptotic BH3-only Bcl-2 family members Puma and Noxa [17], suggested the straightforward model that p53 prevents the development of cancer by inducing apoptosis. Unfortunately, it turned out to be much more complicated.

Although it is clear that p53 can cause arrest of the cell cycle by inducing the cyclin inhibitor p21, and can activate cell death by turning on Puma and Noxa, mice with a form of p53 that cannot drive transcription of Puma, Noxa or p21 do not develop spontaneous tumours as $p53^{-/-}$ mice do. Therefore the key tumour suppressor function of p53 is something other than through the induction of apoptosis [18, 19].

6.1.3 IAPs Are Over-Expressed in Many Cancer Types

Another class of cell death regulators that have been implicated in cancer are the inhibitor of apoptosis (IAP) proteins, which were first identified in viruses that infect insect cells. All IAPs bear from one to three bacluoviral IAP repeat domains (BIRs), so human IAPs are also known as "BIR-containing" proteins or BIRCs. Insect IAPs are required to inhibit cell death during development and are antagonised by small pro-apoptotic proteins that bind to their BIR domains. Mammalian IAPs cIAP1, cIAP2, XIAP and ML-IAP all bear BIR domains and also a carboxy-terminal RING domain that allows them to function as ubiquitin E3 ligases. While deletion of genes for cIAP1, cIAP2 or XIAP individually results in only a very mild phenotype, deletion of genes for cIAP1 together with those for cIAP2 or XIAP results in embryonic lethality at E10 [20].

The smallest of the BIRCs is survivin. As it is expressed by cancer cells of all types, but is rarely detectable in normal tissues, it was initially assumed that survivin is an oncogene that promotes cancer by inhibiting cell death [21]. However, genetic and biochemical studies have shown that rather than being a cell death inhibitor, survivin acts together with aurora kinase B, INCENP and borealin to allow proper segregation of the chromosomes during mitosis [22, 23]. Because survivin is only expressed during mitosis, it is only present at detectable levels in tissues with a high proportion of dividing cells, such as tissues in the embryo and in cancer cells. The correlation between presence of survivin and cancer is not because survivin is an oncogene, but it is because survivin is needed for cell division. As survivin is ubiquitylated and degraded at the end of telophase, screens for drugs that reduce levels of survivin hit on any drug that reduces the rate of cell division and might not directly target survivin at all [24].

However, even though survivin is not an oncogene, and not an inhibitor of cell death, it may nevertheless be a bona fide chemotherapeutic target, because if survivin is blocked, it leads to death of cells by mitotic catastrophe because they are unable to segregate their chromosomes.

Like survivin, BRUCE (BIRC6) appears to have a role in cell division, rather than cell death [25], and NAIP (BIRC1) is involved in innate immune responses to intracellular pathogens [26, 27].

In contrast, there is abundant evidence implicating the other BIRCs, i.e. XIAP, cIAP1, cIAP2 and ML-IAP in cancer, but this evidence is largely correlative. For example, the locus bearing the closely linked cIAP1 and cIAP2 genes is often amplified in human cancers [28–30], the cIAP2 gene is commonly translocated in MALT lymphomas [31], and ML-IAP was first identified because it is expressed at high levels in melanomas [32]. However, to date no one has reported an IAP transgenic mouse that spontaneously develops cancer, thus providing formal proof that IAPs can act as oncogenes. Furthermore, the fact that mutations that inactivate cIAP1 are sometimes found in multiple myeloma raises the possibility that in some cell types IAPs might act as tumour suppressors rather than oncogenes [33].

6.1.4 Function of IAPs

XIAP (but not other IAPs) is able to bind to processed caspases 9, 3 and 7 and block their proteolytic activity. Therefore, cells with high levels of XIAP might be able to prolong their survival after the apoptosome is activated. However, as the apoptosome is activated after cytochrome c is released from the mitochondria and binds to Apaf-1, these cells might be doomed already due to loss of mitochondrial function [34]. This, and the fact that deletion of XIAP genes has much less effect than deletion of genes for cIAP1 and cIAP2 [20], suggests that XIAP will have a weaker oncogenic effect than the cIAPs, and targeting it alone would be unlikely to have a strong therapeutic effect.

cIAP1 and cIAP2 bind to TNF receptor-associated factors (TRAFs) 1 and 2 and are required for efficient activation of canonical (p65/RelA) NF-κB by TNF [35, 36]. By interacting with TRAF2 and TRAF3, cIAPs keep the levels of NIK low and thus limit processing of p100 NF-kB2 to the active form, p50 NF-κB2. If genes for cIAP1, TRAF2 or TRAF3 are deleted, levels of NIK rise, and there is spontaneous activation of p50 NF-κB2, even in untreated cells. Thus, cIAP1 and, to a lesser extent, cIAP2 tend to switch signalling away from non-canonical NF-κB towards canonical (p65/RelA) NF-κB.

One of the key substrates that is ubiquitylated by cIAP1 is RIPK1 [35–37]. In doing so, cIAP1 helps RIPK1 form a platform that allows activation of NEMO and the IKK complex which leads to ubiquitylation of IκB. In the absence of cIAPs, RIPK1 is not ubiquitylated following TNF treatment, IκB is not degraded and p65/RelA NF-κB is not activated, but the un-ubiquitylated RIPK1 is able to activate FADD and caspase 8 to cause the death of some types of cells.

6.2 Cell Death in Cancer Therapy

6.2.1 The Goal of Cancer Therapy Is to Remove or Kill All Malignant Cells

All cells are mortal. Any living cell will die if a process that is required for its ongoing survival is blocked (Fig. 6.1). As there are many complicated mechanisms and processes needed for a cell to survive, such as the ability to maintain integrity of the plasma membrane, a need for a source of energy for metabolism and the ability to produce new proteins, there is a vast array of physical and chemical insults that given for enough time and at sufficient intensity will kill a cell. It is therefore easy to come up with toxins that can kill cells, including cancer cells.

The challenge of cancer therapies is to find treatments that kill the cancer cells but do not kill so many normal cells that the patient also dies.

In order to have a "therapeutic window", in other words a range of levels at which cancer cells are killed but normal cells survive, it is necessary that there are molecular differences between the cancer cells and the normal cells. These differences might be differences in surface proteins that can be recognised by antibodies, differences in dependence on metabolic substrates, differences in the ability to use alternative pathways or differences in the ability to mount an effective stress response and restore homeostasis.

6.2.2 Cells Are Complex Systems That Endeavor to Maintain Homeostasis

Normal cells can act autonomously to respond to chemical and physical changes by mounting the so-called stress response that helps them restore homeostasis. For example, if a cell is heated above 37 °C, proteins begin to unfold and denature. Cells have mechanisms to detect misfolded proteins and produce heat-shock response proteins to refold them correctly or to target them to the proteasome to be destroyed. In addition to production of heat-shock proteins, metallothioneins, chaperones, proteases and DNA repair enzymes, if a cellular stress is severe or prolonged, many cells respond by activating their physiological cell death mechanism and commit suicide. Although the details of what causes a cell to switch from trying to save itself to giving up and committing suicide are not known in detail, over-expression of Bcl-2 can often prolong survival of cells exposed to a wide variety of stressors, so the predominant mechanism of suicide is the Bax/Bak-dependent pathway that Bcl-2 can block. Consistent with this notion, many BH3-only members of the Bcl-2 family become activated during oxidative stress, ER stress, heat stress or DNA damage [38, 39].

As they divide, cancer cells undergo a Darwinian-like process of selection for sub-clones that can produce the most offspring. If a cancer cell stops making proteins—such as secreted or structural proteins—that are not required for it to divide,

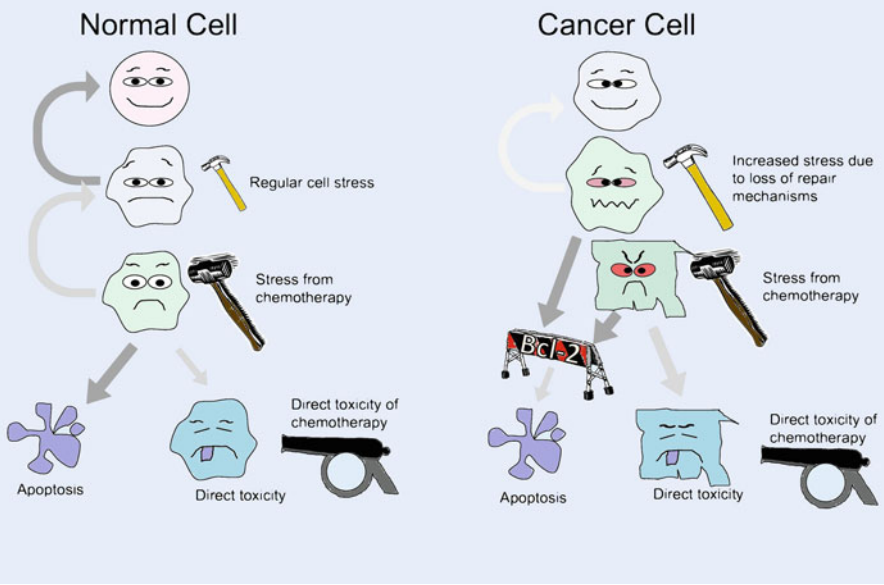

Fig. 6.1 Killing normal and cancer cells. All cells are mortal and will die if a process is stopped that is required for their ongoing survival, such as when cells are exposed to very high doses of chemotherapy (*canons*). Unfortunately, such treatment will kill normal cells and result in death of the patient. Rather than being killed by some external agent, the fate of most normal cells is to kill themselves by a physiological process that exists for that purpose. In addition, normal cells respond to cell stresses (*small hammer*) that arise due to protein misfolding, etc. by activating heat-shock and repair mechanisms and restore homeostasis. If there is a much stronger stress, for example due to chemotherapy (*sledgehammer*), normal cells may undergo apoptosis as a stress response. This is the cause of many of the side effects of chemotherapy. Due to genetic instability and Darwinian selection, cancer cells often lose components of their repair and stress response mechanisms and can accumulate misfolded proteins. Those cancer cells with intact apoptotic pathways die, but those that have higher levels of anti-apoptotic proteins (*road block*) have a selective advantage. Novel chemotherapeutic agents remove the block imposed by anti-apoptotic proteins to allow cancer cells to undergo apoptosis due to accumulated endogenous stress (*claw hammer*) as well as in response to chemotherapy (*sledgehammer*)

it might be able to proliferate more rapidly. If it loses the ability to produce p53 or DNA repair enzymes, its genome may become unstable, and it may be able to mutate and evolve even more rapidly. Cells that have lost the ability to coordinate and maintain intracellular homeostasis might activate their stress-induced cell death pathways and undergo apoptosis, but selective pressure would favour clones that have lost components of the cell death pathway or have abnormally high levels of cell death inhibitors and are thereby able to survive.

Consistent with this, amplification of genes for cell death inhibitors or abnormally high levels of their mRNA or protein have been observed in many types of cancer. For example, high levels of Bcl-2 are seen in chronic lymphocytic leukaemia, and amplification of the cIAP genes is seen in oesophageal cancers.

6.2.3 *Conventional Cancer Therapies Cause Cell Death Both by Direct Toxicity and by Inducing Cell Suicide*

Empirically discovered cancer treatments such as ionising radiation, inhibitors of DNA replication or inhibitors of nucleic acid or protein synthesis can cause tumour cells to die by interfering with a process needed for them to survive. These agents have severe side effects, because they target normal dividing cells as well as cancer cells. Where they do have efficacy, it is often because the normal cells have resting (non-cycling) stem cells that are thereby resistant to the drugs but can subsequently activate and replace the cells that were damaged. For example, chemotherapy designed to kill leukaemia cells also kills many normal blood cells, but if the dose is chosen correctly, after treatment quiescent bone marrow stem cells become active and the blood cells recover.

As well as killing cancer (and normal) cells in this way, exposure to chemotherapeutic drugs at lower doses (that might be insufficient to directly kill a cell) can also cause cell death by causing cell stress and triggering apoptosis as a stress response. Therefore, drugs that antagonise cell death inhibitory proteins such as Bcl-2 family members or IAPs would be expected to lower the threshold at which tumour cells respond to stress induced by conventional treatments and undergo apoptosis. Preclinical data in which Bcl-2 or IAP antagonists (see below) have been combined with chemotherapeutic agents reveal strongly synergistic effects, consistent with this model.

6.2.4 *Cell Death and Resistance to Chemotherapy*

6.2.4.1 Novel Treatments That Use Our New Understanding of Physiological Cell Death

Bcl-2 Inhibitors

The strong evidence linking over-expression of Bcl-2 to follicular lymphoma suggested that inhibiting Bcl-2 or reducing its expression might be a good treatment for this disease. An antisense DNA approach was the first to be tried in the form of Genasense/Oblimersen [40], but its performance in clinical trials has not yet justified its approval for clinical use (Table 6.2).

Several small molecules have been developed that bind to and inhibit anti-apoptotic Bcl-2 family members. Development of obatoclax [41] and gossypol and its derivatives, such as AT-101 [42], are thought to target a wide range of anti-apoptotic Bcl-2 family members and act like BH3-only proteins to inhibit them and thereby allow apoptosis to occur. As they show some ability to kill Bax/Bak double-knockout cells, it is likely that they also have some additional cytotoxic activity that is independent of their ability to antagonise Bcl-2 family members [42].

Table 6.2 Candidate therapeutic agents that target cell death pathways

Anti-cancer therapies that target cell death pathways	Function	Comments	Further information
Bcl-2 antagonists ABT-263 (navitoclax)	Inhibits Bcl-2, Bcl-xl, Bcl-w	In phase II trials. Causes temporary platelet depletion	[43, 45]
ABT-199	Inhibits Bcl-2	In phase II trials, promising for CLL	
Genasense	Antisense to Bcl-2	Failed phase III trial for melanoma	[66, 67]
TRAIL	Activates FADD–caspase 8 death pathway	Has undergone clinical trials, but not yet approved	[68]
TNF	Activates FADD–caspase 8 death pathway	Used in isolated limb perfusion for soft tissue sarcoma and melanoma	[69]
CD95L	Activates FADD–caspase 8 death pathway	Caused too much liver toxicity to be used clinically	[70]
Smac mimetics	Binds to cIAP1 causing it to degrade; binds to XIAP displacing caspases	In phase I and phase II trials	[71, 72]
XIAP antisense	XIAP antisense	AEG35156 has been used in phase 1/2 clinical trial	[73, 74]
Nutlin	Binds p53 preferentially to mdm2	Aims to increase the levels of p53 protein	[56]
Phikan083	Stabilises mutant p53	Binds to a common mutant form of p53	[75]
Steroids	Induce expression of Bim and Puma	Induce apoptosis of lymphoid cells	[76]
Radiation	Causes DNA damage and induces p53		[16, 77]
Conventional chemotherapy	Disrupts essential processes to directly cause cell death and, at lower doses, induces an apoptotic stress response		[78]

Both conventional treatments and novel agents that directly target apoptotic pathways mainly cause tumour cell death by activating physiological cell death mechanisms

The most exciting Bcl-2 family antagonist is ABT-263 (navitoclax) [43]. As Bcl-xl was shown to determine platelet lifespan [44], the finding that treatment with navitoclax causes a drop in platelet levels was a strong indication of a specific on-target effect, even though it was a dose-limiting side effect [45]. Currently there are many trials underway testing navitoclax and other related Bcl-2 family antagonists either as single agents or in combination with conventional chemotherapeutics [46, 47].

Smac Mimetics

Mammalian IAP-binding proteins were identified by immunoprecipitating proteins bound to XIAP or in assays for proteins that could release caspase activity [48, 49]. The similarity of the amino-terminal residues of the mammalian IAP-binding proteins such as Smac/Diablo and HtrA2/Omi with those of the insect IAP antagonists suggested that the first four amino-acids of Smac/Diablo were responsible for binding to the BIRs of XIAP to displace active caspases and bound to the BIRs of cIAPs to antagonise them [50, 51]. This prompted several companies to develop "smac-mimetic" compounds that bind to the IAPs in a similar way. When smac-mimetic compounds are added to cells, they can bind to the BIRs of XIAP and displace active caspases, but when they bind to the BIRs of cIAP1 they cause it to auto-ubiquitylate and be degraded by the proteasome [35, 36, 52, 53].

Smac-mimetic compounds produced by several companies, including Novartis, Tetralogic, Genentech and Ascenta, have entered phase 1 clinical trials [54].

P53-Activating Drugs

As p53 is the most commonly mutated gene in human cancer, but some of the mutations do not prevent production of p53 but just make it unstable, several groups have developed compounds that either bind to p53 and stabilise it [55] or prevent it from being ubiquitylated by MDM2 [56]. Another approach is to exploit synthetic lethality—because many tumour cells lack p53, they are unable to induce p21 and arrest so that damaged DNA can be repaired efficiently. Thus, a combination of a drug that induces a p53-dependent arrest in normal cells, with a drug that kills dividing cells, might improve the specificity of the cytotoxic effect [57].

6.3 Conclusions

The explosion in understanding the biology and mechanisms of apoptosis has provided new understanding of how cancers arise, how they respond to conventional chemotherapy and how they acquire resistance. It has also led to the development of novel pro-apoptotic compounds that might be used alone or in combination with conventional anti-cancer treatments. The next steps will involve empirically testing these agents in the clinic.

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Chapter 7
The DNA Damage Response Mediates Apoptosis and Tumor Suppression

Katherine Baran, Diego Rodriguez, and Douglas Green

Abstract Cells encounter stress on a daily basis that can damage their DNA and promote malignant transformation, yet the latter rarely occurs. The DNA damage response (DDR) is a highly coordinated signaling pathway that functions to detect and repair DNA damage in cells, inhibiting transformation. However, when DNA damage is so severe that it cannot be repaired, the DDR promotes apoptosis, thus preventing the propagation of abnormal cells. The tumor suppressor protein, p53, is one of the most essential molecules keeping DNA damage in check. Here, we discuss the signaling cascades that activate p53 upon DNA damage and the molecular mechanisms that mediate p53-dependent and -independent apoptosis. Moreover, we discuss the signals that trigger the DDR during malignant propagation and the importance of DNA damage-mediated apoptosis in preventing tumorigenesis.

Keywords DNA damage • p53 • ATM • ATR • Chk1 • Chk2 • Apoptosis • Mitochondria • Tumor suppression

7.1 DNA Damage Induced Cell Death

This chapter is aimed at understanding the molecular processes that drive cell death in response to DNA damage. We briefly explore the DNA damage response (DDR) pathway, and how cells translate DNA damage signals into appropriate biological outcomes. The mechanism by which the tumor suppressor, p53, induces apoptosis in response to DNA damage is identified, as are other p53-independent pathways. Finally the role of DNA-damage induced apoptosis in the prevention of cancer is explored.

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7.2 The DNA Damage Response

Every day, cells encounter numerous stresses and toxins that damage DNA, yet these cells rarely become tumorigenic. This is primarily due to the DDR which functions to detect and repair DNA damage in cells, to arrest the cell cycle, and to remove the cells if the damage cannot be repaired, through senescence or apoptosis [1–4].

DNA lesions can be generated through normal physiological processes such as DNA replication which can result in DNA mismatches and DNA double-strand breaks (DSB) as well as free oxygen radicals generated as a result of cellular metabolism [5, 6]. In addition, DNA can also be damaged by exposure to environmental stresses such as ultraviolet (UV) light and ionizing radiation (IR) [7]. Lesions are repaired through repair mechanisms such as homologous recombination (HR), non-homologous end joining (NHEJ), or mismatch repair (MMR) [8]. During the process, cells undergo cycle arrest to allow time for optimal repair and to prevent the damage from being passed on to future generations [8]. Should the extent of DNA damage be too severe, the cells undergo permanent arrest (senescence) or death (apoptosis). A tightly coordinated molecular signaling cascade determines these cellular responses (Fig. 7.1). This section will focus primarily of the signaling events of the DDR.

The signal transduction pathway of the DDR is controlled by the PI(3)K (phosphatidyl-inositol-3-OH kinase)-related kinases (PIKKs): ATM (ataxia telangiectasia mutated) and ATR (ATM- and Rad3-related) [9]. ATM kinase is induced after DNA damage, whereas ATR kinase is induced as a result of stalled replication forks during DNA replication [10, 11]. Here we will discuss the three different stages in the ATM/ATR-dependent signal transduction cascade: (1) Identification of DNA damage results in the activation of the ATM /ATR kinases and their recruitment to the site of damage. (2) Checkpoint mediator proteins enhance ATM/ATR kinase function. (3) ATM/ATR-phosphorylated effector proteins mediate cellular responses to damage.

Sensing DNA damage: In unstressed cells ATM kinase activity is low since the protein exists as an inactive dimer, blocking the active kinase domain [12]. DNA damage-induced DSBs mediate a conformational change in ATM, resulting in its auto-phosphorylation and dissociation of the dimer complex to an active monomeric form [12]. Recently, a complex of the proteins Mre11–Rad50–Nbs1 (MRN) has been shown to act as a sensor of DSBs, aiding in the recruitment of ATM to the site of DNA damage [13]. ATR does not require a conformational change to mediate its kinase activity. Instead ATR remains inactive through its association with ATR interacting protein (ATRIP), and becomes activated when recruited to the site of a stalled replication fork through its interaction with the single-strand DNA-binding protein, replication protein A (RPA) [14, 15]. Once activated and recruited to the site of DNA damage, ATM and ATR can phosphorylate their relative substrates, which include hundreds of proteins [16]. Effective ATM/ATR function is dependent on checkpoint mediator proteins, which create a scaffold for amplification of kinase function.

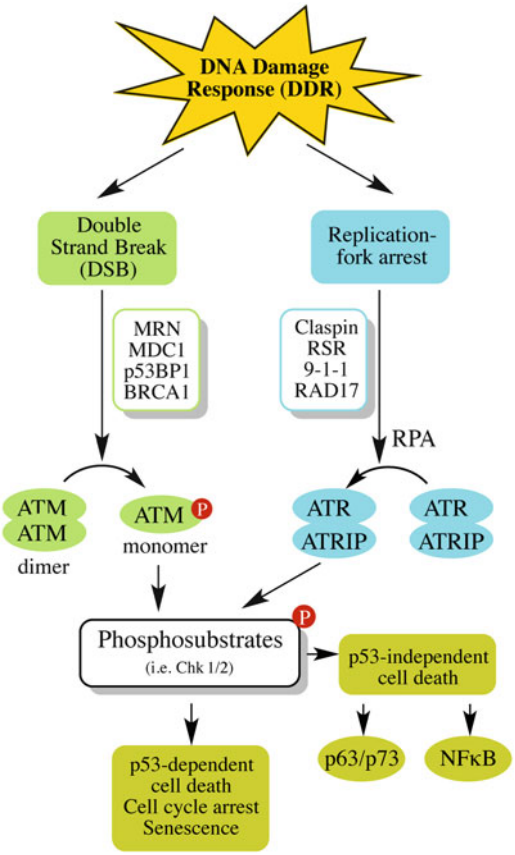

Fig. 7.1 The DNA Damage Response (DDR): DNA stress in the form of double-strand breaks (DSB) or stalled replication forks trigger ATM and ATR kinases. DNA lesions promote the auto-phosphorylation of ATM and a shift from an inactive dimer to active monomer. ATM is recruited to the site of damage by MRN complex (Mre11–Rad50–Nbs1). A complex between ATR and ATRIP renders ATR inactive, and it is activated through RPA recruitment to the site of a stalled replication fork. The kinase activities of ATM and ATR are amplified in DNA damage associated foci formed by the phosphorylation of H2AX. This scaffold promotes the recruitment of checkpoint mediator proteins that amplify the kinase activity of ATM (Mdc1, p53BP1 and Brca1) and ATR (Claspin, RSR, and the 9-1-1 complex). Active ATM and ATR phosphorylate numerous target proteins; the most commonly recognized are Chk1, Chk2, and p53 that function in regulating the cell cycle and inducing apoptosis or senescence. ATM and ATR can also phosphorylate other proteins that mediate apoptosis in a p53-independent manner

Checkpoint mediator proteins enhance the kinase activity of ATM/ATR: The variant histone, H2AX, is phosphorylated at the site of DNA damage by ATM and ATR, creating a scaffold for the recruitment and binding of numerous checkpoint mediator proteins, resulting in the formation of DNA damage associated foci [17, 18]. The exact function of these damage-associated foci are unclear, but they may serve

to enhance the signal transduction pathway [19]. Instead, phosphorylated H2AX and the associated damage foci are most commonly used to identify cells with damaged DNA. The ATM-related mediator proteins include: Mdc1 (mediator of DNA damage checkpoint 1), p53BP1 (p53 binding protein 1), and Brac1 (breast cancer type 1 susceptibility protein) [20–25]. ATR-related mediator Claspin, RSR (RAD17-containing complex), and the 9-1-1-complex (RAD9-RAD1-HUS1) all serve to amplify ATR kinase activity in DNA-damage foci [26–29]. Cells lacking mediator proteins show defective DDR and cancer susceptibility, indicating their importance for propagating ATM/ATR kinase activity [2].

Effector proteins mediate the cellular response to DNA damage: More than 700 proteins are phosphorylated in an ATM/ATR-dependent manner; however, the checkpoint-transducer serine/threonine kinases, Chk2 and Chk1, are by far the most prominent downstream targets of ATM and ATR respectively [16, 30]. The major function of Chk1 and Chk2 is to induce cell cycle arrest at the G1–S, S, and G2–M phases of the cell cycle by inhibiting the CDC25 (cell division cycle) family of phosphatases (CDC25a, CDC25b, and CDC25c) that normally activate the cyclin-dependent kinases (CDKs) [2, 31]. Chk1 and Chk2 also mediate the regulation of various proteins directly involved in DNA repair through transcriptional and post-translational mechanisms. Thus, these two very important functions of the Chk proteins facilitate DNA repair in a rapid and efficient manner. Another key target of both ATM and Chk2 is the tumor suppressor protein, p53 and its negative regulator MDM2 (murine double minute) [32, 33]. p53 functions as a transcription factor that mediates the expression of proteins involved in cell cycle arrest, senescence, and apoptosis (Fig. 7.3) [34, 35]. Expression of p21/WAF1 is induced by p53, and this cyclin-dependent kinase inhibitor 1 (or CDK-interacting protein 1) functions to mediate G1 cell cycle arrest by binding to and inhibiting the activity of cyclins [36]. This process takes longer, but aids in maintaining the G1 arrest initiated by CDC25 inhibition, further facilitating DNA repair. If the damage to the DNA is irreparable, cells can undergo permanent cell cycle arrest (senescence) or apoptosis. P53 can facilitate these responses, but other p53-independent yet ATM-modulated proteins can also be involved.

7.3 Death in the Absence of DNA Damage

Interestingly, although most chemotherapeutic agents induce a DDR and apoptosis in tumor cells, cisplatin can also induce apoptosis independently of DNA damage. In this context, cisplatin induces endoplasmic reticulum (ER) stress-mediated apoptosis [37, 38]. Moreover, Poly(ADP-ribose) polymerase (PARP) proteins which detect single-strand DNA breaks and recruit repair machineries, may also promote an ER stress response independently of DNA [39, 40]. ER stress promotes an accumulation of unfolded proteins, inducing the unfolded protein response (UPR) [41]. The UPR is a complex and dynamic network of signaling pathways that function to maintain cellular homeostasis by reducing the levels of unfolded proteins. If ER

stress is prolonged the UPR triggers apoptosis. Below, we will focus primarily on cellular responses to DNA damage per se.

The role of p53-dependent and independent mechanisms of apoptosis in response to DNA damage will be discussed in the section below.

7.4 DNA-Damage Mediated Apoptosis

Apoptosis is an active cell death process that functions during normal development and cellular homeostasis and is important in tumor suppression, maintaining host defense to pathogens, and the response to cellular stress [42]. A family of cysteine proteases called caspases orchestrates apoptosis, and a subset of these, the executioner caspases, cleaves hundreds of substrate proteins, resulting in cell shrinkage, chromatin condensation, and membrane blebbing [43, 44].

Caspases can be activated by several pathways of apoptosis; the best characterized of these are the death receptor pathway and the mitochondrial pathway of apoptosis. Although the exact molecular signals are not entirely clear, DNA damage can trigger both of these pathways of apoptosis through p53-dependent and independent means. This section will focus on the molecular pathways of p53-dependent and -independent apoptosis induced by the DDR.

The death receptor pathway of apoptosis: This is a tightly regulated signal transduction pathway that is mediated by a subset of the tumor necrosis factor receptor (TNFR) super family, the death receptors [45]. Signals to die are generated once membrane-bound death receptors such as TNFR1, CD95 (Fas), and TRAIL-R are recognized by their specific death ligands (TNF, CD95L, TRAIL) [46]. Trimerization of the receptor follows, with accompanying recruitment of adaptor molecules such as Fas associated death domain (FADD) through a DD–DD homotypic interaction [47]. Adaptor molecules recruit the initiator caspases (8 and 10) through their shared death effector domains (DED), thus forming the death inducing signaling complex (DISC) [48, 49]. The DISC functions to activate the initiator capsases through their respective dimerization and intramolecular cleavage [50]. Once active, initiator caspases cleave and activate the executioner caspases (3 and 7) as well as BID, which stimulates the mitochondrial cell death pathway (Fig. 7.2) [44].

The mitochondrial pathway of apoptosis: This pathway is defined by the permeabilization of mitochondria and release of proteins, including cytochrome c, from the inner mitochondrial space (IMS) into the cytosol [51, 52]. Cytochrome c binds to and triggers the oligomerization of apoptotic protease activating factor-1 (APAF-1) [53, 54]. This provides a signaling platform called the apoptosome for the activation of the initiator caspase-9 and consequent executioner caspases-3 and -7 [55, 56]. The BCL-2 (B cell CLL/lymphona-2) family consists of both pro- and anti-apoptotic proteins that regulate the integrity of the outer mitochondrial membrane (OMM) [57, 58]. The anti-apoptotic proteins such as BCL-2, BCL-xL (BCL-2-related gene, long isoform), MCL-1 (myeloid cell leukemia 1), and A1 (BCL-2 related gene A1) are generally localized on the OMM to promote membrane integrity by inhibiting

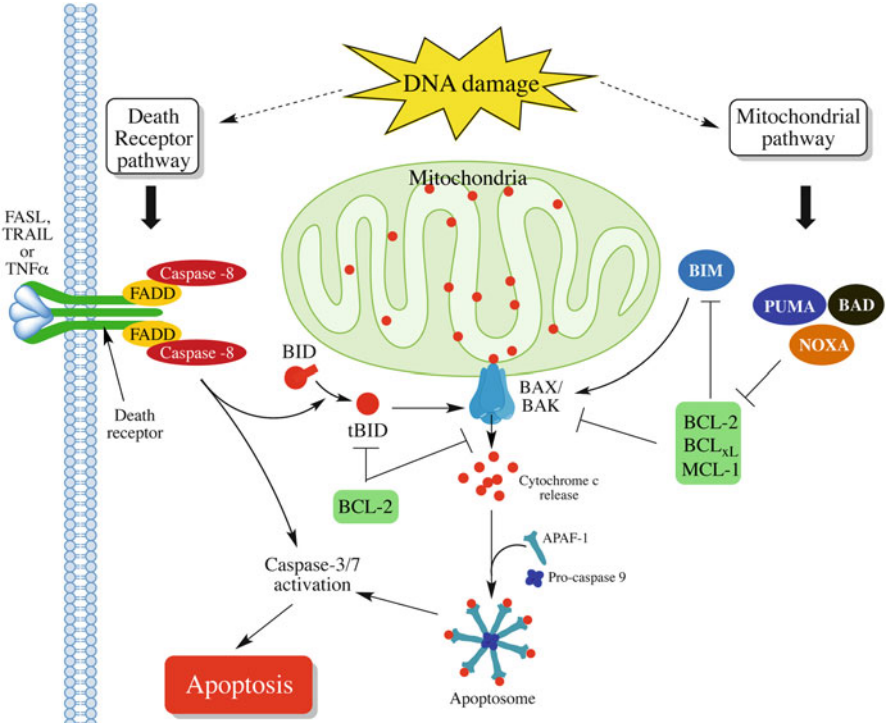

Fig. 7.2 The Death Receptor and Mitochondrial pathways of Apoptosis: DNA damage triggers the death receptor and mitochondrial apoptotic pathways. Death ligands that bind to their respective receptors trigger the recruitment of adaptor molecules (FADD), and subsequent recruitment and activation of caspases to mediate apoptosis. Activated caspases can also cleave Bid to promote the mitochondrial pathway of apoptosis. This pathway is defined by mitochondrial outer membrane permeabilization (MOMP) and is regulated by the BCL-2 proteins. Pro-apoptotic proteins such as BID or BIM promote BAX or BAK homo oligomerization in the mitochondrial membrane, whereas anti-apoptotic proteins such as BCL-2 and BCL-xL inhibit this process. De-repressor proteins BAD, PUMA or NOXA bind the anti-apoptotic proteins and reduce the threshold for BAX/BAK activation. MOMP results in cytochrome c release into the cytosol, which promotes APAF-1 oligomerization, caspase activation, and apoptosis

the pro-apoptotic family members [59]. The pro-apoptotic proteins consist of two groups that promote cell death. Upon activation, the effector proteins BAX (BCL-2 associated x proteins) and BAK (BCL-2 antagonist killer 1), homo-oligomerize and cause mitochondrial outer membrane permeabilization (MOMP) [60, 61]. The BH3-only "direct activator" pro-apoptotic proteins, such as BID or BIM, function to directly activate BAX or BAK [58, 62, 63]. In contrast, the BH3-only "de-repressor or sensitizer" pro-apoptotic proteins such as Bad, PUMA (p53-upregulated modulator of apoptosis), and NOXA bind to anti-apoptotic proteins and lower the threshold required for BAX or BAK activation [58, 64]. Thus, the specific interactions between the pro- and anti-apoptotic proteins directly mediate MOMP (Fig. 7.2).

7.5 p53-Dependendent Pathways of Apoptosis in Response to DNA Damage

p53 has been termed "guardian of the genome" because it prevents the malignant transformation of cells by inducing a variety of cellular effects such as cell cycle arrest, senescence, and apoptosis (Fig. 7.3) [65, 66]. P53 loss predisposes the host to a variety of spontaneous and induced tumors [67]. Moreover, tumor cells routinely induce p53 instability and loss with more than 50 % of tumors lacking p53 function [68]. This highlights the importance of p53 as a tumor suppressor protein. P53 is a transcription factor whose targets are largely responsible for these effects.

In unstressed cells, p53 levels remain low due to its constant degradation through the ubiquitin–proteasome pathway. MDM2 is an E3 ubiquitin ligase that regulates p53 levels via ubiquitylation [69]. MDMX/MDM4 (murine double minute x/4) is

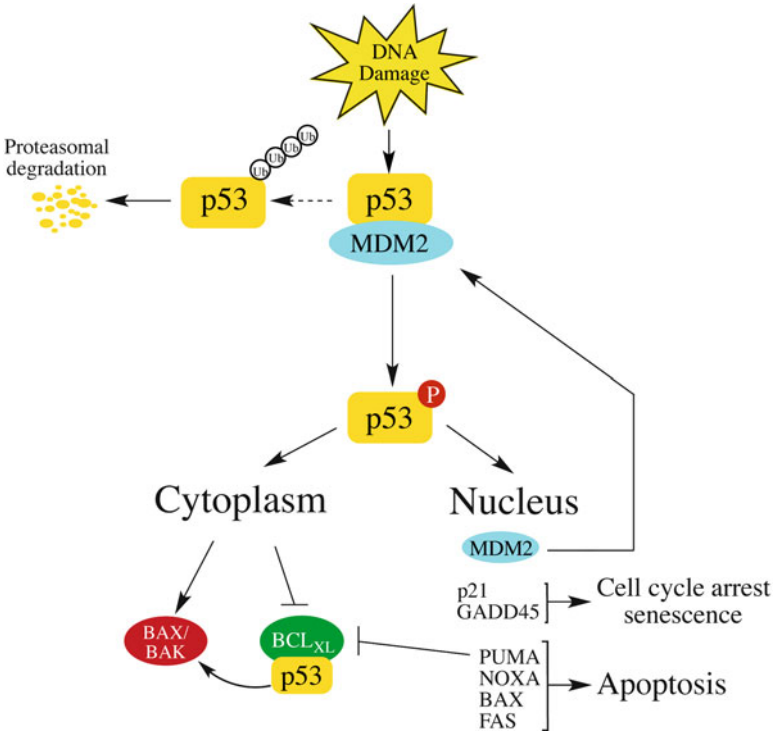

Fig. 7.3 DNA damage induced p53 promotes apoptosis in a transcription-dependent and -independent manner. In unstressed cells p53 levels are low due to its interaction with MDM2 where MDM2 polyubiquitylates p53, promoting its proteasomal degradation. Upon DNA damage residues within p53 and MDM2 are posttranslationally modified generating a fully functional, stabilized p53 protein. P53 binds to the promoter regions and transactivates numerous genes that control the cell cycle, senescence, and apoptosis. P53 can have transcription-independent functions in the cytosol and act analogously to a pro-apoptotic BH3 only protein by promoting MOMP

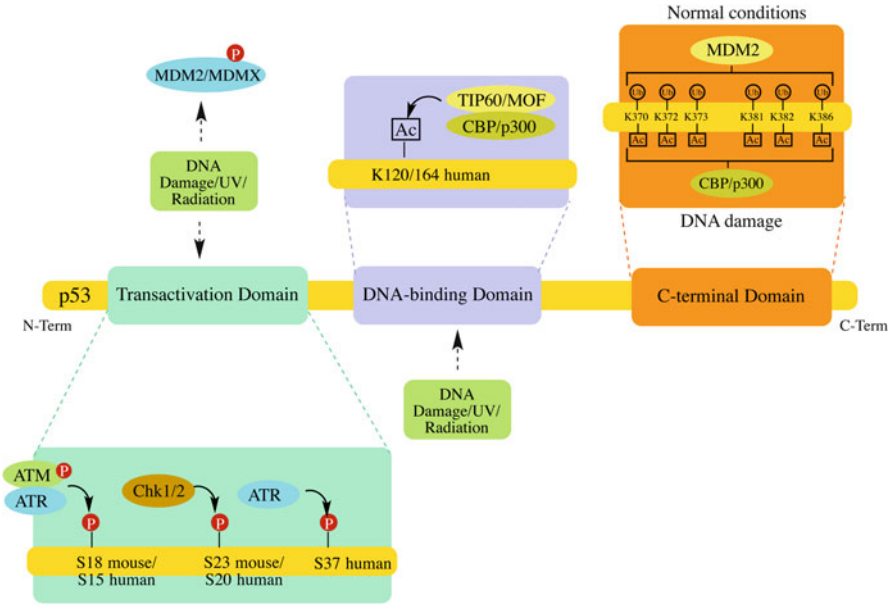

Fig. 7.4 DNA damage results in posttranslational modifications of p53 and its negative regulators, MDM2 and MDMX, to promote apoptosis: After DNA damage the N-terminus of p53 is phosphorylated by ATM, ATR, CHK1, and CHK2 at different serine residues. This serves to inhibit the interaction of p53 with its negative regulators MDM2 and MDMX and promote its transcription function. MDM2 and MDMX are also phosphorylated by ATM resulting in the stabilization of p53 levels through its inability to be exported to the cytosol and polyubiquitylated at its C-terminus. Upon DNA damage, residues in the C-terminus of p53 that are normally ubiquitylated by MDM2 are now acetylated, sumoylated, or neddylated to induce sequence-specific DNA binding and promote p53-dependent apoptosis. Acetylation of lysines (K120/K164) in the DBD of p53 can potentiate the apoptotic function of p53 after DNA damage

also an E3 ubiquitin ligase, and an additional negative regulator of p53, although this function is not via p53 ubiquitylation but through MDM2 stabilization [70]. MDM2 is a transcriptional target of p53, and thus controls its protein level by a simple feedback loop (Fig. 7.3) [71, 72]. Upon DNA damage, p53 becomes active in two ways: the inhibition of the interaction of p53 with its negative regulators, and through various posttranslational modifications that promote its transcription function (Fig. 7.4) [32, 35]. The specific regulation of p53 function will be discussed in greater detail below. Stabilized and active p53 binds to the promoter region of numerous pro-apoptotic target genes of the mitochondrial cell death pathway such as BAX, PUMA, NOXA, and BID and represses the expression of both BCL-2 and BCL-xL [35, 73]. P53 also mediates the expression of CD95 and TRAIL-R1, to facilitate the death receptor pathway of apoptosis (Fig. 7.3) [35, 73].

In addition to its transcription function, studies have shown that p53 can mediate death through transcription-independent means (Fig. 7.3) [34, 74, 75]. In this setting,

p53 functions analogously to a BH3-only pro-apoptotic member of the BCL-2 family, although the exact mechanism by which it mediates MOMP remains unclear. Cytosolic p53 can directly activate and oligomerize BAX [76, 77]. Additionally, mitochondrially localized p53 can promote BAK oligomerization to induce MOMP and cytochrome c release [78, 79]. Other studies suggest that cytosolic p53 can bind and neutralize the anti-apoptotic function of BCL-2 and BCL-xL, promoting MOMP in an indirect manner [80, 81]. Further studies suggest that the cytoplasmic function of p53 may also require its transcription function [82]. BCL-xL binds and inhibits cytosolic p53, which can only be released by PUMA [83]. PUMA-BCL-xL binding promotes p53 release and subsequent MOMP via BAX oligomerization, due to a very precise interaction between a tryptophan residue near the PUMA BH3 region (W71) and a histidine residue within the BCL-2 groove of BCL-xL (H113). The biological significance of cytoplasmic p53-mediated death remains unknown. Experimental evidence suggesting a role in tumor suppression is still lacking, but remains a very interesting topic to be investigated. The exact mechanism by which transcription-independent p53 is regulated by DNA damage is also unclear and will be discussed in detail below.

7.6 The Regulation of p53 Levels in Response to DNA Damage

One of the most interesting topics regarding p53 function is how the cell determines whether to undergo repair, cell cycle arrest, senescence, or apoptosis in response to DNA damage. There is some suggestion that the strength and duration of the damage signal is important, as is the tissue type or compartment undergoing stress. However, what is probably the clearest requirement for a differential p53 response are the posttranslational modifications of various residues within the protein.

P53 is a 393-amino-acid protein (390 in mouse) consisting of distinct functional domains (Fig. 7.4) [84]. The N-terminus contains the transactivation domain (TAD) where transcription co-activators p300 and CBP (CREB-binding protein) bind to promote the transcription function of p53 [85]. MDM2 and MDMX also bind in this region to regulate p53 levels [69, 86]. A proline-rich domain, of unclear function, follows this region [87]. The core region of p53 consists of its DNA-binding domain (DBD) and is also the region in which many cancer-associated mutations are found [88]. The tetramerization/oligomerization (OG) domain promotes oligomerization and the C-terminal region is the regulatory domain that can be either ubiquitylated to mediate proteasomal degradation of p53 or undergo a variety of posttranslational modifications such as acetylation, phosphorylation, neddylation, sumoylation, and methylation to regulate the cellular function of p53 [86, 89, 90].

N-terminal p53 regulation: N-terminal phosphorylation is necessary to inhibit MDM2 binding and promote p53 stabilization [91–93]. Following DNA damage, ATM, ATR, Chk1, and Chk2 all contribute to phosphorylate a number of serine and

threonine residues in the N-terminus (Fig. 7.4) [33, 94, 95]. It remains unclear whether an individual kinase is responsible for phosphorylating one or more residues upon DNA damage, or whether numerous kinases are required. It also remains unclear whether one or more residues must be phosphorylated to inhibit MDM2 interaction. A knockin mouse containing individual phosphorylation mutants S18A (human S15) and S23A (human S20) or combined S18/23A mutations was generated to address this question. Individual mutations showed little difference in p53 stabilization upon DNA damage in numerous tissues [96, 97]. However, p53 was unable to be stabilized in response to DNA damage in the combined S18/23A mutant mouse [98]. Although there are other serine residues that are phosphorylated in the N-terminal region upon DNA damage, their specific function in vivo remains unclear. It is possible that in addition to stabilizing p53, N-terminal phosphorylation may be important for the recruitment and binding of transcription co-factors to specific gene promoter regions, thus influencing cellular response to stress.

Modification of MDM2 and MDMX by ATM stabilizes p53: DNA damage induced kinases stabilize p53 not only through modification of p53 residues, but also through posttranslational modification of residues within the negative regulators, MDM2 and MDMX (Fig. 7.4). Numerous residues near the C-terminal RING domain of HDM2 are modified by ATM in vitro including; S395, T419, S425, and S429 [99]. A knockin mouse containing an S394A (S395 human) mutation, demonstrated that phosphorylation of this residue is important for p53 stabilization after DNA damage in vivo [100]. This mechanism of p53 stabilization remains unclear; some experiments suggest that S395 phosphorylation prevents the nuclear export of p53 and its subsequent ubiquitylation and degradation, while other experiments suggest that ring domain oligomerization is inhibited, thus inhibiting the ubiquitylation function of MDM2 [101, 102]. Interestingly another study proposed that ATM-dependent phosphorylation of MDM2 switches its function from a negative to positive regulator of p53 by binding p53 mRNA and promoting the translation of p53 [103].

Recently ATM has also been shown to decrease the levels of the deubiquitylation enzyme USP7/HAUSP (Ubiquitin-specific-processing protease 7/herpesvirus-associated ubiquitin-specific protease) [104]. Since MDM2 levels are determined through self-ubiquitylation, diminished USP7 can stabilize p53 [105, 106]. It has long been thought that MDM2 regulates the levels of MDMX through ubiquitylation; however, it is becoming more apparent that DNA damage induced kinases can also directly modify residues within the C-terminal region of MDMX, thus inhibiting its function and promoting p53 stabilization. MDMX can be phosphorylated by ATM and Chk2 kinase on S342, S367 and S403, enhancing its specificity for MDM2-mediated binding and degradation [107, 108]. Other E3 ligases such as COP-1 (constitutive photomorphogenetic 1), Pirh2 (p53-induced RING-H2 protein), and HUWE1 (HECT-domain ubiquitin ligase) can mediate low p53 levels through ubiquitylation [109–111]. DNA damage can also regulate these proteins and stabilize p53; for instance ATM can phosphorylate COP-1 on S387 and stimulate its rapid degradation [109].

C-terminal p53 regulation: The C-terminal domain of p53 contains numerous lysine residues that are heavily modified upon DNA damage through acetylation, sumoylation, methylation, and neddylation (Fig. 7.4). In non-stressed cells, the lysine residues in this region are constantly ubiquitylated by MDM2 to promote p53 degradation [69, 86]. In stressed cells, modification of these same residues may help to promote sequence-specific DNA binding, thus influencing the cellular response to stress [90]. However, recent studies using p53 knockin mice where 6 or 7 of the extreme C-terminal lysines were modified, showed little difference in p53 stability or response to stress [112, 113]. This suggests that perhaps other residues in the DNA-binding site (K120 or K164) can contribute to p53 stability and function [114, 115]. Either way, the exact role of C-terminal modifications after DNA damage in mediating p53 cellular effects remains unclear. Perhaps the most interesting new development in this area of research pertains to the idea that p53 is bound to DNA and fully active in the absence of DNA damage and is held in a "repressed state" by it negative regulators MDM2/MDMX [116]. This idea is based on the observation that MDM2 and MDMX deficient mice are embryonic lethal due to p53-mediated death and this lethality can be rescued upon p53 deletion [117–119]. Furthermore, a knockin mouse, $p53^{QS}$, that retains DNA-binding potential but cannot interact with its negative regulators MDM2/MDMX is also embryonic lethal [120]. The various posttranslational modifications that are required to release p53 from this repressed state are currently being elucidated.

Posttranslational modifications of p53 that mediate its transcription-independent function: The posttranslational modifications of p53 that mediate its cytosolic function remain unclear. MDM2 and the E3 ligase MSL2 (male specific lethal 2) are both proposed to monoubiquitylate p53 to promote mitochondrial localization [121–123]. These E3 ligases do not directly shuttle p53 to the mitochondria; instead this function is performed by the mitochondrial chaperone, Tid1 [124]. Once localized to the mitochondrial membrane, HAUSP deubiquitylates p53 to promote MOMP [125]. A recent study has identified that K351 mutation in p53 cannot be monoubiquitylated by MDM2 or MSL2 and cannot be localized to the mitochondria, suggesting that this is the critical residue for modification and subsequent cytoplasmic p53 function [126]. Furthermore, MDM2-mediated monoubiquitylation can promote other modifications of the p53 C-terminal lysine residues, such as sumoylation of K386 by PIASy (protein inhibitor of activated STAT protein gamma) [101]. These modifications not only expose the p53 nuclear export signal (NES) but also release MDM2, further promoting nuclear export. The acetylation of K120 by Tip60/hMOF histone acetyl transferases (HATs) in the p53 DNA-binding domain may promote transcription-independent cell death by displacing Mcl-1 from BAK [127]. However, another study proposed that acetylation of C-terminal K320/K373/K382 mediates p53 binding to the DNA repair protein, Ku, thus inhibiting its interaction with BAX and inducing cell death [128]. Many tumors exhibit p53 mutations within the DNA-binding domain resulting in a transcription-deficient p53. Identifying the mechanism by which DNA damage induces and promotes p53 nuclear export and cytoplasmic/mitochondrial function may be of great value for future neoplastic therapeutics.

7.7 p53-Independent Pathways of Apoptosis in Response to DNA Damage

Although p53 clearly plays an important role in mediating death in response to DNA damage, there are many other proteins that can promote the mitochondrial and death receptor apoptotic pathways in the absence of p53. This is particularly important during tumor therapy since many tumors have mutated or nonfunctional p53. Some of these proteins will be discussed below.

p63 and p73: These proteins are part of the p53 family of transcription factors and they share similar homology, and some transcriptional targets and functions with p53 [129]. Following DNA damage all three family members have the potential to mediate cell cycle arrest and apoptosis [129]. Therefore, it is probable that in response to DNA damage p63 and p73 can compensate for lack of p53 function and mediate death in some settings. Similar to p53, p63 and p73 contain an N-terminal TAD, a central DBD and an OG domain. Unlike p53, they contain a sterile alpha motif (SAM) in their C-terminal regions that promotes protein binding. Two different promoters at the N-terminus of the gene generate two different isoforms; Transcriptional activator (TA)-containing isoform (TAp63 and TAp73) and N-terminal truncated isoform (ΔNp63 and ΔNp73) [130]. Alternative splicing at the C-terminus gives further rise to numerous isoforms of p63 and p73 [130]. TAp63 and TAp73 mediate their cellular effects primarily through transcription-dependent means, whereas ΔNp63 and ΔNp73 function as dominant negative proteins since they can bind to the promoter binding sites but are unable to transactivate gene expression [131]. Like p53, levels of TAp63 and TAp73 increase upon DNA damage and their transactivation function is mediated primarily by posttranslational modifications, although the exact nature and regulation of these modifications still remain to be fully elucidated [132–134]. p63 and p73 transactivate and/or increase the expression of BAX, PUMA, NOXA, BAD, APAF1, caspase-3, -8, and -9, CD95, TNFR, and TRAIL-R1 to influence cell death pathways [135–137]. In addition, a transcription-independent function has been attributed to p73, but not p63. Full length TAp73 or a caspase-cleaved p73 fragment were found localized to the mitochondria, and mediated apoptosis [138, 139]. Interestingly, purified p73 could also induce MOMP on isolated mitochondrial fractions [138]. This function of p73 is still quite controversial and requires further validation in vivo.

TAp63-induced death may be tissue specific since TAp63 is expressed strongly in oocytes and mice deficient for TAp63 were resistant to DNA damage mediated oocyte death [140]. In response to DNA damage, TAp63 induced primordial follicle oocyte death through the induction of PUMA and NOXA [141]. Therefore, p63 may play an important role in female reproduction and oocyte maintenance independently of p53 function.

p63 and p73 may also display tumor suppressor functions. Mice heterozygous for both proteins display spontaneous tumor formation at a rate that is only slightly longer than that seen with p53 heterozygous mice [142]. Furthermore, mice lacking only the TAp73 isoforms displayed a high incidence of spontaneous tumor formation [143]. Unlike p53, however, p63 and p73 are rarely mutated in cancers, but the

N-terminal truncated version of both proteins is very commonly over-expressed [144]. These results all indicate a role in tumor suppression for p63 and p73.

NF-κB transcription factor is induced by DNA damage: NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) mediates a large array of biological processes such as immune regulation and cellular growth but it can also be activated upon DNA damage to promote apoptosis [145–148]. NF-κB is a dimeric transcription factor that belongs to the Rel family of proteins that consists of RelA (p65), c-Rel, or RelB as one half, and p50 (NFKB1) or p52 (NFKB2) as the other [149]. NF-κB is inactive in the cytoplasm by virtue of its interaction with inhibitory IkB proteins, such as IkBα. The IkB kinase complex (IKK) is made up of three proteins; two catalytically active kinases IKKα and IKKβ and the regulatory IKKγ (NEMO) protein that functions to phosphorylate IkBα, inducing its ubiquitylation and subsequent proteasomal degradation, thus allowing the now unbound NF-kB to translocate to the nucleus and mediate is transcription function (Fig. 7.5) [150, 151].

NF-κB can be activated through one of two pathways, the canonical or noncanonical pathways [149]. Here we will briefly discuss the canonical pathway, since NF-κB activation by DNA damage converges with the canonical pathway of NF-kB activation at the point of IKK activation and Ikβ degradation [148, 149].

Bacterial or viral infections or pro-inflammatory cytokines such as TNFα engage the TNFR1, triggering the recruitment of TRADD (TNF receptor associated death domain), FADD, and RIP1 (receptor interacting protein 1) all through their respective death domains (DD). TRADD also binds the E3 ubiquitin ligase, TRAF2 (TNF receptor associated factor 2) that subsequently binds the cellular inhibitors of apoptosis 1 and 2 (cIAP1 and cIAP2). Together, this group of proteins makes the core of the TNFR1 signaling complex, also called complex-I. RIP1 ubiquitylation by cIAP1 recruits the kinase TAK1 (transforming growth factor β-activated kinase 1), which phosphorylates IKKβ, activating the IKK complex, resulting in the degradation of IκBα and an active NF-κB, composed mainly of p50/p65 dimers.

The exact mechanism by which nuclear ATM can activate a complex localized in the cytoplasm is unclear, but IKK-independent functions of NEMO may provide the key to this conundrum [152]. Posttranslational modifications of NEMO mediate its role in transducing DNA damage signals. NEMO is firstly SUMOylated by PIASY-1 (protein inhibitor of activated STAT protein gamma) to promote its nuclear localization and is subsequently phosphorylated by ATM on serine 85 [153, 154]. NEMO is then monoubiquitylated by cIAP1 and exported to the cytoplasm along with a fraction of ATM, upon which TAK1 becomes phosphorylated, thus activating IKK [155–157]. Nuclear translocated NF-κB transcribes genes involved in cell survival such as the anti-apoptotic genes, cIAP1&2, XIAP, $FLIP_L$, BCL-2, A1, and BCL-xL, presumably to allow the cells time to mediate DNA repair [150, 151, 158]. In some cases NF-κB can induce the expression of genes to promote cell death, such as CD95L in T cells exposed to stress [159].

DNA damage can promote cell death through the formation of two cytoplasmically localized protein complexes, complex-II and the ripoptosome (Fig. 7.5). TNF-mediated signaling induces the formation of complex-II. This complex consists of RIP1, FADD, caspase-8, and $FLIP_L$ and forms secondary to the membrane-associated

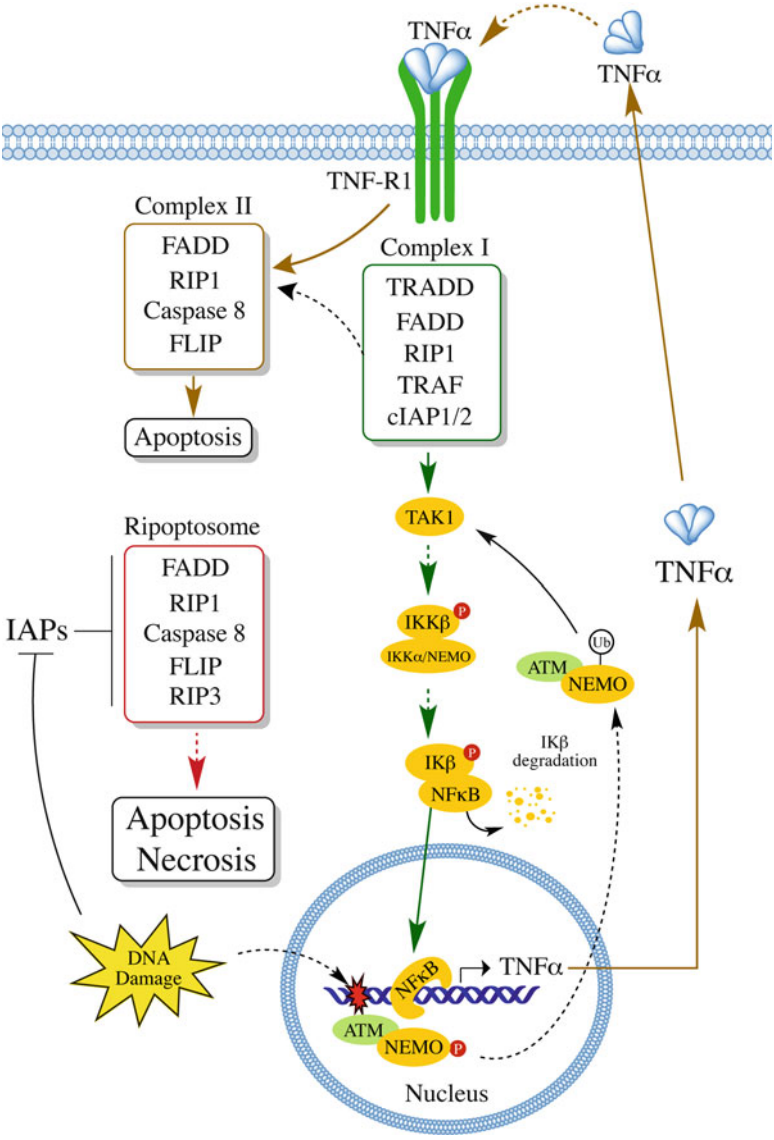

Fig. 7.5 DNA damage promotes NF-κB mediated apoptosis: DNA damage results in ATM phosphorylation of nuclear localized NEMO. The ATM/NEMO complex is subsequently translocated to the cytosol resulting in TAK1 phosphorylation and activation. TAK1 phosphorylates IKKβ resulting in IκB degradation and NF-κB activation. Persistent DNA damage promotes an NF-κB mediated TNFα feed-forward loop, resulting in conversion of complex I to the apoptotic complex II. DNA damage reduces the levels of IAPs, promoting the formation of a ripoptosome complex that promotes either apoptosis or programmed necrosis

complex-I, hours after initial TNF ligation to the TNF-R1 [160]. The molecular mechanism for transition from complex-I to complex-II remains unclear, but a recent study suggests that that strength and duration of DNA damage promotes apoptosis through a TNFα-feed forward mechanism [161]. Low levels of DNA damage induced ATM-dependent NF-κB induction of pro-survival genes, including the expression of TNFα. Newly synthesized TNFα engaged TNFR1 resulting in the formation of complex-II. Here, RIP1 is autophosphorylated resulting in FADD recruitment and caspase 8 activation and apoptosis [161]. Another study has identified the formation NEMO, RIP1, and PIDD (p53-induced death domain) complex upon DNA damage. PIDD promotes NF-κB activation through NEMO sumoylation and ubiquitylation [162]. Caspase-2 can also bind PIDD to form the "PIDDosome" and induce apoptosis [163]. Whether or not a feed forward mechanism of caspase-2 dependent apoptotic signaling can also exist remains unclear, but caspase-2 deficient cells show aberrant death after DNA damage, suggesting that maybe it does [164].

DNA damage can also mediate death independently of TNF signaling through the down regulation of cIAP1, cIAP2, and XIAP [165, 166]. In the absence of IAPs a spontaneous complex is formed in the cytosol called the ripoptosome that can induce both apoptotic and programmed necrotic cell death pathways [166]. The ripoptosome is made up of RIP1/FADD/caspase-8 with additional association of $FLIP_L$ and RIP3. The ripoptosome requires the kinase activity of RIP1 and is negatively regulated by the IAP's through the ubiquitin-mediated proteasomal degradation of RIP1 [166]. Although $FLIP_L$ is also a negative regulator of the ripoptosome, DNA damage can significantly decrease the levels of $FLIP_L$ in a cell [167]. Since FLIP can form heterodimers with caspase 8 and inhibit both apoptosis and programmed necrosis, decreased FLIP levels allow both caspase 8 homodimerization and RIP1/RIP3 association to induce apoptosis and necrosis, respectively, upon DNA damage and ripoptosome formation [168, 169].

This form of cell death may be extremely important in cancer therapy since it was shown to kill tumor cells that were resistant to apoptosis [166].

7.8 DNA Damage Response and p53-Mediated Apoptosis in the Prevention of Tumorigenesis

Aberrant proliferation is a feature commonly seen in tumor cells and is the combined result of active oncogenes and dysfunctional tumor suppressor genes [170]. p53-mediated apoptosis is one the most important mechanisms by which cells prevent oncogene dependent malignant transformation [171, 172]. The DDR pathway is crucial to control initial damage signals to the DNA that can induce genomic instability, but importantly, there is evidence that the DDR is also induced by oncogenic transformation of a cell, thus preventing propagation of early malignant cells.

In this section we discuss the signals that trigger the DDR during malignant propagation and the importance of the DDR in preventing tumorigenesis. Finally we will determine whether tumor-suppression by p53 is mediated through the DDR pathway or whether ARF (**a**lternate **r**eading **f**rame of the INK4a/ARF locus)-dependent mechanisms are preferentially utilized.

Oncogene-mediated DDR: The idea that the DDR acts as a barrier against genomic instability and cancer development came about after histochemical observations in very early neoplastic lung, colon, bladder, and skin tissue. Pre-neoplastic tissue was associated with DNA damage foci and high levels of apoptosis and senescence [173, 174]. Markers of an active DDR, such as phosphorylated H2AX, accumulation of p53BP foci and Chk2 phosphorylation were visible in pre-neoplastic and neoplastic tissue but not in normal tissue [173, 175]. Thus, DNA damage checkpoints could be activated in response to oncogenes during the early stages of malignant progression to promote apoptosis.

The mechanism by which oncogenes promote the DDR in these precancerous tissues remains unclear. Oncogenes promote cellular hyper-proliferation that is accompanied by DNA hyper-replication, resulting in replication stress [176]. Oncogene expressing cells displayed high cyclin E levels, DNA hyper-replication, increased numbers of active replicons, increased replication fork instability and LOH in common fragile sites [175, 177, 178]. These DNA damage signals promoted a specific ATR/Chk1 response with ATR and RPA found to be co-localized in H2AX foci in cells [15, 173].

Another mechanism by which oncogenes can trigger a DDR is through telomere erosion [179, 180]. Telomeres are repetitive nucleotide sequences that protect the very ends of chromosomes from instability. Neoplastic cells exhibit telomere erosion and unless lengthened by telomerase, are recognized as DSBs, promoting a DDR [181]. The shelterin complex caps telomeres for further chromosomal protection and facilitates telomerase function. Oncogene-induced mutations in this complex or telomerase can also contribute to telomere erosion and promote a DDR [180, 182].

Although it still remains unclear exactly how the DDR is induced in tumor cells, DNA damage dependent signaling pathways seem to be a crucial factor in inhibiting tumorigenesis.

DDR suppresses tumorigenesis: Mutations in DNA-damage responses allow the continued cell growth and proliferation of cells with damaged DNA and genomic alterations, thus enhancing the propensity for oncogenic transformation [183]. This is evident in cells over-expressing oncogenic Ras. Cells lacking ATM or Chk2 did not stop proliferating, were more susceptible to transformation, and formed tumors in recipient mice, whereas DDR competent cells arrested [178].

The importance of the DDR in preventing malignancy is further highlighted in mouse genetic models and human conditions in which proteins of the DDR are nonfunctional. Patients displaying defects in genes that mediate NHEJ and HR repair have syndromes such as LIG4 syndrome and FANCD1 (Fanconi Anemia) and a predisposition to developing lymphoma and leukemia [184, 185]. By the same token, genetic defects in proteins that mediate DNA damage signaling pathways

also predispose individuals to tumors. Patients with mutated ATM have a disorder called ataxia telangiectasia (AT) [186]. These patients are sensitive to γ-irradiation (IR) and are predisposed to developing leukemias and lymphomas. Furthermore, mutations in genes that encode downstream targets of ATM also predispose patients to cancers, such as Mre11 (A-T like disorder, ATLD), NBS1 (Nijmegen breakage syndrome, NBS), Brca1 and Brca2 (familial breast, ovarian carcinoma syndrome), Chk2 and p53 (Li–Fraumeni syndrome) [187–192]. In contrast, patients with an ATR mutation (Seckel syndrome) are not predisposed to cancer but display dwarfism, developmental delay, and microencephaly [193]. Similarly, mouse models of a defective DDR also show susceptibility to tumor development. Mice lacking ATM are born viable but are strongly predisposed to developing T cell lymphoma at 2–4 months of age [194]. Mice lacking the checkpoint mediator proteins H2AX and p53BP1 are also predisposed to spontaneous tumor development [195, 196].

DNA damage as an effective therapy against cancer: It may seem paradoxical that the initiation of tumorigenesis may be due to a defective DDR, yet the most common therapy used to treat malignancies involves chemotherapeutic drugs that induce DNA damage. Tumor cells usually have defective repair pathways and proliferate much faster than nonmalignant cells, making them more susceptible to treatment [197]. Without a fully functional repair process, DNA damage preferentially promotes apoptosis. Since effective DNA repair can promote resistance to therapy, inhibitors of DNA repair are currently being explored in the clinic as treatment against cancers resistant to traditional chemotherapy [198, 199].

Another reason why tumor cells may respond to better to chemotherapy is because they are "primed to die" [200]. Most normal cells that encounter unregulated oncogene expression induce an apoptotic response, but some cells survive and grow because they express high levels of anti-apoptotic proteins such as BCL-2 [201]. Importantly, the anti-apoptotic proteins in these cells are occupied with pro-apoptotic proteins such as BIM [202, 203]. These tumor cells are therefore "primed" for death with pro-apoptotic molecules and are more sensitive to conventional chemotherapy that tips the balance of this fragile equilibrium towards cell death [204].

Is the tumor suppressor function of p53 due solely to the DNA damage response? Tumorigenesis in animals lacking a functional DDR may be primarily due to a lack of p53 function. Cells deficient for ATM generally show an inability to stabilize and induce p53-mediated cellular effects after IR, suggesting the importance of ATM-mediated p53 effects in tumor suppression [205]. Furthermore p53 knock in mice that are unable to be stabilized and activated by ATM (combined S18/23A mutations in p53) develop late-onset B cell lymphomas [98, 206]. However, these lymphomas do not develop at the same rate and intensity as is seen with p53 knockout (KO) mice. This suggests that other mechanisms are still mediating p53 activation and function.

Oncogene-mediated hyper-proliferation signals can also stabilize p53 through ARF (p14ARF in human and p19ARF in mouse), and this occurs in a DNA damage-independent manner [207]. ARF is transcribed through the alternate reading frame of the INK4a/ARF locus, which also encodes p16INK4a, a cyclin-dependent

kinase inhibitor. ARF stabilizes p53 by direct binding and sequestration of its negative regulator MDM2 [208]. Mice lacking ARF are prone to developing tumors, and tumors that have high levels of ARF have silenced p53, whereas tumors that retain WT p53 function have nonfunctional ARF [209, 210]. Furthermore, mice null for both p53 and ATM show dramatic acceleration of tumor formation relative to mice deficient for one gene only, thus indicating an alternative mechanism of p53 induction in tumors [211].

Elegant studies performed in knock in mice containing a p53 estrogen receptor fusion protein (p53ERTam) that can restore p53 function upon 4-hydroxytamoxifen (4OHT) administration, have supported the idea that p53-dependent tumor suppression is attributed to ARF and not the DDR [212, 213]. In this model, p53 was required to prohibit radiation-induced lymphoma; however, the time of p53 restoration after the acute radiation response determined its tumor-suppressor function [213]. When p53 was restored in mice before IR, the mice displayed all the features of an effective DDR coinciding with high levels of apoptosis, but this did not prevent lymphoma development. However, when p53 was restored after the acute DDR was cleared, a significant delay in lymphomagenesis was observed. This effect was lost in an ARF null background, suggesting that ARF engaged the tumor suppressor function of p53 in response to the outgrowth of malignant cells after IR [213].

Furthermore, mice that contain an additional copy of the p53 gene ($p53^{Super}$) show a superior response to DNA damage inducing high levels of apoptosis and are highly resistant to tumors [214]. These mice do not confer protection against tumor development the absence of ARF.

It is possible however that the tumor suppressor function of p53 stabilized through DNA damage or ARF is not mutually exclusive. ARF-deficient cells are unable to mount an effective DDR and ARF levels can be increased upon DNA damage [215]. Therefore, additional studies are require to further elucidate the mechanism by which p53 can mediate its tumor-suppressor capabilities.

p53-mediated apoptosis in tumor suppression: The apoptotic function of p53 is primarily mediated through PUMA and NOXA. Mice deficient for PUMA have thymocytes that are highly resistant to IR, yet mice do not develop spontaneous tumors [216, 217]. However, in an Eμ-MYC model of lymphomagenesis, PUMA deficiently accelerated tumor formation [218]. NOXA-deficient mice also show a defect in DNA damage induced apoptosis, although this is specific for mouse embryonic fibroblasts (MEFs) and not thymocytes [217]. They are also resistant to spontaneous tumor formation and fail to accelerate Eμ-MYC tumorigenesis [219]. Mice lacking both PUMA and NOXA phenocopy mice deficient for PUMA alone and are resistant to tumors [220].

Mice deficient for p53-target genes that mediate the death receptor cell death pathway such as TRAIL-R1 and CD95 are also not prone to spontaneous tumors, but may accelerate tumorigenesis under some conditions [221, 222]. These experiments suggest that there may be a redundancy for p53-mediated apoptosis for tumor suppression and that the cell cycle arrest or senescent functions of p53 can compensate

in the absence of apoptosis and mediate tumor suppression. Mice deficient for p21 are generally not tumor prone, but p21 deficiency can accelerate tumorigenesis in some settings [223, 224]. Importantly, however, mice lacking PUMA, NOXA, and p21 display no spontaneous tumor formation although these studies have not been performed in the presence of a cellular stress [225]. This study indicates that the tumor suppressor function of p53 is not dependent on apoptosis or cell cycle arrest. Mice that contain mutations within the TAD of p53 are unable to mediate apoptosis or cell cycle arrest in response to DNA damage. However, p53 with a mutated TAD maintains some transactivation function and can mediate the expression of genes involved in senescence. Importantly, the expression of these genes is enough to mediate tumor suppression in these mice [225, 226]. Another study generated mice with mutations in the lysines of the DBD of p53. In this scenario, p53 was unable to express proteins involved in apoptosis, cell cycle arrest or senescence, yet these mice were also resistant to tumors [227]. A metabolic function of p53 was proposed to be tumor-suppressive in this model, representing a very interesting theme that is being explored in the p53 field at present [228].

It remains unclear why some tumor cells respond to DNA damage by mediating p53 dependent-apoptosis, senescence, or even metabolic effects. Whether it depends on the type or strength of DNA damage, or the tissue type, or the posttranslational modifications of p53 is unknown but requires further identification [229].

7.9 DNA Damage Response Promotes p53-Mediated Cell Competition in HSCs and May Promote Tumorigenesis

Recent evidence suggests that the DDR can not only mediate a cellular response to stress, such apoptosis or cell cycle arrest, but can also regulate the self-renewal and differentiation function of stem cells in a p53-dependent manner. DNA damage mediated cellular effects and DNA damage mediated stem cell effects represent two independent functions of p53 that may have opposing roles in the development of cancer. Here we will discuss how a DDR can mediate a form of cellular competition in the hematopoietic compartment by regulating HSC population. We will also discuss the contribution that the modified stem cell compartment may have in cancer progression.

Cell competition describes the clonal survival of developmentally identical cells in a compartment that has been exposed to a type of stress, such as DNA damage [230]. The “fittest” cells survive the stress and repopulate the cellular compartment. For example, when bone marrow was combined in equal measures from IR and non-IR mice and injected into lethally irradiated mice, the non-irradiated HSCs repopulated the bone marrow and therefore represent the fittest population [231]. In short, undamaged cells outcompete the damaged counterparts.

Reports suggest that DDR mediated cellular competition in the hematopoietic compartment is p53-dependent [231, 232]. HSCs with lower p53 levels displayed

greater self-renewal capacity and outcompete high p53 expressing HSCs after DNA damage. Cell competition is distinct from the p53-mediated DDR, since cells become out-competed at a much later time point after all the DNA damage has been cleared. The outcompeted cells in this scenario did not die by apoptosis but instead saw decreased proliferative capacity and a senescent phenotype, whereas the fitter cells showed better proliferation. Since high levels of DNA damage can deplete stem cell number, lower levels of p53 may be beneficial for long-term stem cell survival. However, increased self-renewal in stem cells is also associated in tumors [233]. Moreover, HSCs with mutated p53 also displayed lower levels of p53 and better competition and this may also contribute to the outgrowth of cells that contribute to malignancy [234, 235].

Another report proposed that survival of HSCs after DNA damage was dependent on ATM-mediated phosphorylation of BID [236]. ATM-dependent phosphorylation of BID at residues S61 and S78 resulted in cell cycle arrest in the S and G2 phase suggesting that BID may have a pro-survival function in response to low levels of DNA damage [237, 238]. Phosphorylated BID was unable to localize to the mitochondria and promote oxidative stress and apoptosis, thus maintaining the quiescence of HSCs [236].

Therefore, although the DDR may be crucial to inhibit cancer progression and induce tumor suppression in tissues, it may however promote the survival of tumor-initiating stem cells that repopulate the organism [239]. A clearer understanding of the mechanisms by which p53 contributes to cell competition in the hematopoietic compartment in response to DNA damage is necessary.

7.10 Concluding Remarks

We have discussed the many ways by which the DDR can trigger both mitochondrial and death receptor cell death pathways in a p53-dependent and -independent manner. The kinases of the DDR mediate detailed posttranslational modifications of p53 and its negative regulators to promote its transcription-dependent and -independent functions. DNA damage kinases can also modulate other pathways to induce apoptosis in a p53-independent manner. This represents an important “backup” for tumor suppression when p53 is deleted or dysfunctional. Although p53-mediated apoptosis is crucial for the prevention of tumorigenesis, whether this is induced through the DDR or ARF-dependent means is still unclear. Interestingly, the apoptotic effects of p53 may not be solely responsible for tumor suppression after DNA damage—senescence or even metabolic effects may play a part, thus presenting a redundancy in mechanism of p53-dependent tumor suppression. Finally, DNA damage mediated tumor suppression in cells may actually promote cellular competition in the hematopoietic compartment and induce the differentiation of stem cells to a more malignant phenotype.

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Chapter 8
Neuronal Death Mechanisms in Development and Disease

Corey L. Cusack, Ryan P. Annis, Adam J. Kole, and Mohanish Deshmukh

Abstract In this chapter we cover several distinct aspects of neuronal cell death. Apoptosis, or programmed cell death, is incredibly important for shaping a properly organized nervous system during development. However, unlike most other cell types, neurons must survive for the lifetime of the organism and therefore possess multiple, unique ways to tightly regulate cell death pathways. Neurons employ several distinct "brakes" within the apoptotic pathway to prevent unwanted cell loss and utilize signaling pathways mediated by dependence receptors and the p75 Neurotrophin Receptor to fine-tune survival and death outcomes during development. In addition, neurons undergo dynamic changes to restrict the apoptotic pathway as they mature and become fully integrated into the adult nervous system. Interestingly, several components of cell death machinery also have critical nonapoptotic roles in neurons, such as modulating synaptic plasticity or mediating neurite pruning to ensure the establishment of precise neuronal circuitry. Lastly, we provide a broad overview of the complex and multiple cell death mechanisms seen in neurons after injury and in neurodegenerative diseases.

Keywords Neuron • Nervous system • Apoptosis • Development • Apaf-1 • Bax • NGF • Neuronal maturation • Trophic factors • Dependence receptors • p75 Neurotrophic receptor • Axon pruning • Dendrite pruning • Caspases • Excitotoxicity • Alzheimer's disease • Parkinson's disease • Huntington's disease • Amyotrophic lateral sclerosis (ALS) • Stroke

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8.1 Apoptosis in Developing Neurons

As one of the longest-lived cell types in mammals, it may be unexpected that a significant number of neurons undergo programmed cell death during nervous system development. However, the activation of apoptosis is one of the crucial steps for shaping both the central (CNS) and peripheral nervous systems (PNS) during development [1].

Seminal work by Viktor Hamburger and Rita Levi-Montalcini described the surprising observation that healthy neurons routinely die during normal development [2]. Their studies ultimately led to the discovery of the first neurotrophic factor (NGF, nerve growth factor) [3–6] and the neurotrophic hypothesis, which provided insight into the basic mechanism that initiates cell death in developing neurons. In its most basic form, the neurotrophic hypothesis states that during development, an abundance of neurons are generated that must compete for limited amounts of survival-promoting growth factors secreted by target cells [7, 8]. Those neurons that reach and properly innervate their target will survive and incorporate into the developed nervous system, while those without adequate tropic factor support undergo programmed cell death and are eliminated. As many as 50 % of neurons undergo apoptosis during this developmental period, elegantly matching the number of target cells to a precise population of neurons. Multiple neurotrophins and their receptors that signal to promote survival have now been identified. These include the classical neurotrophins NGF, BDNF (Brain-Derived Neurotrophic Factor), NT3 (Neurotrophin 3), and their respective ligands TrkA, TrkB, and TrkC [9, 10]. Other neurotrophins include the GDNF (Glial-Derived Neurotrophic factor) family (e.g., GDNF, Neurturin, Artemin, Persephin) [9, 11] and various cytokines (e.g., LIF, CNTF) [12]. These various neurotrophins promote the survival of distinct neuronal populations depending on the expression patterns of their receptors.

More recently, our understanding of programmed cell death during development of the nervous system has expanded to include at least two other functions [1]. First, programmed cell death during neurogenesis limits the number of proliferating neuronal precursors in germinal regions of the developing CNS and PNS [13, 14]. Though the evolutionary advantage of limiting the number of proliferating neural progenitors is not entirely clear, this process is thought to secondarily affect the final number of neurons in the mature nervous system. Second, programmed cell death functions to correct any errors that may have occurred during the developmental process, most notably by eliminating neurons with defects in migration or axon outgrowth [15].

While the neurotrophic hypothesis correctly predicted that developing neurons undergo cell death in the absence of survival-promoting growth factors, for many years it was assumed that this death was a passive process simply due to a *lack* of survival signals rather than *active* cell death signals. We now understand, however, that neurons undergo programmed cell death via the tremendously active pathway of apoptosis, which requires cellular functions such as signal transduction, gene transcription, and translation [8, 16].

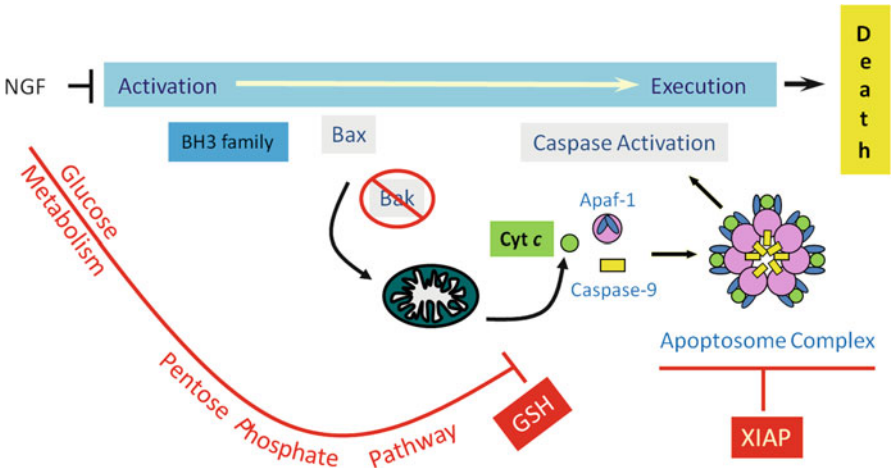

Fig. 8.1 Schematic of the apoptotic pathway in neurons (based on the model of NGF deprivation in sympathetic neurons). Apoptosis is initiated when the BH3-only members of the Bcl-2 family proteins promote Bax activation and its translocation to the mitochondria to induce the release of cytochrome *c* (cyt *c*). Cytosolic cyt *c* binds to Apaf-1 and procaspase-9 to form the apoptosome complex, resulting in caspase activation and neuronal death. Neurons engage mechanisms to inhibit this pathway at multiple points as described in the text (*highlighted in red*)

Some of the best insights into the molecular mechanisms responsible for neurodevelopmental apoptosis have been gathered from in vitro studies of mouse neurons. Models using cultured sympathetic neurons deprived of NGF to mimic the loss of neurotrophic growth factor signaling during development have found that a pathway involving mixed lineage kinase (MLK), c-Jun N-terminal kinase (JNK), and the transcription factor c-Jun leads to the upregulation of at least four genes in the pro-apoptotic BH3-only family (Bim, Hrk/DP5, Puma, Bmf) [17–25]. These BH3-only proteins activate pro-apoptotic Bax, which permeabilizes the mitochondrial outer membrane, leading to the release of cytochrome *c* from mitochondria to the cytosol [26, 27]. Following its translocation from mitochondria, cytochrome *c* binds to Apaf-1 and, together with caspase-9, assembles the apoptosome complex [28, 29]. This scaffolding complex activates caspase-3, the executioner caspase protease responsible for the downstream cleavage of several cellular substrates, ultimately leading to the cell's demise (Fig. 8.1). While the apoptotic pathway in neurons may be best-characterized in sympathetic neurons, other populations of CNS and PNS neurons also utilize the mitochondrial pathway of apoptosis and continue to be the focus of active investigation. A recent study, for example, found that approximately 40 % of developing cortical interneurons undergo programmed cell death in a Bax-dependent manner [30]. However, unlike the situation in PNS neurons where exogenous factors such as neurotrophic factors regulate apoptosis, developmental death of cortical interneurons appears to be regulated by a cell autonomous mechanism. These results highlight how developmental apoptosis in CNS and PNS neurons is likely regulated by distinct mechanisms.

The importance of programmed cell death for the nervous system is best appreciated when examining neural development in the absence of apoptosis. The failure to undergo cell death has dramatic consequences, as has been shown in mouse studies using genetic deletion of critical mediators of apoptosis. Mice lacking genes for Apaf-1, caspase-9, or caspase-3 exhibit embryonic lethality and show marked deformities of the nervous system [31–33]. The most notable morphological defects include large overgrowths of the forebrain, known as exencephaly. Interestingly, although mice deficient in Apaf-1, caspase-9, or caspase-3 show a significant excess of neurons in the forebrain, other regions such as the spinal cord and brain stem appear to develop normally, suggesting that neuronal death may still occur in these models via an atypical, nonapoptotic pathway.

8.2 Unique Molecular Features of Apoptosis Regulation in Neurons

a. *Strict regulation at multiple points*

Though neurons utilize the core apoptotic machinery to undergo cell death during development, there are several points of regulation in the apoptotic pathway that appear to be unique to neurons. Not surprisingly, these mechanisms have predominantly been found to inhibit the apoptotic pathway, providing neurons and other long-lived post-mitotic cells with the necessary "brakes" on apoptosis to support their long-term survival [34]. These restrictions on apoptosis in neurons have been found both before and after the point of mitochondrial permeabilization (Fig. 8.1).

In most mitotic cells, two pro-apoptotic proteins in the Bcl-2 family, Bax and Bak, can directly induce the permeabilization of the mitochondrial outer membrane. As such, the genetic deletion of either Bax or Bak alone is not sufficient to prevent cytochrome *c* release in most mitotic cells. In post-mitotic neurons, however, the deletion of Bax alone completely blocks cytochrome *c* release and is sufficient to inhibit apoptosis [26]. Interestingly, it has been discovered that a unique splice variant of Bak mRNA is present in neurons (Neuronal-Bak, N-Bak), which lacks the transmembrane domain required for mitochondrial membrane insertion and permeabilization [35–37]. Rather, the domain structure predicts that N-Bak functions instead as a BH3-only protein. While little is known about the function of N-Bak, redundancy between Bax and Bak is clearly eliminated in neurons, which may allow these cells to more tightly regulate and prevent the release of cytochrome *c* until absolutely necessary.

Neurons utilize at least two mechanisms to regulate cell death after the point of cytochrome *c* release. First, the activity of cytochrome *c* itself has been found to be regulated by its redox state, where oxidized cytochrome *c* is a more potent activator of caspases than the reduced form. Healthy sympathetic neurons maintain a highly reducing cellular environment that is restrictive for cytochrome *c*-mediated caspase activation [38]. For example, even when released from mitochondria directly by the Bax activator tBid, cytochrome *c* is unable to induce cell death in sympathetic

neurons despite its translocation to the cytosol. However, when the environment in neurons becomes more oxidizing, such as after growth factor deprivation [39], the conditions become more permissive for cytochrome *c* to activate caspases and induce neuronal death.

Second, caspase activation in neurons is also strictly regulated by the X-linked inhibitor of apoptosis protein (XIAP), an endogenous inhibitor of caspase-9 and caspase-3 [40]. In neurons, the ability of XIAP to inhibit apoptosis is enhanced due to the relatively low levels of Apaf-1 and the subsequent inefficient caspase activation. In contrast, the high Apaf-1 levels in many mitotic cells results in efficient caspase activation that is less effectively inhibited by XIAP [41].

For most cells, the release of cytochrome *c* from mitochondria and the concurrent loss of mitochondrial membrane potential mark the "point of no return," since cells often die from energetic failure even if caspase proteases are inhibited. The existence of post-mitochondrial brakes in neurons suggests that neurons may have the capability to survive and recover even after the point of cytochrome *c* release. Indeed, sympathetic neurons can maintain mitochondrial membrane potential for a short period even after the release of cytochrome *c* if caspase activation is prevented. Studies have shown that in the presence of caspase inhibitors, neurons can recover from NGF deprivation-induced apoptosis as long as NGF is reintroduced prior to the loss of mitochondrial membrane potential [27, 42]. This provides neurons with a window of time during which the apoptotic process can be reversed even following cytochrome *c* release.

Despite the mechanisms described above that restrict the pathway of apoptosis, neurons undergoing physiological programmed cell death during development are able to bypass these brakes and permit apoptosis. For example, upon tropic factor deprivation in neurons, the cellular environment becomes more oxidizing [38], creating an environment where cytochrome *c* is more apoptotically active and capable of inducing cell death. In addition, sympathetic neurons are known to degrade XIAP following NGF withdrawal, thereby removing its inhibitory effect on caspase activation to allow cell death [40]. However, as will be described in the next section, neurons continue to engage additional brakes on the apoptotic pathway as they mature beyond the developmental stage. These additional brakes are particularly important since neurons must survive for the rest of an organism's life following the very active period of developmental programmed cell death.

b. *Dependence receptors*

Classically, transmembrane receptors, such as growth factor receptors, are activated when bound by their respective ligands and remain relatively inactive in their unbound state. Thus, the classical model of neuronal programmed cell death assumed that any cell death resulting from the lack of receptor activation was simply due to the loss of associated positive survival signals [43]. However, data obtained over the past two decades supports a novel form of signal transduction initiated by special receptors called dependence receptors. While dependence receptors support cell survival in the presence of their ligand just as seen with other transmembrane receptors, these receptors switch to actively inducing cell death in the absence of ligand [44]. Thus, dependence receptors create a state of cellular

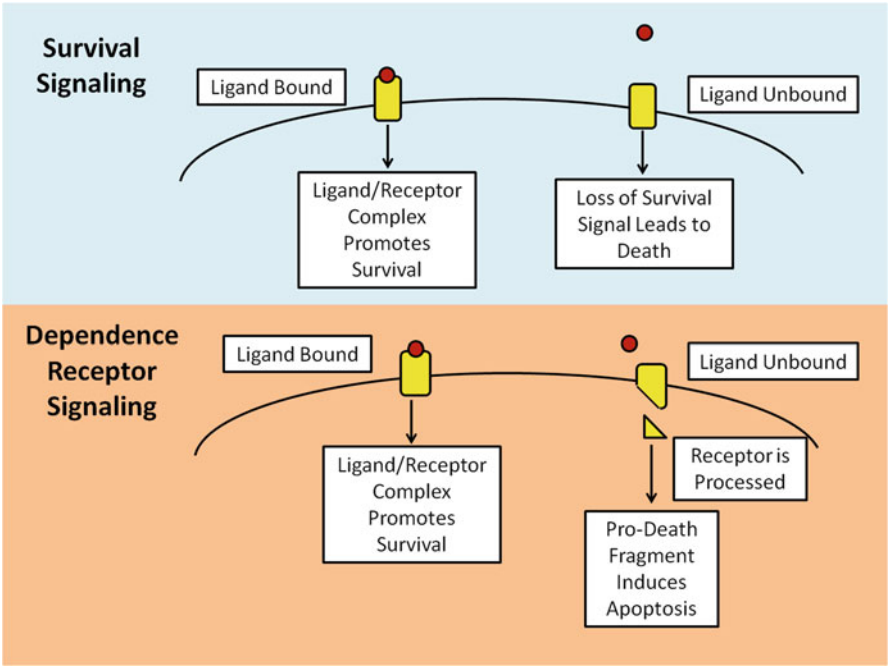

Fig. 8.2 Illustration that highlights the differences between classical death signaling triggered upon ligand removal versus that induced by dependence receptors. In classical death signaling, the pathway is initiated upon the loss of pro-survival signals. In contrast, dependence receptors convert from promoting survival to actively promoting death in the absence of the ligand. This conversion is usually accompanied by proteolytic processing of the receptor

dependence on their respective ligand. At least 15 dependence receptors have been identified thus far, though more likely exist that have not yet been discovered [45, 46] (Fig. 8.2).

Among the first dependence receptors to be characterized is the protein Deleted in Colorectal Carcinoma (DCC). DCC was discovered to promote apoptosis in the absence of its ligand, netrin, while inhibiting apoptosis when bound to netrin [47]. Curiously, the death induced by netrin is believed to be dependent on caspase-3 and caspase-9 but independent of Apaf-1 or cytochrome *c* [48]. Although, as its name implies, DCC was first discovered due to its association with cancer, it also plays an important role in neurons. Netrin–DCC signaling is crucial for axon guidance during nervous system development, and subsequent studies have found that it also plays a role in promoting the survival of some populations of neurons [49].

Another example of a dependence receptor that plays a role in neuronal development and survival is the Ret receptor. Ret complexes with the GFRα (glial cell line-derived neurotrophic factor family receptor α) receptors, of which there are four, to mediate pro-survival signals from a range of neurotrophins, including Glial Derived Neurotrophic Factor (GDNF), artemin, and neurturin [11]. While the Ret receptor's role in neuronal survival is well known, it came as a surprise when

overexpression of Ret alone in cell lines induced an apoptotic death that was rescued by treating the Ret-overexpressing cells with GDNF [50].

Many fundamental questions about dependence receptors and their function still exist. It is unclear at this point what has caused some receptors to evolve as dependence receptors while others have evolved as classic neurotrophin receptors. Indeed, recent work has found that the receptor tyrosine kinases TrkA and TrkC function as dependence receptors, while TrkB, which is closely related, behaves as a classical neurotrophin receptor [51]. In many cases it is also unclear how the absence of ligand stimulation of these receptors leads to apoptosis. Answers to these questions could have interesting implications for multiple fields, such as neural development, neurodegeneration, and cancer research.

c. *p75Neurotrophic Receptor (p75NTR)*

Another mechanism utilized by neurons to regulate apoptosis is the p75 Neurotrophin Receptor (p75NTR). A member of the Tumor Necrosis Factor (TNF) family of receptors, p75NTR is expressed in many populations of neurons during development, including sensory, sympathetic, motor, cortical, cerebellar, hippocampal, basal forebrain, and caudate putamen neurons [52]. p75NTR was initially identified as a neurotrophin coreceptor that was associated with equal affinity with all three classical neurotrophin receptors (e.g., TrkA, TrkB, TrkC) [9, 53]. In this context, p75NTR helps fine-tune the survival signals mediated via the neurotrophin ligand binding to its respective receptor (e.g., NGF binding to TrkA, BDNF binding to TrkB, and NT3 binding to TrkC) [53, 54].

Surprisingly, however, the binding of the neurotrophic ligands to p75NTR alone (in the absence of any Trk receptors) has been found to *promote* neuronal cell death. For example, stimulation of sympathetic neurons with BDNF results in p75NTR-mediated apoptosis (as these neurons lack TrkB expression) [55]. This finding was confirmed in vivo when it was discovered that p75NTR deficient mice have increased numbers of neurons [55] (Fig. 8.3).

p75NTR-stimulated apoptosis occurs through the intrinsic apoptotic pathway and requires gamma-secretase-mediated cleavage of the p75NTR intracellular domain. This domain moves from the membrane to the cytosol and forms a complex with the proteins Traf6 and NRIF. This complex promotes the nuclear translocation of NRIF and the stimulation of JNK3, which lead to apoptosis [56, 57]. Thus, p75 can have both pro-survival and pro-apoptotic effects on neurons depending on the surrounding conditions. For example, if an NGF-responsive neuron encounters BDNF during migration, the fact that this neuron expresses TrkA but not TrkB will result in BDNF-mediated apoptosis via the p75NTR. In these contexts, p75NTR-mediated apoptosis could be utilized to eliminate neurons that aberrantly end up in the wrong place at the wrong time.

While in vitro data have shown that direct binding of p75NTR by neurotrophins such as NGF, BDNF, NT-3, and NT-4 can stimulate apoptosis, there was some skepticism that direct binding of p75 by these ligands was responsible for cell death in vivo because the concentrations of neurotrophins used were thought to be far above those encountered by cells in the body of an organism. An explanation for this discrepancy was provided when researchers found that unprocessed neurotrophin

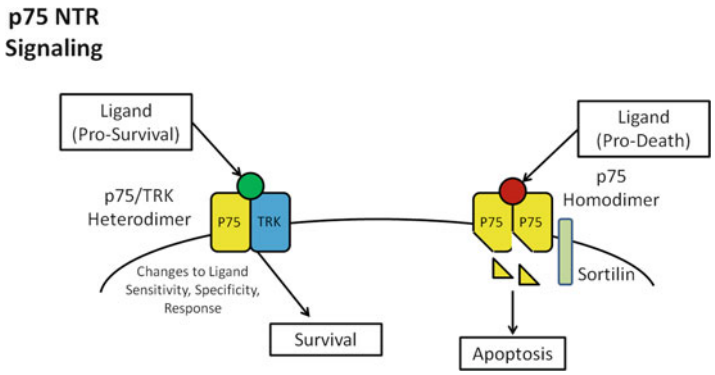

Fig. 8.3 Schematic showing the dual roles of p75 receptor in neurons. The association of p75 with Trk receptors enhances survival signaling whereas stimulation of p75 in the absence of Trk receptors promotes death signaling. In the context of pro-neurotrophic factor-induced death signaling, p75 is often associated with sortilin

precursors, known as pro-NGF and pro-BDNF, were capable of binding p75NTR with higher affinity than their mature, fully processed counterparts. Pro-NGF and pro-BDNF were found to stimulate p75NTR-dependent apoptosis but not TRK receptor-dependent survival [58]. Further work has found that the binding of these pro-neurotrophins to p75NTR is mediated by an adaptor protein called sortilin. Sortilin recognizes the pro-domain of the unprocessed neurotrophins and increases their binding affinity for p75NTR [59].

While p75NTR is expressed in many neuronal populations early in development, it is often downregulated as neurons mature, possibly to prevent the pro-apoptotic functions of p75NTR from inducing apoptosis in neurons that cannot be replaced. Intriguingly, it has been found that p75NTR expression is switched back on in a number of cases of neurodegenerative disease or injury. These include axotomy, Amyotrophic Lateral Sclerosis (ALS), ischemia, Alzheimer's disease, and excitotoxicity. Given the well-documented role of p75NTR as a pro-apoptotic receptor, this protein remains an active area of research for the prevention of neurodegenerative disease [60].

8.3 Restriction of Apoptosis with Neuronal Maturation

The apoptotic pathway has been well known to undergo dynamic changes with neuronal maturation whereby it becomes highly restricted once neurons fully develop and are integrated into the nervous system. Initial studies by Dr. Rita Levi-Montalcini demonstrated that while injection of a neutralizing antibody that inhibited NGF function in neonatal mice led to the near complete destruction of their sympathetic ganglia, similar injections had little effect on sympathetic ganglia in adult mice [61, 62]. This phenomenon can also be recapitulated in vitro, where

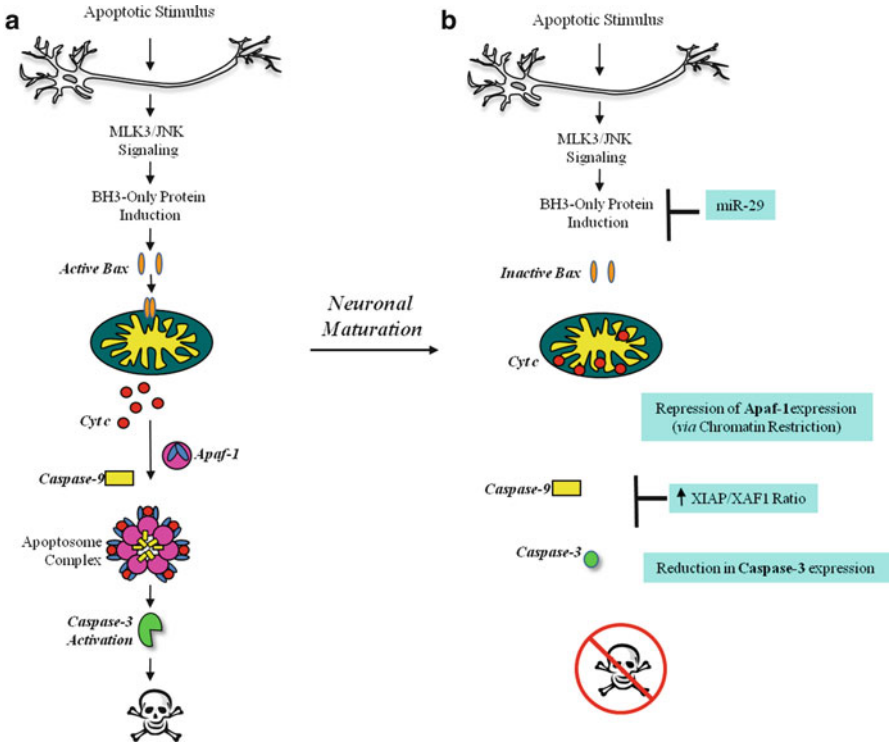

Fig. 8.4 Illustration showing the dynamic changes engaged by mature neurons to restrict the apoptotic pathway. These include the upregulation of miR-29, the transcription repression of Apaf-1, the changes in XIAP/XAF ratio (seen in motor neurons), and the reduction in casapase-3 (seen in many CNS neurons)

sympathetic neurons isolated from neonatal mice undergo apoptosis when deprived of NGF but become resistant to apoptosis with increasing time in culture. This in vitro model has been instrumental in elucidating the mechanisms engaged by neurons to restrict apoptosis as they mature. The emerging evidence indicates that mature neurons develop apoptosis restrictions at multiple points. While mature neurons deprived of NGF went through the initial steps of the apoptotic pathway, such as c-jun phosphorylation, Bax failed to become activated and cytochrome *c* was not released from the mitochondria [63]. One clue that may explain why Bax failed to be activated came from a study that identified a microRNA, miR-29, to be strongly upregulated in mature sympathetic neurons. Interestingly, miR-29 was found to downregulate multiple redundant members of the pro-apoptotic BH3-only domain proteins, thus potentially preventing them from activating Bax and triggering apoptosis [64]. In addition, mature neurons also shut down Apaf-1 transcription at the chromatin level [65], which prevents the formation of the apoptosome in the event of cytochrome *c* release (Fig. 8.4).

While the process by which maturing neurons restrict apoptosis has been best characterized in the sympathetic neuronal model, studies performed on other neuronal populations suggest that they, too, restrict apoptosis as they mature. Similar to sympathetic neurons, many other neuronal types have also been found to restrict Apaf-1 levels with increasing maturation, including sympathetic, cerebellar, photoreceptor, and cortical neurons [65–69]. However, other mechanisms by which apoptosis is restricted with maturation appear to differ between neuron types. For example, many neurons have been found to downregulate the key pro-apoptotic protein caspase-3 with increasing maturation, while sympathetic neurons do not [66–68, 70]. Also, motor neurons downregulate the endogenous XIAP inhibitor XAF, allowing XIAP to more potently inhibit apoptosis [71].

An interesting unanswered question is why would the cell not simply shut down production of all apoptotic proteins in order to maintain its long term survival? The likely answer to this is that many of the proteins in the apoptotic pathway have functions outside of their role in cell death. Cytochrome *c*, for example, has long been known to play a role in oxidative phosphorylation. More recently, nonapoptotic roles have been identified for Bax (mitochondrial fission) and caspase-3 (neuronal synaptic plasticity). Importantly, while these apoptosis restrictions are important for maintaining the long term survival of mature neurons, these brakes can be reversed or circumvented in catastrophic situations of injury or disease, as described later in this chapter.

8.4 Nonapoptotic Functions of Caspases in Neurons: Neurite Pruning and Synaptic Plasticity

In addition to apoptosis, where the entire neuron dies, neurons can also activate a program where only the neurites degenerate and the neurons survive. This process, also known as pruning, removes unnecessary dendrite and axon branches to refine neuronal connections. Neurite-specific degeneration is also required beyond development as neurons must maintain the ability to remove individual connections throughout our lifetime; this ability allows us to continually process and learn new information (Fig. 8.5).

Evidence from multiple model systems indicate that the pruning of axons and dendrites is also mediated by caspases. This was unexpected because caspase activation can put neurons at risk of undergoing apoptosis. However, emerging evidence indicates that neurons engage elegant mechanisms to spatially localize and limit caspase activation. During metamorphosis of *Drosophila melanogaster*, for example, dendritic pruning requires localized caspase activation (in this case, the upstream caspase Dronc) that is tightly and spatially regulated by the proteasome-mediated degradation of DIAP1 (Drosophila inhibitor of apoptosis protein, the fly homolog of XIAP) [72–74]. Dendritic caspase activation also appears to play a key role in learning and memory in zebra finch songbirds [75]. Specifically, active caspase-3 is located in dendritic spines of the auditory forebrain. Here, preactivated

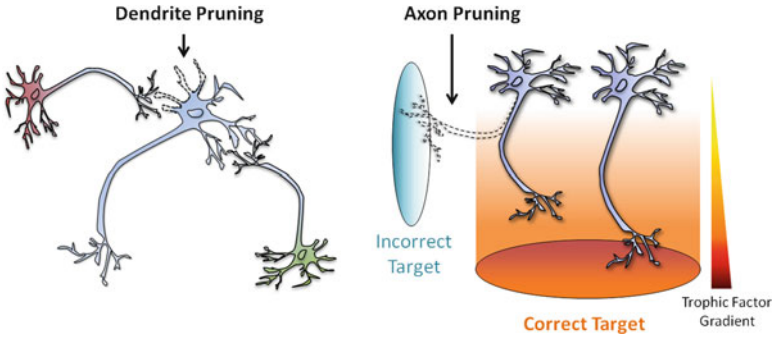

Fig. 8.5 Illustration of developmental neurite pruning that refines neuronal circuitry by removing excessive or misprojecting dendrites or axons

caspase-3 is held in check by being bound to XIAP, and appropriate stimuli trigger its release to induce synaptic remodeling.

Axon pruning removes individual axons and, when necessary, occurs on a larger scale to remove entire collateral branches. While developmental axon degeneration is central to the process that generates the adult patterns of projections in the nervous system, it is particularly important for facilitating overall system flexibility and the progressive processes of neuronal specification [76, 77]. As the induction of specific signaling and fate-determining pathways is highly regulated by which factors are encountered by a projecting axon during development, removing misguided axons is just as important as maintaining correctly projecting ones to establish proper neuronal identity.

Interestingly, the Bcl-2 family proteins have recently been shown to modulate the axon degeneration that occurs during pruning as deficiency of Bax or overexpression of anti-apoptotic protein Bcl-XL both protect axons following axon-specific nerve growth factor (NGF) deprivation [78–80]. Mice deficient for another anti-apoptotic Bcl-2 family member, Bcl-w, exhibit progressive degeneration of small fiber innervations in the skin without cell body loss, suggesting that Bcl-w is selectively induced in the axons of sensory neurons in response to tropic factor stimulation [81–83]. Furthermore, consistent with the idea that apoptosis and axon pruning pathways overlap and are strictly spatially regulated, several critical caspases—specifically caspase-9, caspase-3, and caspase-6—have all been implicated in axon-specific degeneration [78–80, 84–86].

The idea that caspase activation occurs in the absence of cell death is further supported by the recent finding that low levels of caspase-3 activation are necessary for long-term depression (LTD) [87], a synaptic change underlying memory that causes the removal of synapses and the spines in which they reside. Indeed, Bax, Bad, caspase-9, and caspase-3 have all been shown to be required for LTD in mice [87, 88]. Therefore, there appears to be an evolutionarily conserved role for site-specific and tightly regulated caspase activation in post-synaptic remodeling.

These observations raise several interesting points. If the same effectors mediate apoptosis and axon degeneration, how do neurons undergo axon-selective degeneration or eliminate specific synapses without triggering apoptosis? Alternatively, are there critical undiscovered differences that allow neurons to activate one pathway but not the other? Such critical questions are under investigation as research reveals the growing number of non-death functions of classically apoptotic proteins.

8.5 Neuronal Cell Death in Disease and Injury

Neuronal death is a fundamental characteristic of multiple neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease, Amyotrophic Lateral Sclerosis, and stroke, among others. The extremely limited regenerative capacity of the central nervous system makes neurodegenerative diseases particularly devastating, resulting in worsening symptoms and a declining quality of life as neurodegeneration progresses over time.

Neurodegenerative disorders are complex and can be caused by multi-factorial stimuli. These include genetic and environmental factors, metabolic dysregulation, excitotoxicity, neuroinflammation, cellular stressors such as oxidative stress, disrupted calcium regulation, mitochondrial dysfunction, interrupted cell transport, sustained activation of microglia (the brain's immune cells), and the accumulation of misfolded or toxic proteins. Delineating the causes of neuronal cell death in degeneration and injury is complicated by the fact that one stimulus can trigger multiple death pathways depending on its degree, duration, cell type, brain region, and the affected neuron's bioenergetic state [89]. Several different pathways of neuronal cell death are implicated in neurodegeneration, including apoptosis, necrosis, glial injury, excitotoxicity, and toxicity resulting from damaging reactive oxygen species [90]. Indeed, it is not unusual to observe more than one type of cell death pathway occurring in neurodegenerative states; necrotic and apoptotic markers have even been observed in the same neuron following ischemia, indicating that more than one cell death mechanism can be concomitantly active in a cell [91]. A brief overview of neuronal cell death across several of the most prevalent neurodegenerative diseases and stroke is described here (Fig. 8.6).

Alzheimer's disease (AD) is the most common form of neurodegeneration worldwide, and the vast majority of AD cases are sporadic. This disease is characterized by the loss of neurons and synapses in the cerebral cortex and several subcortical regions, resulting in massive brain atrophy of these areas. AD is most often diagnosed in people over 65 years of age, although the less prevalent early-onset form occurs much earlier. AD is progressive, and as neurodegeneration spreads throughout the brain, bodily functions are eventually lost, ultimately leading to death.

There is currently no known cure for AD, and understanding the causes and progression of AD is an area of intense investigation. The clinical hallmarks of AD include extracellular senile plaques, which are mostly composed of amyloid-β (Aβ) peptides, and neurofibrillary tangles composed of intracellular, hyperphosphorylated

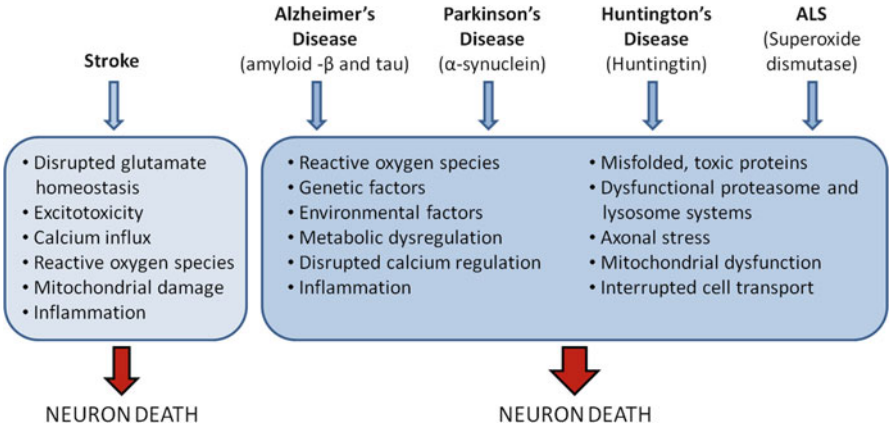

Fig. 8.6 General summary of various factors that have been implicated in promoting neuronal death during stroke or neurodegenerative diseases

microtubule-associated tau protein. Aberrant build-up of these proteins interferes with proper neuronal processes, such as intracellular transport, calcium ion homeostasis, and mitochondria dynamics; these aggregates also induce inflammation [92].

The direct relationship between aggregate lesions, their toxic soluble monomers, and neuronal loss in AD is still unclear, but evidence of caspase activation in AD may provide a link. Both initiator and executioner caspases and executioner caspases are upregulated in the brains of patients with AD [93], and caspases mediate the cleavage of multiple targets associated with AD pathology [90, 94–99]. In particular, caspase-6 has been shown to cleave both Aβ and tau, and caspase activation plays a role in the synaptic loss associated with Aβ toxicity [97–99]. There is now evidence that neurons can tolerate chronic low levels of caspase activation without dying in a mouse model of AD [100], and caspase-mediated cleavage of Aβ and tau produces toxic protein aggregates [101–103]. These observations may help to explain how caspase activation—a normally acute, rapid event during apoptosis—can occur at sub-apoptotic levels for long periods of time, thus long-preceding but culminating in the progressive neurodegeneration of AD.

Parkinson's disease (PD) is a degenerative movement disorder caused by the death of dopaminergic neurons in the substantia nigra, a region of the midbrain that plays an important role in motor planning. Early symptoms of PD are movement-related and include shaking, rigidity, slowness, and unstable posture. More advanced stages of the disease involve dementia as well as cognitive, sleep-related, and behavioral issues. PD symptoms are considered to be the direct result of the progressive dysfunction and loss of dopaminergic neurons, yet how and why these particular neurons are targeted for neurodegeneration remains unclear.

The pathology of PD is strongly linked to the progressive accumulation of alpha-synuclein into inclusions called Lewy bodies in neurons. The formation of Lewy bodies is a leading theory as the development and spread of these inclusions appears to

correspond with disease progression and region-specific degeneration [104]; however, in some cases alpha-synuclein has been shown to be protective [105, 106]. Alpha-synuclein can be secreted by neurons, partake in cell-to-cell transmission, induce neurotoxicity, make neurons more sensitive to apoptosis, and trigger inflammatory glial responses [104, 107, 108]. Inflammation and dysfunction of the proteasomal and lysosomal systems also contribute to PD pathologies, and defects in mitochondrial quality-control processes such as mitochondrial fission, fusion, and autophagy have also been linked to substantia nigral neuron degeneration [109, 110].

Caspase-dependent and -independent death pathways contribute to neurodegeneration in PD [108, 111]. Positive labeling for numerous apoptotic markers in postmortem human tissue samples confirm the presence of apoptotic nigrostriatal dopaminergic neurons, and similar observations have been replicated in multiple mouse models of PD. Also, Bax suppression, overexpression of Bcl-2 family members, and overexpression of XIAP, respectively, have been shown to protect nigrostriatal neurons in certain PD mouse models [111]. Inhibiting the opening of the mitochondrial permeability transition pore or overexpressing the E3 ubiquitin ligase Parkin to facilitate the removal of damaged mitochondria also promote survival in dopaminergic neurons [109, 111].

There is also evidence of paraptosis, a caspase-independent death pathway, in PD. Paraptosis depends on the activation of poly(ADP-ribose)polymerase (PARP-1), a DNA repair enzyme that, along with p53, is induced by genotoxic stress and DNA damage. PARP-1 activation leads to the translocation of apoptosis inducing factor (AIF) from the inner mitochondrial membrane to the nucleus where it participates in chromatin condensation and large-scale DNA fragmentation [112]. Nuclear AIF is highly observed in postmortem PD patient brain tissue [113]. Additionally, PARP-1 inhibition blocks alpha-synuclein cytotoxicity as well as cell death in toxic (MPTP/MPP^+) models of PD [111]. Thus, multiple points of intervention have been identified that could be developed into effective therapies for PD.

Amyotrophic lateral sclerosis (ALS) is characterized by the progressive loss of motor neurons throughout the brain, brainstem, and spinal cord. Onset of this disease normally occurs during one's 50s, and the average life expectancy after clinical onset is 4 years. Unfortunately, the causes of almost all ALS cases remain unknown. Several of the mutant genes found in familial forms of ALS (e.g., superoxide dismutase 1) are involved in mitochondrial maintenance, and mitochondrial damage and dysfunction are widely observed in patients with ALS and in mouse models of the disease. Disrupted axonal transport, impaired mitochondrial fusion, decreased mitochondrial size and density, and defective mitochondrial membrane potential are also present in degenerating motor neurons of ALS mouse models [114].

Excitotoxicity also contributes to ALS pathology. Glutamate-mediated excitotoxicity caused by repetitive neuronal firing and/or elevation of intracellular calcium levels by calcium-permeable glutamate receptors is known to cause neuronal death. In normal physiological conditions, glutamate is the primary excitatory neurotransmitter in the nervous system. The release of glutamate into the synaptic cleft activated glutamate receptors, which leads to the influx of calcium and sodium into

the post-synaptic neuron to trigger its depolarization. The removal of synaptic glutamate by glutamate transporters is required to prevent repetitive firing, and the glial glutamate transporter EAAT2 is responsible for approximately 90 % of glutamate clearance in motor neurons [115, 116]. Elevated levels of glutamate are found in 40 % of sporadic ALS patients [117], and loss of EAAT2 protein causes significantly reduced glutamate transport in brain regions affected in ALS [118]. The abundance of data indicating dysfunctional astroglia in both sporadic and familial forms of ALS suggests that restoring astrocyte health and glutamate transport capabilities may be a very effective therapy for this neurodegenerative disease [119].

Despite the combined contributions of oxidative damage, axonal stress, and toxicity to motor neuron dysfunction in ALS, caspase-3 activation appears to be the final event in the death cascade of these neurons [119]. Other evidence for the role of apoptosis in ALS includes the observation that administration of a pan-caspase inhibitor to ALS mice is neuroprotective and significantly extends survival, indicating a role for caspase-1 and caspase-3 in ALS [120]. As ALS mice age, there is a progressive transcriptional upregulation of caspase-1 and caspase-3 messenger RNA (mRNA) [120, 121]. These findings support that clinical relevance of mouse models of ALS as both caspase-1 and caspase-3 activation have been found in spinal cord samples from human ALS patients. In addition, other central markers of apoptosis—including caspase-9 activation, the release of cytochrome c from mitochondria, and pro-apoptotic changes in the Bcl-2 family—have also been found in the spinal cords of ALS mice, and ALS mice that overexpress the anti-apoptotic Bcl-2 gene survive longer than other ALS mice [122].

Huntington's Disease (HD) is a universally fatal autosomal dominant neurodegenerative disease in which neostriatal medium spiny neurons and eventually certain regions of the cortex progressively waste away. Onset typically occurs during the mid-30s and 40s, and persons with HD usually die within 15–20 years. Symptoms include abnormal involuntary movements, cognitive decline, and psychiatric problems. The disease is caused by a genetic mutation encoding for the aberrant expansion of CAG-encoded polyglutamine repeat regions in a protein called Huntingtin (Htt), resulting in mutant huntingtin protein (mHtt). Posttranslational modifications of mHtt result in cleavage of the protein that leaves behind toxic, shorter fragments that form damaging protein aggregates instead of folding properly into functional proteins. These aggregates, called inclusion bodies, accumulate over time and ultimately interfere with proper neuronal functions. There is also extensive evidence for deficient energy metabolism, mitochondrial dysfunction, and glutamate-mediated excitotoxicity in HD [123, 124]. caspases, particularly caspase-6, have been strongly implicated in HD [125–127]. Caspase-mediated cleavage of Htt appears to be one factor initiating striatal degeneration, and inhibiting such cleavage blocks Htt-mediated toxicity [126, 127].

Stroke is an acute, rapid form of neurodegeneration that is caused by ischemia (lack of blood supply) or hemorrhage. Both the severity and duration of the insult induce complex metabolic changes that then determine the type and extent of cell death in stroke [128]. Irreversible neuron loss is caused by necrosis in the core

tissue of the stroke lesion. However, the region surrounding the necrotic core, called the penumbra, is damaged by apoptotic processes, and this region retains enough structural integrity to support functional restoration following therapeutic interventions.

Cell death mechanisms induced by ischemic injury occur on a dynamic timescale. At early time points, neurons within the core infarct area undergo necrotic death [129]. The mechanism of neuronal death in this region is largely due to the massive disruption of glutamate homeostasis resulting in excitotoxicity. The unregulated accumulation of glutamate in the synaptic space over-activates glutamate receptors and causes a large influx of calcium, the production of reactive oxygen and nitrogen species, and mitochondrial damage, all of which lead to neuron death. Secondary neuronal death then occurs due to the inflammation caused by ROS and cytokines released by dying neurons that activate surrounding microglia, which secrete their own ROS and cytokines that only exacerbate the damage [130–132]. Future therapies for stroke may therefore involve an appropriate combination of treatments that prevent apoptosis, antagonize glutamate, and reduce oxidative stress.

In the penumbral region, which develops at later time points, neurons undergo a caspase-mediated death [90]. Bcl-2 protein family members play a significant role in neuronal death following ischemia as the overexpression of anti-apoptotic Bcl-2 protein [133] and the loss of Bid [134], a BH3-only protein, respectively protect against ischemic injury by reducing mitochondrial damage. Importantly, caspase inhibitors have been shown to improve functional outcomes in ischemic mouse models [85, 135].

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Chapter 9
The Complex Interplay Between Metabolism and Apoptosis

Kelly Lindblom and Sally Kornbluth

Abstract Energy balance is essential for cells to function properly and proliferate. Sufficient nutrient quantities are required for energy and the synthesis of building blocks of cellular structures such as lipid membranes, proteins, and nucleic acids. In contrast, nutrient excess can increase ER stress and reactive oxygen species, leading to cellular damage and apoptosis. In this chapter, we detail three important aspects of the interplay between metabolism and apoptosis: the mechanisms by which metabolic imbalances regulate major signaling effectors in apoptosis, how metabolism itself can be controlled by apoptotic proteins, and the major disease states affected when perturbations in metabolism modulate apoptosis.

Keywords Apoptosis • Bcl-2 family • Caspase • Metabolism • Glucose • Lipotoxicity

Abbreviations

AMPK	AMP-activated protein kinase
COX	Cytochrome oxidase
CPT-1	Carnitine palmitoyltransferase-1
FBPase-2	Fructose bisphosphatase-2
FFA	Free fatty acid
Fru-2,6-P_2	Fructose 2,6-bisphosphate
GAMT	Guanidinoacetate methyltransferase

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HK	Hexokinase
IMM	Inner mitochondrial membrane
MOMP	Mitochondrial outer membrane permeabilization
NAFLD	Nonalcoholic fatty liver disease
PFK-1	Phosphofructokinase
PPP	Pentose phosphate pathway
ROS	Reactive oxygen species

9.1 Introduction

Throughout life, multicellular organisms must eliminate cells that are no longer needed or are damaged. This is largely accomplished through apoptosis, or programmed cell death—a highly regulated set of reactions that neatly eliminate unwanted cells. In order for cells to survive and proliferate, they must be provided with the proper balance of nutrients. Sufficient nutrients are necessary for synthesizing cellular membrane structural components as well as new proteins and nucleic acids. However, an excess of sugars and fats can increase ER stress and reactive oxygen species (ROS), leading to cellular damage and apoptosis. Here, we discuss the mechanisms by which an imbalance in metabolism modulates some of the major signaling effectors in apoptosis. In addition, we explore the ways in which metabolism itself can be modulated by apoptotic proteins.

The relationship between metabolism and apoptosis has relevance for many major diseases in the Western world, including cancer, diabetes, and nonalcoholic fatty liver disease (NAFLD). First, we will briefly review the basics of metabolism and apoptosis before taking a closer look at the research linking excessive energy intake or nutrient starvation to protein regulation of apoptosis and survival. Finally, we will discuss the relevance of these processes for common disease states.

9.2 Brief Overview of Metabolism

The term "metabolism" refers to the set of intracellular reactions necessary for two fundamental processes: the breakdown of environmental chemical sources for energy (catabolic metabolism) and the synthesis of macromolecules necessary for survival, growth, and proliferation (anabolic metabolism). The most prominent catabolic pathways include glycolysis (which oxidizes glucose to pyruvate), the citric acid cycle (which oxidizes acetyl-CoA), and fatty acid oxidation; these pathways all provide reducing equivalents (NADH, $FADH_2$) for the electron transport chain to produce ATP. The pentose phosphate pathway (PPP) also oxidizes glucose in order to produce nucleotide precursors and the NADPH that is necessary for anabolic reactions. The major anabolic pathway we discuss here is fatty acid synthesis; this pathway utilizes NADPH and acetyl-CoA to produce long chain fatty acids. For a summary of these pathways, see Fig. 9.1.

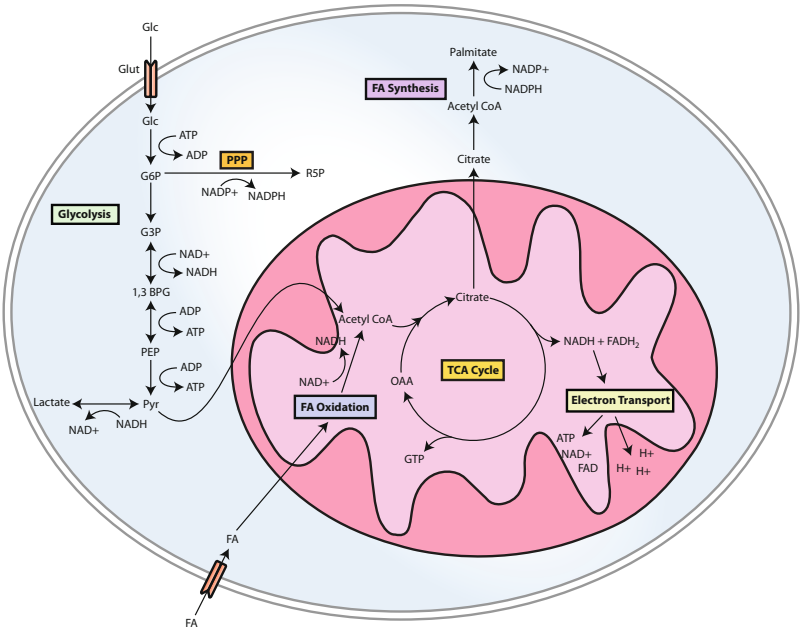

Fig. 9.1 Glucose enters the cell through specialized membrane transporters, and once phosphorylated to glucose 6-phosphate (G6P), it is then used in the pentose phosphate pathway (PPP) to form ribose 5-phosphate and produce NADPH or is used in glycolysis. In the glycolytic pathway, G6P is converted to glyceraldehyde 3-phosphate (G3P) through a series of steps, which is then converted to 1,3-bisphosphoglycerate (1,3-BPG) in a redox reaction that also forms NADH from NAD+. 1,3-BPG is then converted to phosphoenolpyruvate (PEP) in a series of reactions, one of which produces ATP, and then PEP is irreversibly converted into pyruvate (pyr), again producing ATP. Pyr can then be shuttled into the mitochondrial matrix to irreversibly form acetyl-CoA for the citric acid cycle (TCA cycle), or pyr can be converted to lactate to regenerate NAD+. In the TCA cycle, acetyl-CoA and oxaloacetate (OAA) condense to from citrate, which then undergoes a series of reactions that produce NADH, FADH2 (which can be used in the electron transport chain (ETC) to form ATP, NAD+, and FAD+), and GTP, eventually reforming OAA for subsequent cycles. The β−oxidation of fatty acids also occurs in the mitochondrial matrix and generates acetyl-CoA for the TCA cycle and NADH that can be oxidized by the ETC. Citrate can also be exported out of the mitochondria and converted to acetyl-CoA and eventually palmitate through the NADPH-dependent reactions in the fatty acid synthesis pathway

9.3 Apoptosis and Survival Signaling

9.3.1 The Core Apoptotic Machinery

Apoptosis is the major form of programmed cell death and is characterized by distinct morphological and biochemical events; these include membrane blebbing, cell shrinkage, chromatin condensation, and DNA fragmentation. Apoptosis can be subdivided into two widely recognized pathways characterized by the origin of the apoptotic stimulus: the extrinsic and intrinsic signaling pathways, both of which can be mediated by caspases and Bcl-2 family proteins. The caspase family of

proteolytic enzymes mediates apoptosis through cleavage of specific substrates to break down the cell and sustain apoptotic signaling. Caspases are normally synthesized as inactive zymogens that become active once cleaved. Caspases can be divided into two groups: initiator caspases (caspase-2,-8,-9) and executioner caspases (caspase-3,-6,-7). Initiator caspases respond to cell stress signals by cleaving and activating the executioner caspases. These activated executioner caspases consequently cleave a wide range of substrates in the cell, leading to its demise.

Although caspases share certain biochemical hallmarks, individual initiator caspases are engaged by different cell death stimuli. Caspase-8 activation is most widely studied in response to extrinsic or extracellular death signals mediated through plasma membrane receptors such as CD95, TNFR-1, TRAILR-1, TRAILR-2, and TRAMP [1]. Caspase-8 is recruited to the cell surface along with activating adaptor proteins. Once active it can cleave executioner caspases and/or the Bcl-2 family member Bid, leading to downstream apoptotic events discussed further below. In contrast, caspase-2 is activated mostly in response to intrinsic stimuli including DNA damage, cytoskeletal disruption, and metabolic and oxidative stress [2–5]. In some cases, this activation appears to involve an activation platform consisting of the molecules PIDD and RAIDD, but in many paradigms, the mechanism of caspase-2 activation is not clear. Moreover, although caspase-2 is the most evolutionarily conserved caspase, relatively few substrates have been identified [6]. Caspase-2, like caspase-8, has been shown to cleave Bid to potentiate downstream apoptotic signaling. Caspase-9, unlike caspases -8 and -2, is activated downstream of the mitochondria. Once cytochrome c is released from the mitochondria (see below), caspase-9, Apaf-1, and cytochrome c oligomerize to form the apoptosome, which further potentiates downstream caspase activation.

Bcl-2 family proteins control mitochondrial outer membrane permeabilization (MOMP) and the subsequent release of mitochondrial proteins (such as cytochrome c) into the cytosol. These proteins are classified by function into anti-apoptotic or pro-apoptotic, with the latter group further subdivided into effector proteins or BH3-only proteins [7]. The anti-apoptotic group, which includes Bcl-2, Bcl-x_L, and Mcl-1, acts to preserve mitochondrial outer membrane integrity by inhibiting the pro-apoptotic members. The pro-apoptotic effectors (Bax and Bak) promote MOMP directly, while the pro-apoptotic BH3-only sensitizers (PUMA, Noxa, and BAD) bind and inhibit anti-apoptotic members. Interestingly, Bcl-2 family members Bid and Bim can both inhibit the anti-apoptotic members and activate the pro-apoptotic effectors Bax and Bak.

9.3.2 Signaling Molecules that Modulate the Core Cell Death Machinery

In addition to caspases and Bcl-2 family proteins, there are multiple signaling protein families that impinge upon the decision to live or die. Here we specifically discuss those pathways that either appear to be under tight metabolic regulation, as

discussed later in this chapter, or that serve to regulate both cell survival and metabolic activity. These pathways include p53-associated signaling, the insulin-like signaling cascade, the AMPK survival pathway, and the sirtuin family.

p53 is one of the most well-studied apoptotic modulators due to the high frequency of mutations/deletions of this gene in cancer. This transcription factor is maintained at low levels in normal cells through degradation by the ubiquitin ligase MDM2. Under mild cellular stress, p53 can help revert the cell back to homeostatic conditions by mediating cell cycle arrest. However, under severe or prolonged stress, p53 will induce apoptosis [8]. Bax and PUMA are two Bcl-2 family proteins known to be transcriptionally activated by p53, and p53 has also been shown to activate Bax and promote MOMP through nontranscriptional mechanisms [9].

The Akt kinase is at the crossroads of many intracellular signaling pathways and its activation leads to the modulation of many cellular processes including metabolism, apoptosis, proliferation, and cell adhesion. Akt can be activated in response to survival factors including members of the insulin-like signaling cascade. This cascade is initiated by the IGF family ligands: insulin, IGF1, and IGF2, which bind plasma membrane receptors to induce survival signaling [10]. Receptor activation induces the phosphorylation of cytoplasmic proteins IRS1 and IRS2, which can activate multiple branches of signaling, including the PI3K cascade. Once active, PI3K produces $PI(3, 4)P_2$ and $PI(3,4,5)P_3$. This, in turn, leads to the recruitment of PDK1 and Akt to the plasma membrane. PDK then phosphorylates Akt leading to its activation. Consequent phosphorylation of substrates, including FOXO transcription factors and the Bcl-2 family protein, BAD, promotes cell growth and survival [10].

AMP-activated protein kinase (AMPK) is recognized as one of the key regulators of energy homeostasis, important for promoting cell survival through the maintenance of proper metabolite levels. AMPK is canonically activated by metabolic stresses or drugs that cause intracellular increases in AMP, ADP, or Ca^{2+}, such as glucose or oxygen deprivation, muscle contraction, or metabolic poisons [11]. Other noncanonical activators of AMPK include either ROS, such as H_2O_2, or genotoxic agents, such as etoposide and doxorubicin. Once activated, AMPK acts to restore metabolic homeostasis by both initiating catabolic pathways for the production of ATP and inhibiting anabolic pathways that consume ATP. In addition, AMPK can both enhance the uptake and subsequent breakdown of glucose and fatty acids and increase mitochondrial biogenesis to promote further oxidative capacity and energy generation.

The widely studied sirtuin family is known to play key roles in cell survival in response to metabolic stress. The sirtuins consist of seven enzymes that require NAD+ as a cofactor for their catalytic activity, classically either deacetylation or ADP-ribosylation. More recently, it has also been demonstrated that sirtuins can reverse other less-common protein modifications such as malonylation and succinylation [12]. Sirt1, a primarily nuclear sirtuin, and Sirt3, a mitochondrial sirtuin, are the most intensively studied of the family; these respond to falling nutrient levels (which results in an increased NAD+/NADH ratio) to increase ATP levels and decrease harmful ROS. This is accomplished through modulation of several key

metabolic processes: glycolysis, the citric acid cycle, the electron transport chain, fatty acid metabolism, acetate metabolism, as well as antioxidant defense enzymes and regulators of survival and death [13].

9.4 Interplay Between Metabolism and Apoptosis

All of the metabolic pathways discussed above impinge on apoptotic effectors that can trigger cell death in response to energy imbalance. Additionally, pro-survival proteins are impacted by energy levels to keep cells alive under homeostatic conditions. The fate of glucose has been the primary focus of research into metabolism and cell survival, particularly due to the role of glycolysis in maintaining cancer cell proliferation. However, increased scrutiny is now being given to other types of energy imbalance such as high free fatty acid (FFA) levels, which can also trigger cell death pathways, as discussed below.

It is not yet possible to provide a fully integrated model for understanding the impact of metabolism on pro-apoptotic or pro-survival proteins (or their effects on metabolism). Rather, the relevant literature provides snapshots viewed through the lenses of individual proteins/pathways. We review these here and point out a number of commonalities (see Figs. 9.2 and 9.3) to provide a starting point for establishing regulatory paradigms.

9.4.1 Pro-apoptotic Proteins

9.4.1.1 Caspase-8

Caspase-8 has been implicated in various settings of metabolic stress: both nutrient starvation and nutrient excess and in both death receptor-dependent and -independent models. In a variety of cancer cell lines, caspase-8 is activated more quickly in response to TRAIL or anti-CD95 stimulation when glucose is absent [14]. In this context, caspase-8 is recruited to the DISC complex leading to downstream cytochrome c release and caspase-3 activation. Caspase-8 activity in response to extrinsic stimuli is also enhanced after treatment with the glucose analog 2DG, which cannot enter the glycolytic pathway. Additionally, in multiple contexts of nutrient deprivation, caspase-8 has been activated in a manner that appears to be independent of the full DISC: caspase-8 and its substrate Bid are both cleaved in response to glucose deprivation in HepG2 cells [15]. This effect was independent on the expression of the caspase-8-activating adaptor protein, FADD, a well-characterized DISC component. Caspase-8 knockdown in HeLa cells makes the cells less sensitive to glucose deprivation, while blocking FasL–FasR interactions does not prevent resultant cell death [16]. As suggested by these data, either another receptor system is involved or glucose deprivation activates caspase-8 in a receptor-independent manner.

In contrast, caspase-8 activation results from high levels of glucose in the neuronal cell line PC12, a potential model for diabetic neuropathy [17]. In this setting,

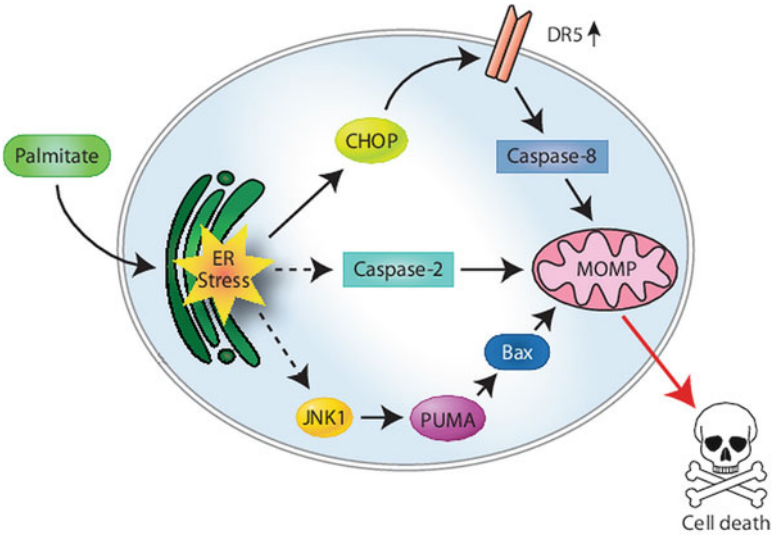

Fig. 9.2 Excess uptake of the long chain saturated fatty acid, palmitate, is toxic to cells partially through its role in promoting ER stress. This chapter describes multiple reports of core apoptotic proteins that are modulated by excess palmitate to promote MOMP and cell death. Although the report on caspase-8 activation is the only report that directly links ER stress (through CHOP activation and DR5 upregulation) to death, it is likely that caspase-2 and PUMA are also downstream of the ER stress response in their roles in palmitate-induced apoptosis. Jnk1 can be activated by ER stress [80] and is required for palmitate-induced PUMA activation, Bax activation, and apoptosis. In addition, caspase-2 has been reported to be activated in response to ER stress [4], and therefore it is also probable that ER stress from excess palmitate uptake is contributing to the apparent activation of caspase-2 and subsequent MOMP

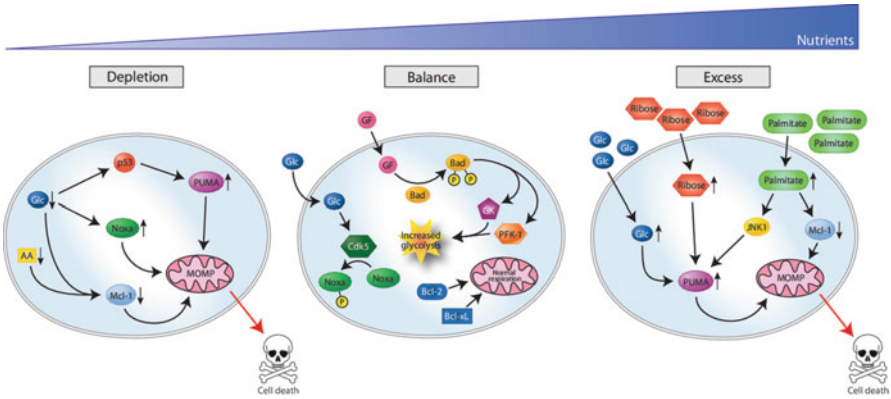

Fig. 9.3 The activity and protein expression levels of Bcl-2 family proteins are modified based on the metabolic state of the cell. In glucose-deprived cells, PUMA and Noxa promote MOMP, while Mcl-1 levels fall (which also occurs with amino acid depletion) in order to diminish its anti-apoptotic effects. In healthy cells, many Bcl-2 family proteins promote metabolic reactions. Growth factor signaling promotes the phosphorylation of BAD, which leads to enhanced activity of the glycolytic enzymes GK and PFK-1. Glucose also promotes the phosphorylation of Noxa by Cdk5, which sequesters its anti-apoptotic activity in the cytosol so it is unable to promote MOMP. Bcl-x_L and Bcl-2 also promote normal mitochondrial respiration by enhancing ETC function. In contrast, PUMA can promote MOMP in response to an excess of multiple nutrients: glucose, ribose, and palmitate. Palmitate also induces MOMP by promoting Mcl-1 degradation

caspase-3 and -9 are also activated, but the upstream events leading to caspase-8 activation remain unclear. Similarly, caspase-8 cleavage has been demonstrated in hepatoma Huh7 cells in response to treatment with high levels of the saturated long chain fatty acid, palmitate, a model of lipoapoptosis [18]. In this setting, caspase-8 activation is downstream of the death receptor DR5, and knockdown of caspase-8 or FADD is able to reduce palmitate-induced death in both these cells and in Jurkat cells. Nonetheless, the precise mechanism underlying palmitate-induced caspase-8 activation is unknown.

9.4.1.2 Caspase-2

Another initiator caspase, caspase-2, is also regulated by changes in cellular metabolic status. Studies in the *Xenopus* egg extract system have demonstrated that metabolite levels influence caspase-2 activation. In this system, caspase-2 can undergo a suppressive phosphorylation mediated by CaMKII. This occurs in response to exogenous pentose phosphate pathway intermediates that promote the production of NADPH (or by addition of NADPH itself) and prevents caspase-2 activation and downstream apoptotic signaling [5]. In addition, phospho-caspase-2 is bound by 14-3-3ζ when NADPH levels are sufficient, protecting caspase-2 from pro-apoptotic dephosphorylation. However, 14-3-3ζ is released over time in response to metabolic changes in the extract, leading to caspase-2 dephosphorylation and activation [19]. This reduced binding inversely correlates with 14-3-3ζ acetylation levels [20]. 14-3-3ζ is a target of the NAD+-dependent deacetylase Sirt1; addition of exogenous NAD+ to promote Sirtuin activity is able to suppress 14-3-3ζ acetylation, while inhibition of Sirt1 with nicotinamide is able to promote the dissociation of caspase-2 and 14-3-3ζ.

Caspase-2 is also reportedly controlled by the intracellular abundance of acetyl-CoA. In mammalian cells with basal levels of acetyl-CoA, caspase-2 is N-terminally acetylated, which increases its affinity for its activating binding partner, RAIDD [21]. However, in cells deficient in the acetyl-CoA producing enzymes, specifically ATP citrate lyase or acetyl-CoA synthetase, caspase-2 is not acetylated at its N-terminus and therefore, its affinity for RAIDD is decreased. More studies are needed to determine both the magnitude of change in caspase-2 activity due to changing acetyl-CoA levels, and the sensitivity of this mechanism over the range of acetyl-CoA levels generated intracellularly.

It has also been suggested that caspase-2 activity increases in response to exogenous palmitic acid or stearic acid in cultured pancreatic beta cells [22]. The method used for quantitation of activity has been criticized as nonspecific because other caspases are believed to cleave this substrate sequence; however, caspase-3 activity was not detected in this study. Further studies will be needed to determine the role of caspase-2 in this context, and if its activity is leading to a noncanonical apoptotic mechanism that does not involve caspase-3.

9.4.1.3 PUMA

PUMA is one of the Bcl-2 family members that acts as a pro-apoptotic sensitizer, binding and sequestering the anti-apoptotic members that inhibit apoptosis. During nonstressed conditions, PUMA is expressed at very low levels. However, upon certain stress stimuli, PUMA transcription is upregulated. This is most notably mediated by the transactivator p53, but also occurs through activity of p73, c-Myc, E2F1, and FoxO3a [23]. PUMA has been implicated in metabolic stress from both nutrient withdrawal and nutrient excess. In the IL-3-dependent hematopoietic precursor cell line, FL5.12, glucose starvation leads to the induction of PUMA expression in a p53-dependent manner, even in the presence of growth factors [24]. In line with this observation, increasing glucose uptake and utilization through overexpression of Glut1, a plasma membrane glucose transport protein, and Hexokinase1 (HK1), a glycolytic enzyme, is able to attenuate PUMA upregulation and cell death in growth factor-withdrawn cells. Moreover, knockdown of PUMA is able to prevent cell death induced from glucose deprivation [25]. However, stimulation of mitochondrial metabolism, and not specifically glucose metabolism, was able to prevent PUMA induction and cell death. Treatment of these cells with the cell permeable analog of pyruvate (the end product of glycolysis), methyl-pyruvate, in the absence of glucose and growth factors was able to prevent the induction of PUMA expression and prevent cell death. In addition, physiological levels of oleate and palmitate can also prevent cell death under these conditions, but neither methyl-pyruvate nor oleate/palmitate can prevent death in the presence of the electron transport chain complex I inhibitor, rotenone. The combination of glucose and rotenone is not toxic to these cells, indicating that in the absence of glucose, stimulation of mitochondrial metabolism is necessary for cell survival.

PUMA has also been implicated in mediating the toxicity from high levels of glucose, ribose, or palmitic acid. Pancreatic islet cells from PUMA −/− mice, in addition to Bim −/− mice, have reduced sensitivity to glucose and ribose toxicity, as characterized by reduced cytochrome c release and less DNA fragmentation compared to controls [26]. In these studies, double knockout islet cells lacking both PUMA and Bim were completely insensitive to high sugar. PUMA transcription is also increased in primary murine hepatocytes in response to the long chain fatty acid palmitate in a Jnk-dependent manner [27]. PUMA induction by palmitate leads to the activation of Bax, caspase-3/7, and subsequent cell death; this can be prevented with PUMA shRNA.

9.4.1.4 Noxa

In response to diverse stress stimuli, multiple transcription factors, such as p53 and HIF1α, induce the expression of another BH3-only Bcl-2 family member, Noxa. This sensitizer protein promotes apoptosis, at least in part, by contributing to the degradation of the anti-apoptotic Mcl-1 protein. In dividing T cells, knockdown of

Noxa abrogates cell death in response to glucose deprivation [28]. In addition, disruption of citric acid cycle function by the aconitase inhibitor SFA kills cells less effectively when Noxa levels are reduced [29]. The 6-phosphogluconate-dehydrogenase inhibitor, 6-AN, which disrupts PPP function, is also a less potent death-inducer in the absence of Noxa. In line with this, Noxa-overexpressing Jurkat cells undergo accelerated apoptosis in response to glucose deprivation [30]. This study also reported that Cdk5 phosphorylates Noxa in the presence of glucose, suppressing its apoptotic function by sequestering the protein in the cytosol. When Cdk5 is knocked down, cells are more sensitive to glucose deprivation. Noxa was also suggested to have a growth stimulatory role: Noxa overexpressing cells are better able to survive in the presence of the glucose analog 2-DG, which can be metabolized through the pentose phosphate pathway, but not glycolysis. Therefore, the authors of this study suggested that Noxa might be able to alter the fate of glucose in order to promote cell growth.

9.4.1.5 BAD

BAD is another Bcl-2 family BH3-only sensitizer known to both regulate and be regulated by metabolism. Many studies have confirmed that hormone and growth factor signaling (from IL-3, GLP-1, IGF-1, and EGF) inhibits BAD through phosphorylation by multiple kinases, including Akt, p70S6K, PKA, and Jnk [31–34]. Four BAD residues, Thr201 and three evolutionarily conserved serine residues, have been identified as phosphorylation sites; one of these, S155, is located within the hydrophobic BH3 domain that binds anti-apoptotic Bcl-2 family partners. Phosphorylation at S155 is believed to serve as a molecular switch, toggling BAD between the metabolic and pro-apoptotic functions [35]. In pancreatic beta cells, phosphorylation of BAD leads to glucokinase activation (discussed below), mitochondrial respiration, and ATP production. Moreover, in BAD-deficient islet cells, insulin release is not changed upon glucose stimulation. However, the reintroduction of WT BAD expression restores glucose-stimulated insulin secretion. Additionally, expression of the S155A mutant has no effect on insulin secretion, suggesting that phosphorylation both precludes BAD-mediated activation of cell death and enhances proliferative metabolism.

BAD is implicated in the regulation of glucokinase, a high K_m form of the glycolytic enzyme hexokinase (HK) with limited expression in hepatocytes, beta cells, and glucose-sensing hypothalamic neurons [36]. In hepatocytes, BAD is necessary for the formation of a holoenzyme complex composed of PKA and PP1 catalytic subunits, WAVE-1 anchoring protein, and glucokinase [37]. BAD-deficient hepatocytes lack this complex and have decreased mitochondrial respiration in response to glucose. BAD also associates with another key glycolytic enzyme, phosphofructokinase-1 (PFK-1) [38]. The activation of PFK-1 is dependent on Jnk1-mediated phosphorylation of BAD on Thr201, although this phosphorylation is not necessary for their physical interaction. BAD −/− MEFs that are reconstituted with a T201V mutant BAD are not able to rescue the decrease in glycolytic rate seen in BAD −/− MEFs.

9.4.1.6 Bid

Bid is a Bcl-2 family BH3-only protein that can both bind anti-apoptotic members in response to stress stimuli and activate pro-apoptotic Bax and Bak directly. Bid is cleaved to tBid in the cytosol and moves to the mitochondrial outer membrane. There it activates Bax, promoting MOMP and mitochondrial cytochrome c release. The glycolytic enzyme HK has been shown to prevent the activation of Bax by binding VDAC on the outer mitochondrial membrane [39], a phenomenon that can be antagonized by active tBid. Upon overexpression of tBid, or in growth factor-deprived cells, tBid promotes the dissociation of HK from the mitochondria and induces subsequent MOMP [40]. In glucose-replete cells, but not in the absence of glucose, Akt is able to prevent the dissociation of HK and tBid-mediated apoptosis.

In addition to altering the localization of the glycolytic HK enzyme, tBid can also affect the activity of carnitine palmitoyltransferase-1 (CPT-1). This outer mitochondrial membrane protein adds carnitine to long chain fatty acids, facilitating their transit across the mitochondrial membrane for subsequent β–oxidation. Addition of tBid to permeabilized hepatocytes leads to a decrease in CPT-1-dependent palmitate oxidation, but not in CPT-1-independent octanoate oxidation [41]. It has been suggested that the reduction in CPT-1 activity observed in the presence of tBid could reflect the ability of tBid to reduce cardiolipin levels in the mitochondria, changing the lipid environment of the membrane. Bcl-2 overexpression can reverse the effect of tBid on lipid oxidation, but this suppression is independent of Bax, Bak, and the major CPT-1 inhibitory molecule, malonyl-CoA.

9.4.1.7 Cytochrome c

Cytochrome c is the quintessential dual-functioning molecule, serving a key metabolic role in the electron transport chain within the mitochondria of healthy cells, while inducing cell death once cytosolic. Upon receipt of apoptotic signals, cytochrome c is released from the intermembrane space of the mitochondria in two phases: first, mobilization or dissociation from intermembrane components, and then translocation into the cytosol. Typically, the majority of cytochrome c is associated with the mitochondrial membrane phospholipid cardiolipin. Upon apoptotic stimulation, cardiolipin is oxidized, promoting dissociation of cytochrome c from intermembrane constituents and allowing for release. Under conditions of excess ROS formation (which occurs with nutrient excess, or experimentally, following hydrogen peroxide treatment), cytochrome c has peroxidase activity toward cardiolipin [42]. The oxidation of cardiolipin leads to the release of apoptogenic mitochondrial proteins, including Smac/Diablo and cytochrome c itself. Release of mitochondrial intermembrane space constituents does not occur in cytochrome c –/– MEFs after treatment with actinomycin D, but this can be rectified by exogenous addition of oxidized cardiolipin. Thus, data supports a role for cytochrome c in cardiolipin oxidation.

In addition, the oxidation of cytochrome c itself appears to be important for its apoptosis-inducing activity: neurons injected with cytochrome c are much more resistant to cell death in response to hydrogen peroxide when supplemented with cell-permeable GSH, a reducing agent [43]. Because addition of ROS can have widespread affects, the authors of this study also altered the redox state of purified cytochrome c before injection into cells. Again, they found that oxidized cytochrome c more potently induces apoptosis. Flux of glucose through the pentose phosphate pathway, increasing NADPH levels, has been suggested to be the mechanism responsible for preventing oxidation of cytochrome c and cell death: inhibition of PPP enzymes sensitizes cells to exogenous tBid or cytochrome c, as does glucose deprivation.

9.4.1.8 p53

Cellular glucose levels and glucose uptake have been shown to affect posttranslational modifications, RNA and protein expression, and activity of the tumor suppressor p53. Although p53 abundance is well known to be regulated through ubiquitination by the E3 ligase MDM2, AMPK, which is activated by a high ADP/ATP ratio, has been shown to induce transcription of p53 mRNA [44]. Moreover, following AMPK overexpression, activation of p53 transcription is even further increased in the absence of glucose. Accordingly, AMPK overexpression both increases p53 protein levels and induces phosphorylation of p53 on Ser46 (an activating site). p53 activity also appears to be altered by glucose metabolism; in FL5.12 cells, overexpression of Glut1 and HK1 suppresses the ability of p53 to induce transcription, even after growth factor withdrawal. These observations indicate that sufficient intracellular glucose levels inhibit p53 activity [24]. In another study using HepG2 cells, low glucose at early time points induced the transcriptional coactivator PGC1-α to bind p53, thus modulating its transactivation of target genes to upregulate metabolic genes such as TIGAR (see below) [45]. However, upon prolonged stress, PGC1-α was degraded, allowing for p53 acetylation and activation of pro-apoptotic p53 target genes such as Bax.

The expression and activity of p53 is not only intimately linked to the metabolic status of the cell; p53 also affects cellular metabolism itself. Multiple lines of evidence have implicated p53 in slowing glycolytic flux. First, luciferase assays in each of three cell lines, osteosarcoma-derived SaOS-2 cells, rhabdomyosarcoma-derived RD cells, and C2C12 myotubes, have all shown that overexpression of p53 decreases GLUT1 and GLUT4 promoter activity, consequently reducing glucose uptake [46]. Through a posttranscriptional mechanism, overexpression of p53 in MEFs also reduces protein levels of phosphoglycerate mutase (PGM), an enzyme in the glycolytic pathway [47]. The p53-inducible gene TIGAR also plays a role in decreasing glycolytic flux: TIGAR acts similarly to fructose bisphosphatase-2 (FBPase-2) by reducing the allosteric effector fructose 2,6-bisphosphate (Fru-2,6-P_2). This results in a decrease in PFK-1 activity and slowed glycolysis [48]. As some reports have suggested, p53 may also play roles in enhancing glycolysis: the promoters of both the muscle-specific PGM gene and type II HK contain p53-responsive elements [49, 50].

p53 is also involved in promoting oxidative phosphorylation. Glutaminase-2, a metabolic enzyme that catalyzes the conversion of glutamine to glutamate, can be upregulated by p53 under both basal and stressed conditions [51]. Glutamate can then be converted to α-ketoglutarate, which enters the citric acid cycle and promotes mitochondrial respiration. p53 expression also promotes transcription of Synthesis of Cytochrome c Oxidase 2 (SCO2), which regulates the electron transport chain complex, cytochrome oxidase (COX), to promote oxygen utilization [52]. Liver mitochondria, from both murine and human cell lines deficient in p53, show lower levels of SCO2 compared to controls. Disruption of the SCO2 allele by homologous recombination in HCT116 cells phenocopies the metabolic defects of p53 loss, leading to decreased oxygen consumption and increased production of lactate.

p53 can also modulate metabolism by regulating fatty acid oxidation. In a variety of diverse cell lines, Guanidinoacetate methyltransferase (GAMT) is induced in a p53-dependent manner in response to glucose deprivation [53]. GAMT is able to upregulate fatty acid oxidation to elevate ATP levels during nutrient stress. Overall, it is clear that p53 plays an important role in modulating metabolism and affecting cell fate decisions in times of metabolic imbalance.

9.4.2 Pro-survival Proteins

9.4.2.1 Bcl-2

Bcl-2 is a key inhibitor of MOMP in the intrinsic apoptosis pathway. It also appears to have significant impact on the control of mitochondrial dynamics and metabolism. Bcl-2 has long been implicated in affecting mitochondrial levels of ROS: Bcl-2 expression reduces lipid peroxidation in 2B4 hybridoma T cells in response to the ROS-generator, dexamethasone [54]. More recently, it was reported that overexpression of Bcl-2 results in an increase in COX activity in the electron transport chain and an increase in COX subunit localization to the inner mitochondrial membrane [55]. As a result, Bcl-2 promotes a slightly pro-oxidant state under normal conditions. However, during times of metabolic stress, such as serum/glucose deprivation, Bcl-2 overexpressing cells decrease their COX activity by decreasing the localization of the COX subunits, COX Va and Vb, to the mitochondria. As a result, the cellular oxidative environment is stabilized to prevent apoptosis [56]. Similarly, Bcl-2 overexpression can protect islet cells from high glucose or ribose toxicity, another metabolic stressor that can increase the production of ROS [26].

9.4.2.2 Bcl-x_L

In addition to its well-known anti-apoptotic role, Bcl-x_L, another Bcl-2 family protein, has been implicated in control of mitochondrial respiration. In FL5.12 cells deprived of growth factors, Bcl-x_L overexpression is able to increase the amount of ADP in the mitochondrial matrix [57]. The increased mitochondrial ADP is then

able to promote ATP synthesis, as shown by an increase in oxygen consumption. Bcl-x_L has also been shown to bind the F_1F_0 ATP synthase, increasing its activity, and thus promoting the production of ATP in neurons [58]. Bcl-x_L overexpression also prevents H+ leakage through the complex, making the reaction more efficient.

In addition to affecting respiration, Bcl-x_L has also been implicated in reducing cellular acetyl-CoA levels [21]. Bcl-x_L-deficient MEFs have increased levels of acetyl-CoA, resulting in either an increase in N-alpha acetylation or acetylation at the N-terminus of proteins. Caspase-2, -3, and -9 all have lower levels of N-alpha acetylation when Bcl-x_L is overexpressed, and as stated above, this results in decreased pro-apoptotic activity. Addition of citrate or acetate to Bcl-x_L–overexpressing cells is able to increase acetyl-CoA levels and sensitize cells to doxorubicin-induced death—thus demonstrating the interdependence of metabolism and apoptosis.

9.4.2.3 Mcl-1

The protein expression, proteolysis, and compartmentalization of another Bcl-2 family protein, Mcl-1, are all regulated by apoptotic stimuli and affected by metabolic stresses, such as glucose deprivation and lipotoxicity. Mcl-1 is said to be part of an "apoptotic rheostat" in dividing T cells, where knockdown of Mcl-1 can increase sensitivity of these cells to low glucose and increase Noxa levels to promote apoptosis [28]. Intracellular glucose levels have been shown to be vital for mitochondrial Mcl-1 expression: IL-3 withdrawal from wild-type or Bcl-x_L overexpressing cells leads to decreased Mcl-1 expression whereas Mcl-1 levels are maintained following similar withdrawal in T cells that overexpress Glut1 or HK [59]. In Glut1/HK cells, suppressive phosphorylation of GSK3 is also increased. Furthermore, genetic depletion of GSK3β from wild-type cells increases Mcl-1 levels. Mcl-1 protein expression has also been found to be generally regulated by metabolic stressors, as decreasing glucose or amino acids leads to downregulation of Mcl-1, while reducing Noxa levels protects cells against the reduction in PPP flux caused by the drug 6-AN [29].

More complex control of Mcl-1 by compartmentalization has been described recently. Three species of Mcl-1 are produced by proteolysis, and each localizes to different regions in the mitochondria to perform specific functions. Species of 38 and 40 kDa reside on the outer leaflet of the outer mitochondrial membrane, whereas a 36 kDa species is associated with the inner mitochondrial membrane and matrix [60]. Targeted constructs demonstrate that an OMM fragment can protect against apoptotic stimuli, while the matrix fragment can stimulate ATP production in galactose-supplemented, glucose-free media. Mcl-1 expression has also been shown to be necessary both for proper expression of COX1 and COX2 and for efficient oligomerization of ATP synthase.

Mcl-1 also plays roles in regulating apoptosis in response to excessive nutrition: Mcl-1 levels are reduced when pancreatic beta cells are exposed to either ER stressors, pro-inflammatory cytokines, or the saturated fatty acid palmitate [61].

Knockdown of Mcl-1 sensitizes these cells to apoptosis, whereas overexpression of Mcl-1 can prevent Bax translocation to the mitochondria, caspase-3 cleavage, and subsequent apoptosis.

9.4.2.4 PI3K-Akt

The PI3K-Akt pathway affects a wide variety of cellular processes including those that connect metabolism and cell death. When the nutrient supply is adequate, Akt promotes cell survival both by enhancing metabolism and protein translation and by more directly inhibiting apoptosis. While transcription of GLUT1 is typically decreased following growth factor withdrawal, transcription levels are maintained under these conditions when constitutively active Akt is overexpressed, leading to an increased capacity for glucose uptake and thus an increased rate of glycolysis and ATP production [62]. By enhancing the activity of HK at the mitochondrial membrane, Akt has also been shown to promote the coupling of glycolysis and oxidative phosphorylation [63]. In the presence of glucose, Akt can act to suppress apoptosis by stabilizing the anti-apoptotic Mcl-1 [64].

As a kinase, Akt is also known to promote survival and metabolic processes by phosphorylating a multitude of substrates. For example, Akt can phosphorylate and inhibit the pro-apoptotic Bcl-2 family proteins Bad and Bax [32, 65]. Additional evidence suggests that Bax can be suppressed through Akt-mediated inhibition of GSK-3, which in turn prevents Bax from localizing to the mitochondrial membrane [66]. Akt also suppresses apoptosis through transcriptional mechanisms: Akt can phosphorylate both FKHRL1 to prevent the transcription of pro-apoptotic genes such as Bim, PUMA, and Fas and FOXO proteins to prevent the expression of pro-apoptotic protein TRAIL [67, 68].

9.4.2.5 AMPK

AMPK is widely known as a stress sensor that acts to restore energy homeostasis, especially in the context of a falling ATP/AMP ratio. AMPK has also been shown to promote survival by regulating the expression of CPT-1, a member of the carnitine acyltransferase family that helps move lipids across the outer mitochondrial membrane for subsequent oxidation [69]. Metformin, an AMPK activator, increases CPT-1 mRNA in MCF-7 breast cancer cells, whereas MEFs with mutations in the AMPK subunits fail to induce CPT-1 expression in response to hypoxia and glucose deprivation. The significance of this effect was clearly shown when mutant CPT-1 increased caspase-3 and caspase-9 activation and enhanced cell death in response to hypoxia/glucose deprivation. In addition to promoting survival by regulating metabolism, AMPK can also more directly affect apoptotic proteins. As mentioned above, AMPK can lead to p53 transcriptional activation and phosphorylation on Ser46 in response to glucose deprivation [44].

9.4.2.6 Sirtuins

Because they are NAD+ dependent, members of the sirtuin family are integrally involved in connecting metabolism and cell fate decisions. Sirt1 has been shown to deacetylate p53, reducing its transactivation activity and promoting survival in response to oxidative stress [70]. In addition, Sirt1 expression controls the localization of p53 in response to stress: in WT cells, oxidative stress causes p53 to move to the mitochondria, but in Sirt −/− mES cells it translocates to the nucleus [71].

Sirt3 utilizes multiple mechanisms to help protect cells against oxidative stress. Sirt3 can deacetylate Ku70 in response to stress. This deacetylation promotes its association with Bax, preventing Bax from triggering MOMP and subsequent cytochrome c release [72], a function that has also been previously attributed to Sirt1 [73]. Sirt3 can also deacetylate the mitochondrial antioxidant enzyme, MnSOD, to counteract oxidative stress [74]. In addition, the reduction in oxidative stress that is seen in response to a calorie-restricted diet is mainly dependent on Sirt3: oxidative stress levels are not reduced by this restricted diet in Sirt3 −/− mice. Sirt3 can also deacetylate the mitochondrial matrix protein IDH2 *in vivo* in response to calorie restriction and in cell culture to protect against oxidative stressors [75]. IDH2 is a major source of NADPH, which helps protect against oxidative stress by reducing glutathione. Acetylated IDH2 has a 44-fold decrease in activity. Importantly, an acetylation resistant mutant, $IDH2^{K413R}$, is able to protect Sirt3 −/− MEFs from oxidative stress [76].

9.5 Metabolism and Apoptosis in Disease

The relationship between metabolism and cell survival in the context of cancer has been studied for decades—ever since Otto Warburg noted that cancer cells undergo high rates of aerobic glycolysis [77] dubbed the "Warburg effect." In addition, evasion from apoptosis has long been considered a key mechanism in cancer development and progression. Therefore, an important research goal has been to understand the metabolism of cancer cells and how we might manipulate its components to either prevent growth/proliferation or promote apoptosis. Today, it is clear that cancer cells not only have high levels of glycolysis, but also that in most cases, their mitochondria are in fact functional [78], contrary to the hypotheses of Warburg. Functional mitochondria are important for cancer cell proliferation because they are necessary to produce the many molecular constituents required to build new cells, such as lipids, proteins, and nucleic acids.

The relationship between metabolism and apoptosis is also highly relevant in diabetes. Due to the extremely high prevalence of this disease, research on its molecular pathogenesis and progression is imperative. Type 2, or adult-onset, diabetes results in a loss of pancreatic beta cell function and mass through apoptosis. Energy dense diets, high in sugars and fats, are a known risk factor for developing Type 2 diabetes; as a result, research focuses on how hyperglycemia and

hyperlipidemia cause apoptosis of beta cells. The main known causes of beta cell apoptosis are thought to be ER stress, increased ROS from increased metabolic activity, and lipotoxicity from ceramide accumulation.

Often concurrent with diabetes, NAFLD also involves the interplay between metabolism and cell death. Insulin resistance increases the levels of FFAs in the blood, overwhelming the liver's ability to esterify FFAs to produce triglycerides. Saturated fatty acids, such as palmitate or stearate, are especially toxic to hepatocytes because they are more difficult to esterify, while unsaturated fatty acids such as oleate or palmitoleate are less toxic [79]. The increased FFAs induce lipoapoptosis of the hepatocytes, likely through mechanisms involving ER stress and ROS. Importantly, NAFLD severity correlates with hepatocyte lipoapoptosis, which is thought to result in increased fibrosis. Because of this relationship, the precise mechanisms of hepatocyte apoptosis are of great interest.

9.6 Conclusion

It is clear that a balanced level of nutrients is necessary for proper cellular function and survival. Yet, how nutrients affect the health of a cell is a matter of intense study. We now have firm evidence that the pathways regulated by, and responding to, nutrient levels loop through the pathways that control cell proliferation and death; not only do apoptotic effectors respond to metabolic stress by promoting cell death, but also these same proteins can modulate metabolism. Given the relevance of this relationship for highly prevalent diseases such as cancer, diabetes, and NAFLD, this area will continue to be intensely studied, leading both to better treatment and prevention strategies and to a more clear understanding of the processes that control cellular fate.

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Chapter 10
Programmed Necrosis/Necroptosis: An Inflammatory Form of Cell Death

Francis Ka-Ming Chan

Abstract It was not long ago when necrosis was thought to be cell injury caused by nonspecific physical trauma. In recent years, a dedicated pathway that triggers necrosis in response to TNF-like death cytokines, certain toll-like receptors, and in response to viral pathogens was described. Signaling adaptors that contain the RIP homotypic interaction motif (RHIM), such as receptor interacting protein kinase (RIPK) 1 and RIPK3, are key inducers for this form of "regulated" necrosis, often referred to as "programmed necrosis" or "necroptosis." Genetic and biochemical experiments show that RIP kinase-dependent necrosis and caspase-dependent apoptosis are intimately linked. Unlike apoptosis, necrosis tends to promote inflammation. Emerging evidence indicates that the pro-inflammatory nature of necrosis is a critical driver in a wide range of disease pathologies. In this chapter, I discuss the molecular pathway that controls necrosis and how it contributes to different inflammatory diseases.

Keywords Programmed necrosis • Necroptosis • RIP1 • RIPK1 • RIP3 • RIPK3 • Mixed lineage kinase domain-like (MLKL) • Pgam5 • Vaccinia virus • Cytomegalovirus • Fadd • Caspase-8 • vFLIPs • Smac mimetics

10.1 Introduction

The balance between cell proliferation and cell death is critical for metazoan homeostasis. Necrotic cell death is characterized by extensive organelle and cell swelling, and rupture of the plasma membrane. These morphological changes are

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distinct from those of apoptosis, which show organelle and cell shrinking, nuclear chromatin condensation, and blebbing of membrane-bound fragments known as apoptotic bodies [1, 2]. Necrosis is considered to be an inflammatory form of cell death because of the release of intracellular contents such as HMGB1 that are collectively termed "danger-associated molecular patterns" (DAMPs) [3]. The released DAMPs can activate toll-like receptors (TLRs) on the surface of innate immune effector cells to promote inflammatory cytokine expression [4, 5]. These observations imply that necrosis is an important cell death module that can alter the quality and magnitude of immune responses.

Necrosis was once considered to be an accidental and unregulated type of cell injury. However, emerging evidence shows that necrosis can be induced in a regulated manner like apoptosis. Regulated necrosis has been called "programmed necrosis" or "necroptosis" to distinguish it from necrosis induced by physical trauma [6]. Programmed necrosis can be induced by plasma membrane-associated death receptors in the TNF receptor (TNFR) superfamily [7–10], by T cell receptor [11–13] and by toll-like receptors (TLRs) [14–16]. In this chapter, I discuss the emerging roles of programmed necrosis in biology with a specific emphasis on its functions in immunity and inflammation. For the sake of simplicity, we will use the term necrosis to refer to regulated programmed necrosis or necroptosis hereafter.

10.2 The Morphology of Necrosis

Organelle and cell swelling is a key distinction of necrotic cells. Cell death with organelle and cell swelling was first described by the German pathologist F. von Recklinghausen as "oncosis" over a 100 years ago [17]. Examination by electron microscopy reveals numerous small vacuoles in early necrotic cells (Fig. 10.1, black arrowhead). These small vacuoles appear to swell or coalesce with each other to form larger vacuoles. In addition to the vacuoles, mitochondrial distension can be readily detected (Fig. 10.1, red arrow). The identity and origin of these vacuoles and whether they are the cause or consequence of necrosis signaling are unknown. It is noteworthy that the larger vacuoles often exhibit some lesions on their membranes (Fig. 10.1, red arrowhead). These intracellular membrane lesions can be detected in cells with intact plasma membranes. Hence, internal membrane damage precedes plasma membrane leakage in necrosis. Based on these morphological changes, necrosis is akin to cell death through "internal bleeding".

10.3 A Defined Signaling Pathway Regulates Necrosis

As its name implies, TNF (tumor necrosis factor) made its mark as a factor that causes necrosis of tumor transplants [18]. Since that time, much of the work has focused on how TNF mediates NF-κB activation and apoptosis. By comparison,

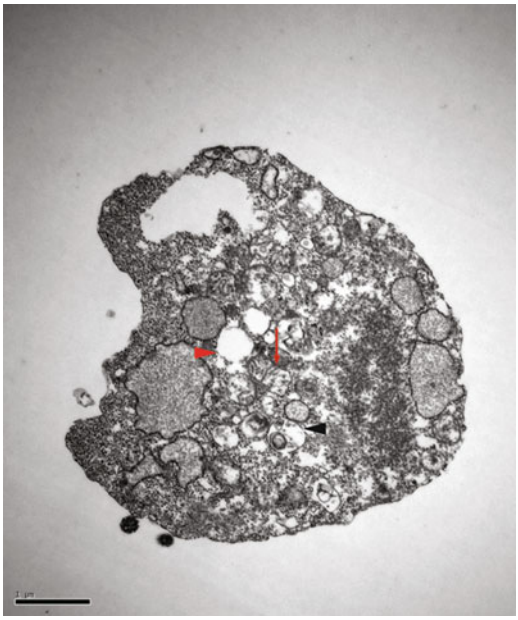

Fig. 10.1 The anatomy of a necrotic cell. Electron micrograph of vaccinia virus-infected mouse embryonic fibroblasts treated with TNF. Note that many vacuoles contain cytoplasmic contents (*black arrowhead*) or exhibit compromised membrane integrity (*red arrowhead*). The *red arrow* indicates a swollen mitochondrion. Plasma membrane rupture is also prominent

necrosis was rendered somewhat of an afterthought. That changed when several recent reports show that a dedicated molecular pathway driven by the receptor-interacting protein kinases RIPK1 and RIPK3 drives necrosis induced by death cytokines such as TNF, Fas ligand (FasL), and TRAIL (TNF-related apoptosis inducing ligand) [10, 19–22]. Because ligation of TNF receptor 1 (TNFR-1/TNFRSF1a/CD120a) is the best-defined pathway that leads to necrosis, we use it to illustrate some salient principles.

10.4 Checkpoint 1: Necrosis is Controlled by Protein Ubiquitination

TNF signaling is controlled by temporally and spatially distinct complexes. When TNF engages TNFR-1, a membrane-associated signaling complex termed "Complex I" is formed [23]. The primary function of Complex I appears to be NF-κB activation, as it contains many adaptors that are crucial for NF-κB activation. These include TNFR-associated death domain (TRADD), RIPK1, cellular inhibitor of apoptosis 1 (cIAP1), cIAP2, TNFR-associated factor 2 (TRAF2), and the linear ubiquitin chain assembly complex (LUBAC). Although early reports show that K63-linked ubiquitination of lysine 377 of RIPK1 is essential for recruitment of NEMO and activation of the IKK complex [24, 25], other types of ubiquitin linkages involving other lysine residues may also promote NF-κB activation [26, 27].

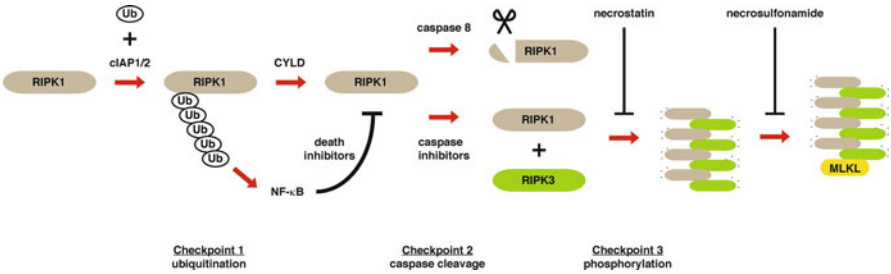

Fig. 10.2 The three major regulatory checkpoints in necrosis signaling pathway. In the first checkpoint, the E3 ligases cIAP1 and cIAP2 mediate RIPK1 ubiquitination (Ub), which inhibits necrosis through NF-κB-dependent and -independent mechanisms. Removal of ubiquitin chains on RIPK1 by the de-ubiquitinase cylindromatosis (CYLD) is important for assembly of the RIPK1-RIPK3 necrosome. In the second checkpoint, active caspase-8 cleaves and inactivates RIPK1, RIPK3, and CYLD (only RIPK1 cleavage is shown for the sake of simplicity). Cleavage of RIPK1 and RIPK3 prevents their trans-phosphorylation and phosphorylation of downstream substrates. When the integrity of RIPK1 and RIPK3 is preserved in the presence of caspase inhibitors, trans-phosphorylation of RIPK1 and RIPK3 promotes their aggregation into amyloidal complexes. This unique architecture of the necrosome promotes recruitment and activation of downstream substrates such as MLKL

NF-κB activates expression of death inhibitory factors such as cFLIP (cellular FLICE (caspase-8)-like inhibitor protein). In addition, the polyubiquitin chains on RIPK1 can sterically hinder binding of downstream effectors such as Fadd/caspase-8 or RIPK3 [28, 29]. In both scenarios, the assembly and activation of the cytoplasmic death-inducing signaling complex termed "Complex II" is impaired [6]. Thus, the ubiquitin chains on RIPK1 prevent cell death through NF-κB-dependent and -independent mechanisms. Consistent with an inhibitory role of RIPK1 ubiquitination in cell death signaling, the ubiquitin hydrolase cylindromatosis (CYLD) is a strong promoter of apoptotic and necrotic responses [30, 31] (Fig. 10.2).

10.5 Checkpoint 2: Caspase-8 is a Negative Regulator of Necrosis

Besides protein ubiquitination, necrosis is also negatively controlled by caspase activity. Early studies show that caspase inhibition by peptide-based inhibitors or virus-encoded inhibitors could often sensitize cells to TNF-induced necrosis [8]. Caspase inhibition also led to necrosis when Fadd, the upstream adaptor for caspase-8, was chemically induced to form dimers [32, 33]. These early reports implicate that the Fadd-caspase-8 apoptotic axis antagonizes necrosis. In agreement, caspase-$8^{-/-}$ or $Fadd^{-/-}$ cells are highly sensitive to TNF-induced necrosis. The requirement of caspase inhibition for optimal induction of necrosis is attributed to the fact that several key necrosis mediators are caspase-8 substrates. For example, caspase-8

cleaves RIPK1 and RIPK3 at the boundary of their respective kinase domains, thereby preventing the phosphorylation of RIPK1 and RIPK3 substrates [19, 22, 34, 35]. Cleavage of another necrosis mediator CYLD compromises protein stability, leading to rapid loss of the protein and accumulation of polyubiquitinated RIPK1 [36] (Fig. 10.2). The dominance of apoptosis over necrosis is likely an important regulatory mechanism to minimize the deleterious effects of necrotic cell death to the organism (see below).

10.6 Checkpoint 3: Phosphorylation Promotes RHIM-Mediated Necrosome Formation

RIPK1 and RIPK3 form a tight filamentous/punctate-like complex termed the "necrosome" that is crucial for necrosis [20, 21]. Necrosome formation requires the RIP homotypic interaction motif (RHIM) that is present in RIPK1, RIPK3, and several other adaptors with important functions in cell death and innate immune signaling [37] (Fig. 10.3). Both RIPK1 and RIPK3 are phosphorylated during necrosis. RIPK1 appears to be the apical kinase in the necrosis pathway, since the RIPK1-specific inhibitor necrostatin-1 abolished RIPK3 phosphorylation and necrosome assembly [20, 38]. Mass spectrometry of overexpressed RIPK1 shows that the kinase domain is heavily phosphorylated [39]. Interestingly, while expression of the full-length proteins results in diffuse cytoplasmic distribution, expression of truncated RIPK1 and RIPK3 lacking the kinase domain leads to spontaneous filamentous aggregation [40]. Because formation of the filamentous aggregates requires the RHIM, the kinase domain may inhibit RHIM-driven aggregation. The charge repulsion that results from phosphorylation of the kinase domains may relieve this inhibition to allow RHIM–RHIM filamentous aggregates to form (Fig. 10.2). Taken together, necrosis is regulated by at least three distinct mechanisms: protein ubiquitination, caspase cleavage, and phosphorylation.

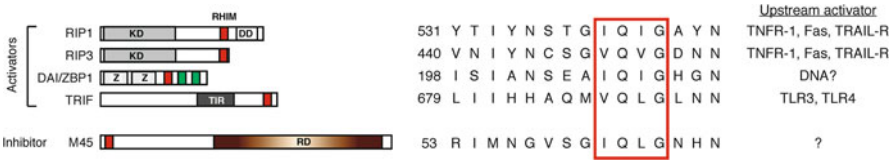

Fig. 10.3 Mammalian RHIM-containing adaptors and their activating receptors. On the *left: schematic diagram* showing the domain organization of different RHIM-containing adaptors. *Red boxes* = RHIM domains, *green boxes* = RHIM-like domains, *KD* = kinase domain, *DD* = death domain, *Z* = z-DNA binding domain, *RD* = ribonucleotide reductase domain. *Middle panel*: Alignment of tetrapeptide RHIM (*red box*) and its flanking sequences. The *numbers on the left* denote the starting amino acid position for the respective sequences. For comparison, the RHIM sequence of vIRA/M45 is shown at the *bottom*

10.7 RHIM-Mediated Amyloidal Fibril Assembly: A Novel Paradigm in Cell Death Signaling

As I have indicated above, the RHIM is an emerging protein–protein interaction domain found in several other cell death and innate immune signaling adaptors including TIR domain-containing adaptor molecule 1 (TICAM1/TRIF) and DNA-dependent activator of interferon regulatory transcription factors (DAI/ZBP1) [41]. The RHIM is defined by a highly conserved tetrapeptide core sequence of mostly hydrophobic residues that are predicted to be β-sheet (Fig. 10.3). Recent biophysical studies show that the filamentous aggregates of the RIPK1-RIPK3 necrosome exhibit properties of amyloid complexes [40]. Mutagenesis of the tetrapeptide RHIM core sequences shows that the RHIM is essential for amyloidal assembly of the necrosome, RIPK1 and RIPK3 kinase activity, and necrosis induction. Proteinaceous amyloidal plaques are toxic to neurons and thought to be the etiological agents in many neurodegenerative conditions such as Alzheimer's disease and Parkinson's disease [42]. Unlike the neurotoxic amyloids, the necrosome appears to be a signaling platform for recruitment of downstream necrosis mediators (see below). Nonetheless, these results raise the tantalizing possibility that necrosis driven by the RIP kinases is involved in the neurodegenerative disorders.

10.8 Downstream Regulators of Necrosis

In contrast to amyloid fibrils that directly trigger cytotoxicity in neurons, the RIPK1–RIPK3 amyloid fibrils appear to have a predominant role in recruitment and activation of downstream RIPK3 substrates. One such substrate is the mixed lineage kinase domain-like (MLKL), which was identified by biochemical purification and by shRNA screen [43, 44]. The significance of MLKL in necrosis is highlighted by the identification of a small molecule inhibitor called "necrosulfonamide" (NSA). NSA inhibits TNF-induced necrosis by covalently modifying human MLKL. Importantly, NSA- or siRNA-mediated silencing of MLKL expression did not interfere with RIPK1–RIPK3 necrosome formation. Hence, MLKL is a key downstream target of RIPK3 [43].

Another potential RIPK3 substrate is the mitochondrial protein phosphoglycerate mutase family member 5 (Pgam5). There are two isoforms of Pgam5, the long (5L) and short form (5s). Biochemical purification identified both isoforms of Pgam5 as RIPK3 binding partners. RIPK3 can phosphorylate Pgam5 in vitro. siRNA-mediated silencing of both isoforms of Pgam5 inhibits TNF-induced necrosis. NSA prevents the recruitment of Pgam5s, but not Pgam5L, to the RIPK1–RIPK3 necrosome, indicating that the activation of Pgam5 is a multi-step process [45]. Interestingly, Pgam5s expression causes mitochondria fragmentation [46], a function that is consistent with its ability to dephosphorylate and activate the mitochondria fission factor Drp-1 [45]. This raises the interesting possibility that the

necrosome can engage the mitochondria fission machinery to execute necrosis. Further experiments, such as those with gene deleted mice, will be vital to confirm the in vivo role of MLKL and Pgam5 in physiological necrosis.

10.9 Role of Necrosis in Innate Inflammatory Responses: Viral Infections

As I have indicated earlier in the chapter, many RHIM-containing protein adaptors are key components of cell death and innate immune signaling pathways. This molecular signature suggests that necrosis is intimately linked to innate inflammatory responses. Consistent with this notion, inflammatory cytokines such as interferons can greatly sensitize cellular necrosis [47] and the release of endogenous adjuvants from necrotic cells is known to be immuno-stimulatory [48]. These observations highlight the possible role for necrosis in innate immunity against microbial pathogens. The first example of this emerging paradigm comes from study of vaccinia virus infection. Vaccinia virus, like other poxviruses, encodes many immune evasion genes, many of which are potent inhibitors of inflammatory cytokines [49–51]. Despite these inhibitory mechanisms, vaccinia virus elicits a strong inflammatory response in mice and in humans.

One of the immune evasion genes encoded by vaccinia virus is B13R or Spi2, a serpin that inhibits caspase-1 and caspase-8 that is functionally similar to the cytokine response modifier A (CrmA) from cowpox virus [52]. Expression of CrmA or B13R/Spi2 inhibits caspase activation and death receptor-induced apoptosis. However, B13R/Spi2 is not sufficient to protect vaccinia virus-infected cells from the cytotoxic effect of TNF [53]. Instead, TNF stimulation of the infected cells causes classical morphology associated with necrosis such as extensive intracellular vacuoles formation and organelle swelling. By contrast, vaccinia virus-infected RIPK1$^{-/-}$ and RIPK3$^{-/-}$ cells are resistant to TNF-induced cytotoxicity [19, 20]. In wild-type mice, vaccinia virus infection causes elevated TNF expression within 24 h post-infection, which coincides with assembly of the RIPK1–RIPK3 necrosome in infected tissues [20]. This indicates that necrosis is an early response to vaccinia virus infection. Consistent with a protective role for necrosis in innate immune defense against vaccinia virus, tissue necrosis, and inflammation are greatly reduced in RIPK3$^{-/-}$ mice. This contributes to greatly increased viral load and mortality of infected RIPK3$^{-/-}$ mice [20]. Collectively, the in vitro and in vivo studies show that necrosis is an important innate immune mechanism that controls the viral factory before virus-specific T- and B-cells are mobilized in high enough number to fully eradicate the virus.

The study on vaccinia virus suggests that host cell necrosis is a crucial innate immune defense mechanism against certain viruses (Fig. 10.4). Because viruses are adept at circumventing host immune responses, it begs the question whether some viruses might have developed strategies to inhibit necrosis as a means to

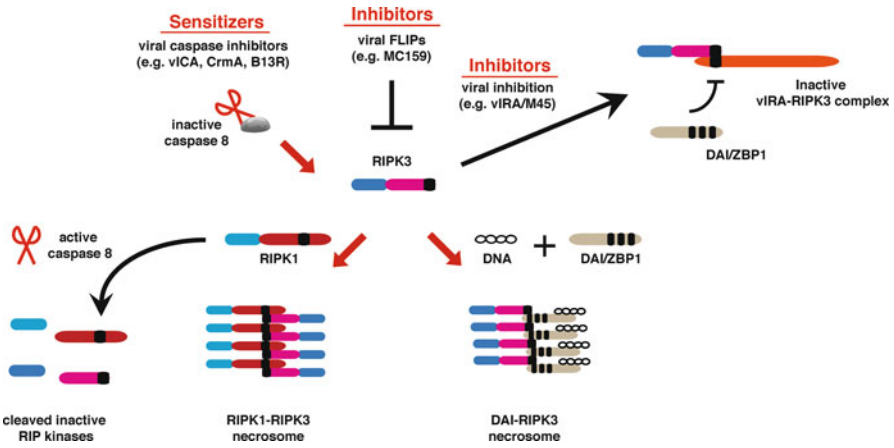

Fig. 10.4 Viral cell death inhibitors that modulate host cell necrosis. Under normal conditions, active caspase-8 cleaves RIPK1 and RIPK3, thereby preventing necrosome assembly and activation. In cells infected by poxvirus, caspase-8 is inhibited and the integrity of RIPK1 and RIPK3 is preserved. This allows assembly of the necrosome to promote host cell necrosis. Hence, certain viral cell death inhibitors are actually "sensitizers" for necrosis. In MCMV infection, vICA inhibits caspase activity. However, necrosis is prevented by vIRA/M45, which binds to RIPK3 and DAI to prevent their activation. In the absence of vIRA, DAI engages RIPK3 to promote necrosis through assembly of a noncanonical necrosome. Unlike vIRA, the mechanism by which vFLIPs inhibit necrosis is poorly understood

escape elimination from the host. Viral FLIPs (FLICE-like inhibitor proteins) are orthologs of cellular caspase-8 and caspase-10 that lack the enzymatic subunits p20 and p10. Binding of vFLIPs to caspase-8 or caspase-10 via their death effector domains (DEDs) inhibits apoptosis induced by TNF-like death cytokines [54–56]. Interestingly, the vFLIPs MC159 from Molluscum contagiosum virus and E8 from Equine herpesvirus are also potent inhibitors of TNF-induced necrosis [19]. Because not all vFLIPs can inhibit necrosis, DED–DED interaction, which is critical for apoptosis inhibition, is not responsible for necrosis inhibition.

Like poxviruses, murine cytomegalovirus (MCMV) is a herpesvirus that encodes many immune evasion genes. The three different types of viral cell death inhibitors encoded by MCMV, vICA (inhibitor of caspase-8-induced apoptosis), vMIA (mitochondria inhibitor of apoptosis) and vIRA (inhibitor of RIP activation) are crucial for productive infection and replication of viral progenies [57]. Interestingly, vIRA or M45 contains a RHIM at the amino terminus that mediates binding to cellular RHIM-containing adaptors including RIPK1, RIPK3, and DNA activator of interferon (DAI/ZBP1) (Fig. 10.3) [58–61]. Recombinant virus expressing a mutant vIRA with tetra-alanine substitutions within the core RHIM sequence fails to establish productive infection in cells and in mice due to premature cell death by necrosis. Productive infection is restored when the RHIM mutant MCMV infects RIPK3$^{-/-}$ mice and, to a lesser extent, DAI$^{-/-}$ mice, but not in RIPK1$^{-/-}$ cells [61, 62].

Hence, necrosis induced in response to mutant MCMV infection is mediated through RIPK3 interaction with another RHIM-containing adaptor DAI. These results support a model in which MCMV infection sensitizes cells to necrosis through vICA. However, in contrast to infection with vaccinia virus, vIRA/M45 inhibits premature host cell necrosis to ensure productive viral replication (Fig. 10.4). Unlike M45, the M45 ortholog of human CMV, UL45, was a weak inhibitor of apoptosis [63]. It will be interesting to determine if similar viral inhibition of necrosis plays any role in the pathogenesis of human CMV infection.

10.10 Necrosis in Bacterial Infections

Lipopolysaccharide (LPS) is a major component of the membrane of Gram-negative bacteria. LPS binding to toll-like receptor 4 (TLR4) on the surface of immune cells such as macrophages leads to expression of TNF and other pro-inflammatory mediators. The cumulative effects of these inflammatory mediators give rise to the systemic symptoms of bacterial septic shock. Strikingly, RIPK3$^{-/-}$ mice were protected from TNF-induced systemic inflammatory response syndrome (SIRS) [64, 65] and cecal ligation puncture-induced sepsis [64]. By contrast, results obtained using the RIPK1 inhibitor necrostatin-1 (Nec-1) were less definitive than those obtained with RIPK3$^{-/-}$ mice. While one group reported protection by Nec-1, another group found that Nec-1 exacerbated TNF-induced SIRS [64, 65]. These opposing observations may be due to off-target effects of high doses of Nec-1 [13]. Unfortunately, since RIPK1$^{-/-}$ mice exhibit neonatal lethality [66], genetic model to assess RIPK1 function in these inflammatory diseases is currently not available. Nonetheless, these results support a role for TNF-induced necrosis in promoting bacterial sepsis.

Although the importance of TNF is undisputed, it is not the only mediator of necrosis in bacterial sepsis. Recent evidence indicates that TLR4 stimulation, especially in the presence of caspase inhibition, could directly trigger macrophage necrosis. The inflammatory cytokine storm and extensive macrophage necrosis induced by LPS and the pan-caspase inhibitor zVAD-fmk was abrogated in RIPK3$^{-/-}$ and TRIF$^{lps2/lps2}$ mutant mice [14]. TRIF is a toll/IL-1 receptor (TIR) domain-containing adaptor that mediates type I interferon response to TLR3 and TLR4 [67, 68]. In addition, TRIF contains a RHIM near the carboxyl terminus through which it interacts with other RHIM-containing adaptors (Fig. 10.3). Mutagenesis experiments show that the RHIMs of TRIF and RIPK3 are similarly required for TLR4-induced macrophage necrosis [14]. Hence, distinct RHIM-containing adaptors can promote necrosis downstream of different receptors. Besides driving necrosis, RHIM-mediated interactions between TRIF, RIPK1, and RIPK3 also regulate apoptosis and NF-κB activation [69, 70]. These results indicate that RHIM–RHIM interaction per se does not necessarily specify cell death by necrosis. Rather, additional structural constraints, such as stoichiometry and tertiary structure (e.g. amyloid folding), are likely to be critical determinants for the signaling outcome.

10.11 Necrosis in Sterile Inflammation

Besides its role in pathogen-induced inflammation, necrosis also promotes sterile inflammation in certain settings. For example, the eye appears to be exquisitely sensitive to RIPK1 or RIPK3-mediated necrotic cell injury. RIPK3 expression was highly elevated in necrotic cone cells in a mouse model of retinitis pigmentosa, and cone cells necrosis was rescued in RIPK3$^{-/-}$ mice [65]. Moreover, retinal detachment-induced photoreceptor necrosis is blocked in RIPK3$^{-/-}$ cells [71]. Repeated doses of cerulein triggers acinar cell necrosis and acute pancreatitis that is rescued in RIPK3$^{-/-}$ mice [21, 22]. Administration of the RIPK1 inhibitor Nec-1 significantly ameliorates tissue damage in animal models of myocardial infarction, ischemia induced brain injury, and renal ischemia/repurfusion injury [38, 72–74], suggesting that RIPK1-dependent necrosis is activated under these conditions in wild-type animals. Although TNF and other inflammatory cytokines are often elevated in ischemia/reperfusion-induced injury [75, 76], the etiological agents that mediate necrosis in these diseases have yet to be determined. It is possible that necrosis can be induced without death receptor engagement in these disease pathologies, such as that observed in "intrinsic" apoptosis induced in response to genotoxic stress.

Similar to caspase inhibition in tissue culture, caspase-8$^{-/-}$ or Fadd$^{-/-}$ cells and mice are highly sensitive to necrosis induction. Germline inactivation of these genes results in extensive necrosis and embryonic lethality on E9.5 [77–79]. Most remarkably, the defect was rescued by deletion of RIPK1 or RIPK3 [80–83]. Similarly, keratinocyte- or intestinal epithelium-specific deletion of Fadd or caspase-8 caused severe inflammation in the respective sites that was corrected by deletion of RIPK3 [84–87]. The current model posits that these inflammatory conditions are caused by increased necrosis. However, since Fadd, caspase-8, RIPK1 and RIPK3 have all been shown to modulate innate immune signaling, the possibility that Fadd and caspase-8 are required to clamp down on uncontrolled inflammatory signaling through RIPK1 or RIPK3 cannot be overlooked (see below) [88, 89].

10.12 Direct Roles for RIPK1 and RIPK3 in Inflammation Signaling

As I have discussed earlier, promoting inflammation via NF-κB was the first function ascribed to RIPK1. In addition to TNF receptor, RIPK1 also mediates NF-κB activation by certain innate immune receptors such as TLR3 [90], TLR4 [91, 92], and RIG-I [88, 93]. In contrast to RIPK1, RIPK3$^{-/-}$ cells exhibit normal NF-κB induction in response to TNFR-1 and several TLR agonists [94]. However, early reports show that overexpression of RIPK3 either inhibits or promotes NF-κB activation [69, 90, 95, 96]. As such, it remains possible that RIPK3 can modulate NF-κB responses in specific situations.

In addition to potentially regulating NF-κB, RIPK3 was recently reported to have a surprising role in driving maturation of the pro-inflammatory cytokine IL-1β [97]. Production of mature IL-1β requires two signals. The first signal, which can be provided by activation of innate immune receptors such as TNFR-1 or TLR4, activates de novo synthesis of pro-IL-1β in a NF-κB-dependent manner. Secretion of mature IL-1β requires a second signal that activates the inflammasome and caspase-1-mediated processing of pro-IL-1β. In addition to caspase-1, noncanonical inflammasome activation can involve processing of pro-IL-1β by caspase-8 and caspase-11 [98–101]. Smac mimetics (SM), which induce proteasomal degradation of cellular inhibitor of apoptosis 1 (cIAP1), cIAP2 and X-linked IAP, potently sensitizes cells to apoptosis and necrosis. Surprisingly, SM also drives IL-1β maturation in LPS-primed macrophages. This effect of SM requires RIPK3, canonical and noncanonical NLRP3 inflammasome activation. Consistent with the effect seen with SM, LPS primed cIAP1$^{-/-}$ cIAP2$^{-/-}$ XIAP$^{-/-}$ macrophages spontaneously produce mature IL-1β [97]. These results suggest the tantalizing possibility that RIPK3 can promote inflammation through multiple routes. On one hand, release of DAMPs from necrotic cells activates TLRs to promote inflammatory gene expression. On the other hand, RIPK3 can directly engage the inflammasome to promote expression of IL-1 like inflammatory cytokines.

10.13 Necrosis in Lymphocyte Tolerance and Immune Homeostasis

The maintenance of immune homeostasis is critically dependent on proper regulation of lymphocyte cell death. T cells recognize antigenic peptides bound to self-MHC (major histocompatibility complex) through their T cell receptors (TCRs). Because TCRs are generated by random gene rearrangement, T cells that express low affinity TCRs that cannot be activated by peptide-MHC complexes or high affinity TCRs that are autoreactive are produced. These lymphocytes are eliminated through thymic "positive selection" and "negative selection," respectively. The cumulative effect of thymic selection is a TCR repertoire that is largely devoid of autoreactive cells.

The mechanism by which developing lymphocytes are eliminated during thymic selection is not fully defined. However, death receptors in the TNF receptor superfamily are dispensable for thymic selection processes. By contrast, the death receptors Fas/CD95/APO-1, TNFR-1, and TNFR-2 play key roles in peripheral tolerance of mature T cells that populate the peripheral organs [102, 103]. Initial stimulation of naïve T cells through their TCRs leads to clonal expansion. However, repeated TCR stimulation of activated T cells causes "activation/restimulation-induced cell death" (AICD or RICD). Repeated TCR stimulation in the presence of the T cell trophic factor IL-2 greatly enhances Fas, Fas ligand (FasL), and TNF expression

[104]. As a result, activated T cells are eliminated through FasL-Fas or TNF–TNFR interaction in a paracrine fashion. This process is critical for mature lymphocyte homeostasis, since mutations in Fas and FasL in mice and humans lead to systemic autoimmune diseases (*lpr/gld* in mice and Autoimmune Lymphoproliferation Syndromes (ALPS) in human) [105–107].

It is widely believed that apoptosis is the cell death module that controls mature lymphocyte cell death in response to FasL and TNF. However, this notion was challenged when mice with T-cell-specific deletion of Fadd or caspase-8 were found to be immuno-deficient rather than developing *lpr*-like autoimmune disease [11, 77, 108]. Similarly, immuno-deficiency rather than ALPS-like systemic autoimmunity is the dominant phenotype of human patients with caspase-8 mutations [109]. Although these defects were originally attributed to defective TCR-induced NF-κB activation [110], subsequent experiments show that TCR-induced NF-κB activation was normal in caspase-8$^{-/-}$ T cells [11]. Further examination revealed that Fadd$^{-/-}$ and caspase-8$^{-/-}$ T cells undergo extensive necrosis in response to TCR stimulation. Consistent with the notion that Fadd$^{-/-}$ and caspase-8$^{-/-}$ T cells suffer from extensive necrosis, the RIPK1 inhibitor Nec-1 restored normal T cell proliferation [111]. Moreover, Fadd$^{-/-}$RIPK1$^{-/-}$ and caspase-8$^{-/-}$ RIPK3$^{-/-}$ T cells show normal TCR-induced proliferation in vitro and virus-induced clonal expansion in vivo [80, 81]. Results obtained from RIPK3$^{-/-}$ mice expressing a dominant negative Fadd also show similar results [112]. Most remarkably, mice with impaired apoptosis and necrosis pathways developed *lpr*-like autoimmune disease [12, 81, 82]. These results revealed an unexpected pro-survival function for Fadd and caspase-8 during T cell clonal expansion. They also underscore the fact that T cell tolerance and immune homeostasis is achieved by the coordinate action of caspase-dependent apoptosis and RIP kinase-dependent necrosis.

In contrast to T cells, B cell proliferation through the antigen receptor or CD40 is unaffected in Fadd$^{-/-}$ and caspase-8$^{-/-}$ B cells [113, 114]. However, TLR3- and TLR4-induced B cell proliferation is impaired in Fadd$^{-/-}$ and caspase-8$^{-/-}$ B cells [113, 114]. Unlike TCR-induced proliferation, defective Fadd$^{-/-}$ B cell proliferation was not restored in Fadd$^{-/-}$ RIPK1$^{-/-}$ B cells [80]. Because RIPK1 is required for NF-κB activation in response to TLR3 and TLR4 [90, 115], the defective TLR3/4-induced proliferation in Fadd$^{-/-}$ RIPK1$^{-/-}$ B cells can be attributed to defective NF-κB signaling. It will be interesting to determine if normal proliferation is restored in Fadd$^{-/-}$ RIPK3$^{-/-}$ B cells. Taken together, these results illustrate that although RIPK1 and RIPK3 have differential roles in regulating antigen receptor-induced proliferation in T and B cells.

10.14 Concluding Remarks

Recent studies have provided a molecular explanation for the age-old observation that inflammation is often accompanied by tissue necrosis. While the necrosis-promoting function of the RIP kinases has been clearly demonstrated, key questions

remained whether they perform functions beyond regulating necrosis. For example, RIPK1 and RIPK3 expression is highly induced during T cell activation [13, 20]. It seems counterintuitive to upregulate expression of death-promoting molecules such as RIPK1 and RIPK3 at a time when clonal expansion of lymphocytes should be the primary goal for the organism. Similarly, the expression of RIPK3 is highly induced during embryogenesis [80]. Why would the organism risk the damaging effects of necrosis when the well-being of the organism is in delicate balance? Under normal circumstances, the deleterious effects of necrosis is tempered in part by caspase-8-mediated cleavage of the RIP kinases. It begs the question that perhaps RIPK1 and RIPK3 have important biological functions other than necrosis. Discovering and deciphering the non-necrotic or normal physiological functions of the RIP kinases will be of critical relevance as the scientific community ponders the possibility of manipulating necrosis as a therapeutic option.

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Chapter 11
Structural Perspectives on BCL-2 Family of Proteins

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Abstract BCL-2 proteins are a family of pro- and anti-apoptotic proteins that regulate a critical step in the mitochondrial apoptotic pathway, the permeabilization of the mitochondrial outer membrane. Because apoptosis and mitochondrial function play an important role in physiology and a number of diseases, intensive investigation over the past two decades has been invested to understand in detail the structure and function of the BCL-2 proteins. Structural biology investigations of BCL-2 proteins have provided tremendous insights into our understanding of their structure–function relationships and models have been proposed to explain how the BCL-2 family members form a network of interactions to control apoptosis signaling. Here, we will review the available structural information of pro- and anti-apoptotic members and the structures of their interaction in homodimerization and heterodimerization. We will discuss the structural insights for each structural domain of BCL-2 proteins that determine their function in the cytosol and the outer mitochondrial membrane. Furthermore, we will discuss the structural details of the interactions between BCL-2 family members and the various structural paradigms that ultimately regulate the activation of apoptosis.

Keywords Apoptosis • BCL-2 • BAX • BH3-only • BH3 domain • Structure • MOMP • Cytochrome c • Mitochondria

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11.1 Introduction

Programmed cell death, or apoptosis, is an essential, genetically controlled process that maintains the normal development and homeostasis of multicellular organisms. Apoptosis is employed to eliminate the body of unwanted or malfunctioning cells [1, 2]. However, when uncontrolled stress surrounding the cell or from inside the cell triggers apoptosis, the balance between cell proliferation and cell death is disrupted. Apoptotic deregulation, which leads to excessive cell death, significantly contributes to diseases such as cardiovascular disease, infectious diseases, stroke, and neurodegenerative disorders [3–5]. In contrast, aberrant signaling that prevents apoptosis and leads to excessive cell proliferation leads to development of cancer and autoimmune disease [6, 7]. Therefore, cell death is an essential component in cellular life and in disease development, and understanding the mechanisms and regulators of the apoptotic process may lead to new therapeutic interventions [8, 9].

The BCL-2 family proteins are master regulators of the intrinsic or mitochondrial pathway of apoptosis, Fig. 11.1 [10]. BCL-2 proteins regulate the integration

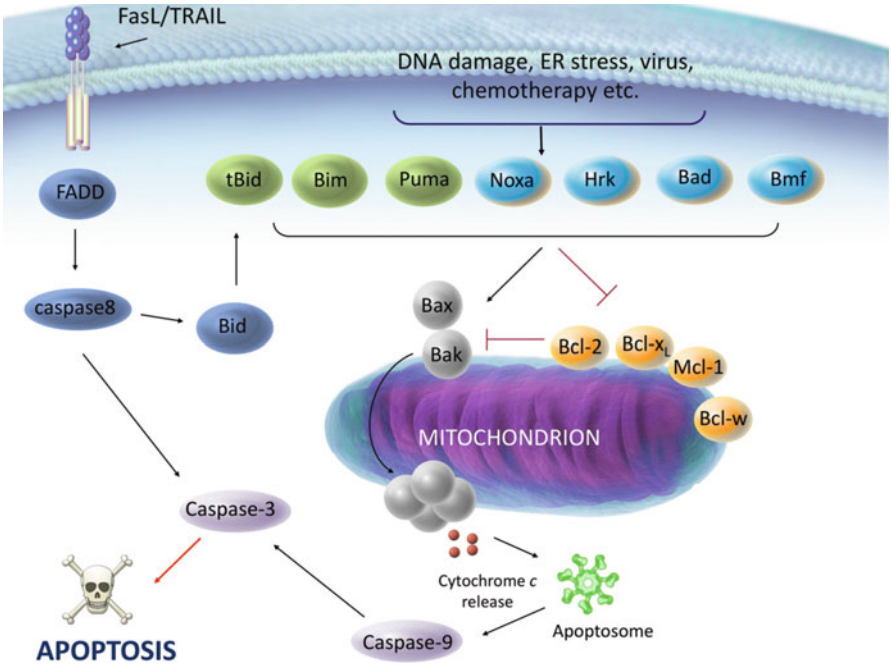

Fig. 11.1 The BCL-2 family is a principal regulator of the intrinsic or mitochondrial apoptotic pathway. The intrinsic pathway is initiated by different stimuli such as DNA damage, viral infection, ER stress, chemotherapy, radiation, and the extrinsic apoptotic pathway through cleavage of Bid protein. Protein interactions among the BCL-2 family proteins govern MOMP and release of apoptotic factors from mitochondria that commit the cell to apoptosis

of stimulatory or stress signals inside the cell and decide whether the mitochondrial outer membrane permeabilization (MOMP) occurs [11]. MOMP is considered to be a point of no return in most cells' commitment to apoptosis [12]. The cell is doomed to die whether MOMP induces the release of cytochrome *c*, SMAC/DIABLO, and other mitochondrial proteins into the cytosol to activate downstream enzymatic effectors of apoptosis that dismantle the cell, or induces mitochondrial dysfunction leading to cessation of ATP production [11]. Structural biology approaches have vastly contributed to our understanding of the BCL-2 family proteins and how they regulate survival and death mechanisms in the cell. Based on the structural insights, BCL-2 family proteins have become prime targets for drug discovery and the first generation of BCL-2 inhibitors are in clinical development as promising cancer therapeutics [13].

11.2 BCL-2 Family of Proteins

The BCL-2 family members are identified by the presence of one to four regions of sequence homology, named BCL-2 homology (BH) domains and are categorized into three different groups depending on their functional role, Fig. 11.2 [10]. The anti-apoptotic BCL-2 proteins (Bcl-2, Bcl-xl, Bcl-w, Mcl-1, A1/Bfl-1, Bcl-b) have four BH domains and are the pro-survival BCL-2 members that promote survival by binding and blocking the pro-apoptotic BCL-2 members [14]. Bax and Bak are the effector pro-apoptotic BCL-2 proteins and are essential to promote MOMP or mitochondrial dysfunction [15]. Bax, Bak, and Bok have four BH domains and surprisingly are structurally similar to the anti-apoptotic BCL-2 proteins. A second group of pro-apoptotic proteins, the BH3-only proteins, have only sequence homology with the BH3 domain of multidomain BCL-2 proteins [16]. BH3-only proteins sense different stress signals from outside or inside the cell and deliver the stress signals to the multi-BH BCL-2 proteins to initiate the mitochondrial apoptotic pathway. There are multiple BH3-only proteins identified such as Bad, Bid, Bim, and Puma and are regulated by multiple mechanisms such as transcriptional regulation, phosphorylation, ubiquitination, and enzymatic cleavage by proteases [16].

Interactions among the BCL-2 members control the commitment of cell to initiate apoptosis through MOMP induction (Fig 11.1). Bax and Bak are the essential pro-apoptotic proteins that are activated to undergo conformational changes and oligomerization at the mitochondrial outer membrane [15, 17]. Three models of Bax and Bak activation have been proposed. The "indirect model" assumes that Bax and Bak are repressed in an activated conformation by the pro-survival BCL-2 proteins and are spontaneously released by the competitive binding of BH3-only proteins to the pro-survival proteins [18, 19]. The "direct activation" model considers that a select group of BH3-only proteins can directly activate Bax and Bak [20, 21]. The pro-survival proteins in this model sequester the "direct activator" BH3-only proteins (Bid, Bim and Puma), however, "sensitizer" or "derepressor" BH3-only proteins (e.g. Bad, Noxa, Bmf) bind to anti-apoptotic BCL-2 proteins and release the direct activators that activate Bax and Bak. The third model, known as the "embedded

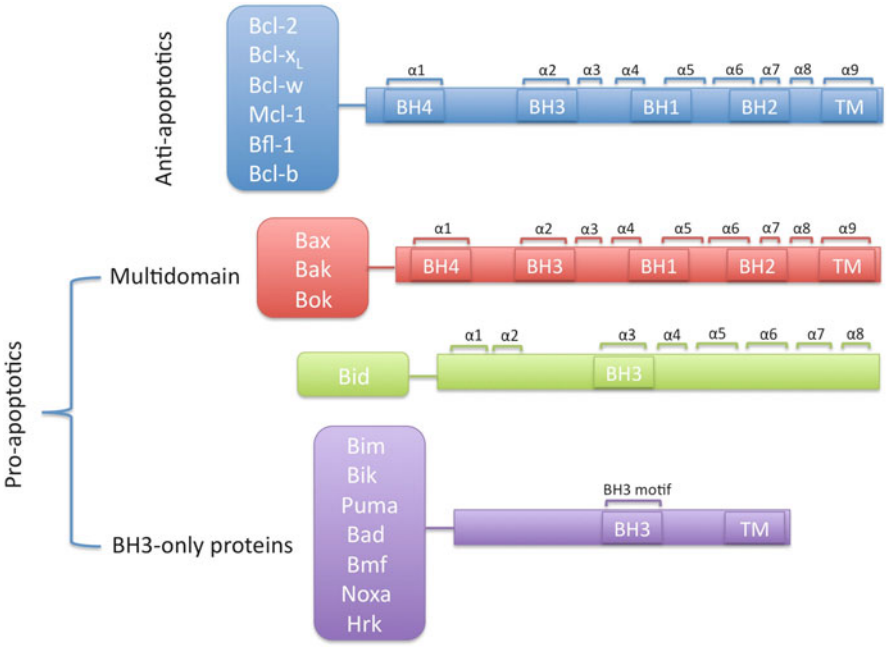

Fig. 11.2 The BCL-2 protein family. The family comprises pro-apoptotic and anti-apoptotic members. BCL-2 family members are also separated by the presence of the conserved Bcl-2 homology domains (BH1-4) as multidomain BCL-2 proteins and BH3-only proteins. Multidomain BCL-2 proteins have very similar structure whereas BH3-only proteins are mostly unstructured and contain only a folded BH3 motif. Bid is an exceptional BH3-only protein, because it has a multidomain BCL-2 fold even though it lacks three BH homology domains. BCl-2 proteins can be anchored to the mitochondrial membrane through their carboxy-terminal transmembrane domain (TM)

together" model, assumes that key binding events between BCL-2 proteins occur at the mitochondrial outer membrane and that anti-apoptotic proteins translocate to the membrane and inhibit MOMP by binding and blocking BH3-only proteins as well as Bax and Bak [22, 23]. Regardless of how Bax or Bak is activated, when there is a sufficient anti-apoptotic BCL-2 reserve, pro-apoptotic proteins are blocked and pro-apoptotic signal is suppressed. However, when the amounts of pro-apoptotic proteins overwhelm the anti-apoptotic resistance, Bax/Bak activation occurs, following oligomerization in the membrane and formation of mitochondrial pores that allow the release of aptogenic proteins to activate the enzymatic effectors of apoptosis [22, 23].

In this chapter, we will review the available structural studies of the BCL-2 family members and their protein–protein complexes. We will underscore the structural features of these proteins and the conformational changes involved in their transition from cytosolic to membrane environment. Furthermore, we will highlight similarities and differences of the various complexes between BCL-2 family proteins and how different interactions control the commitment of the cell to MOMP.

11.3 Structural Insights of Anti-apoptotic BCL-2 Proteins

The structures of six (Bcl-xl, Bcl-2, Bcl-w, Mcl-1, A1/Bfl-1, Bcl-b) anti-apoptotic members have been determined and they all demonstrate an eight α-helical bundle that has loops of varying length that connect the helices, Fig. 11.3a [24–30]. The structures consist of a central hydrophobic helix, α5, that is solvent protected and forms the center of the hydrophobic core of the protein. This central helix is surrounded from the N-terminal side by helices α1, α6, and α2, from the C-terminal side by helices α3 and α4 and from the top side by helices α7 and α8. Distinctively

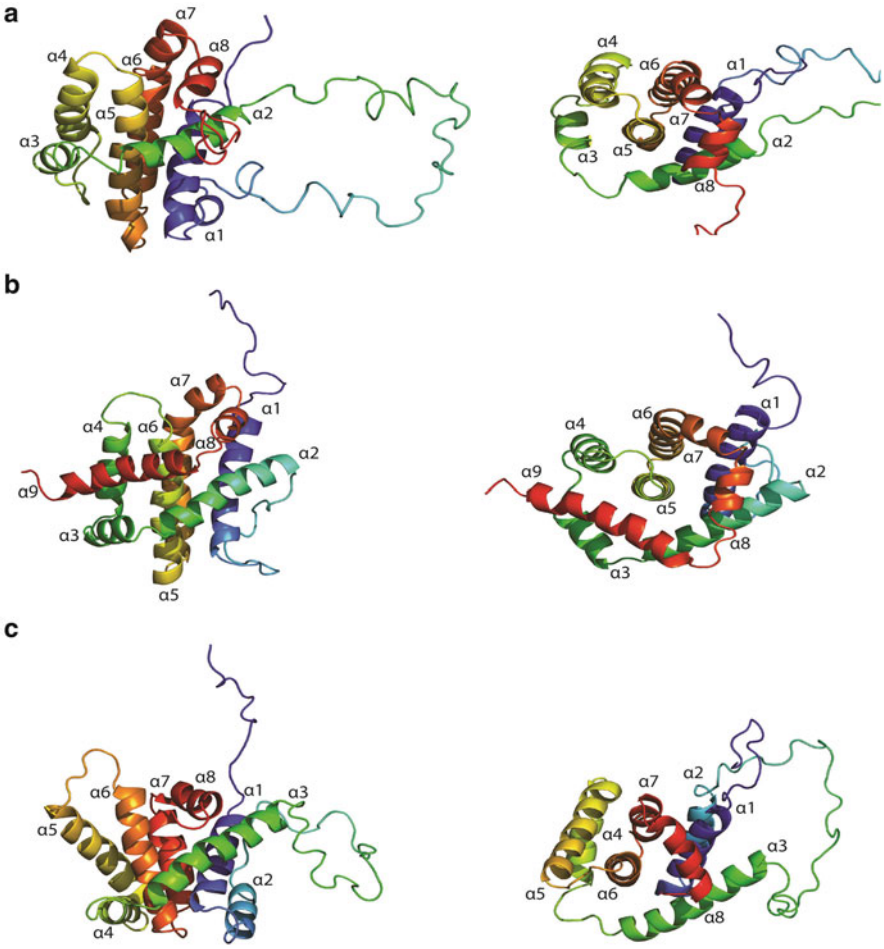

Fig. 11.3 Representative structures of the three groups of BCL-2 family proteins shown in ribbon and in two different views: (**a**) Bcl-x_L α9 truncated structure (PDB ID: 1LXL) (**b**) Full-length Bax structure (PDB ID: 1F16) and (**c**) Full-length Bid structure (PDB ID: 2BID)

from other BCL-2 proteins, MCL-1 has an extended region of ~160 residues at the N-terminus that is likely to be unstructured and contains two PEST (enriched in proline, glutamate, serine, and threonine) sequences that regulate its stability [10].

Due to technical challenges with the solubility and stability of the corresponding full-length recombinant forms, all of the pro-survival BCL-2 structures except Bcl-w have been determined without the C-terminal helix α9, present in all anti-apoptotic BCL-2 sequences. The structures of pro-survival proteins revealed a prominent hydrophobic groove formed by helices α2, α3, α4, α5, and α8 that contains the conserved BH1, BH2, and BH3 domains. This hydrophobic groove is an interaction site for the BH3 domains of pro-apoptotic proteins and it is termed the BH3 groove. Binding to the BH3 grooves by pro-apoptotic BCL-2 proteins results in sequestration of their BH3 domains and inhibition of pro-apoptotic activity. The structures of anti-apoptotic BCL-2 proteins revealed differences in structural topology and electrostatic potential among the different hydrophobic BH3 grooves [24–30]. The chemical and structural context, along with the degree of the structural plasticity, of these BH3 grooves can potentially dictate the different interaction selectivity observed between anti-apoptotic BCL-2 proteins and their pro-apoptotic counterparts.

Another common structural feature revealed by structures of anti-apoptotic BCL-2 proteins is the presence of an unstructured loop region between the α1 and α2 helices. This loop region is present also in pro-apoptotic Bax and Bak [31, 32]. This unstructured region is highly variable in sequence, and its length is about ~15 residues in most BCL-2 proteins but it can be also as long as 60 residues in the case of Bcl-x_L [24]. This unstructured region is compatible with the lack of electron density observed in crystal structure determinations and lack of medium- and long-range NOEs in NMR structures. Although binding and structural studies of anti-apoptotic BCL-2 proteins have established that this unstructured region is not involved in interactions with BH3 helical domains, functional studies in cells have shown that deletion of this unstructured region, posttranslational modifications, or binding by other proteins can regulate the inhibitory activity of the pro-survival proteins [33–35]. Surprisingly, cleavage of this unstructured region by caspases results in converting the anti-apoptotic BCL-2 proteins to molecules with pro-apoptotic function [36, 37]. The structural bases of the regulatory mechanisms involving the loop between α1 and α2 helices for the different BCL-2 proteins have not been elucidated yet.

Between anti-apoptotic BCL-2 structures available, only the solution structure of the Bcl-w contains several residues from the C-terminal helix of the protein [26, 27]. The C-terminal helix residues are bound to the hydrophobic BH3 groove of Bcl-w suggesting that the C-terminal helices of anti-apoptotic BCL-2 proteins can occupy the BH3 groove. The C-terminal helix of Bcl-w can be displaced by the binding of Bid to the BH3 groove and its deletion increases the binding affinity for BH3 helices of Bak and Bid, indicating that the C-terminal helix also regulates access of BH3 helices from pro-apoptotic proteins. The hydrophobic C-terminal helix of BCL-2 proteins is proposed to be an anchoring helix to the outer mitochondrial membrane, thus, the truncated forms of pro-survival proteins preserve the

structural fold and inhibitory activity in solution [24–30]. As a result, inhibitory activity by anti-apoptotic BCL-2 proteins may be regulated by whether the C-terminal helix is bound to the rest of the structure in the cytosol or bound to the outer mitochondrial membrane leading to exposure of the hydrophobic BH3 groove. The structural conformations of pro-survival proteins that include the C-terminal helix, whether bound to the BH3 pocket or to a membrane, are resisting structural determination, preventing structural insights of two important forms of these proteins in the cellular milieu.

A revealing structural feature of the BCL-2 proteins is the presence of the two central hydrophobic helices α5 and α6, that adopt similar overall fold with the membrane insertion domains of diptheria toxins and colicins [24, 38]. Based on this structural similarity, it was subsequently demonstrated that Bcl-2, Bcl-x_L and pro-apoptotic Bax and Bak can all form ion-conducting pores in membranes of synthetic lipid bilayers [39]. However, the existence of proteinaceous pores or channels detected in synthetic lipid bilayers or liposomes has been confronted by cellular studies that suggest that only Bax and Bak oligomerization in the mitochondrial outer membrane causes permeabilization, and anti-apoptotic members inhibit Bax and Bak oligomerization but do not oligomerize in the membrane [40–45].

Based on the distribution of BCL-2 proteins between the cytosol and mitochondrial outer membrane, it is obvious that their cytosolic and aqueous-soluble structures require conformational changes to expose their hydrophobic helices for membrane interactions. In an effort to determine the conformational changes and the structural fold involved upon membrane interaction and insertion, Bcl-xL protein was studied in lipid micelles by NMR spectroscopy [46]. Interestingly, Bcl-xL undergoes a dramatic conformational change as it interacts with detergent micelles. The protein remained monomeric and consists of a number of alpha helices separated by loops, however, the length and position of individual helices is different from those in aqueous solution. Based on NMR data, this study concluded that helices α1, α6, and possibly α5 are buried in the hydrophobic interior of the detergent micelle and other helices are near or outside the surface of the detergent micelle. This is in contrast to the formation of large oligomers by pro-apoptotic Bax and Bak observed in detergent micelles and phospholipid bilayers [40, 44, 45].

Another conformational change was demonstrated by the crystal structure of Bcl-xL forming a domain-swapped dimer in aqueous solvent upon treatment with heat or alkaline [47]. This domain-swapped dimer is formed between alpha helices α5, α6, α7, and α8 of monomer subunits with the hinge region occurring at the hairpin turn between α5 and α6 helices. The crystal structure of a domain-swapped dimer of Bcl-w revealed a different conformational change that involves helices α3 and α4 moving away from the hydrophobic core of the protein [48]. Functional studies also confirmed that a hinge region between helix α4 and α5 regulates the conformational change and function of anti- and pro-apoptotic BCL-2 proteins. Interestingly, the BH3 groove, and indeed the overall structure, of the protomer in both domain-swapped dimer conformations is preserved and enables binding of BH3 domains as in the monomeric structures [47, 48]. While these crystal

structures are determined from truncated constructs and in aqueous solvents, it may be possible that these structures provide insights to possible conformational changes involved with the BCL-2 proteins.

Further structural studies are required to understand the structure and conformational changes involved within the membrane and interactions of anti-apoptotic BCL-2 members with pro-apoptotic Bax and Bak. These structural studies should ultimately reveal why anti-apoptotic BCL-2 members do not undergo oligomerization, block Bax and Bak oligomerization, and prevent mitochondrial outer membrane permeabilization.

11.4 Structural Insights of Pro-apoptotic Bax and Bak

The structures of pro-apoptotic Bax and Bak closely resemble that of pro-survival BCL-2 proteins, Fig. 11.3b. An unresolved mystery in the apoptosis research is how BCL-2 proteins with very similar structure have such an opposite function by promoting or inhibiting mitochondrial outer membrane permeabilization. Both Bax and Bak structures have their amphipathic helices wrapped around the central hydrophobic helix α5 and the length and orientation of all helices are similar to the pro-survival counterparts. Most similarity is observed for helices α5, α6, α7, and α8 [30, 31]. One notable difference is that Bak, even in healthy cells, resides in the mitochondrial outer membrane while Bax resides mostly in the cytosol and translocates to the membrane upon stress stimuli.

The structure of Bax determined by NMR spectroscopy contains all residues of the Bax sequence [30]. Notably, the structure demonstrates that the C-terminal α9 helix occupies Bax's own hydrophobic pocket that corresponds to the anti-apoptotic BH3 groove, burying its most hydrophobic face from the solvent. Moreover, this inactive monomeric Bax structure has the hydrophobic residues of the BH3 domain facing inwards to the hydrophobic core. Consequently, the cytosolic structure of Bax has all the hydrophobic surfaces protected from water, suggesting that its translocation to the mitochondria and interaction with the membrane requires a conformational change to expose its hydrophobic surfaces. A number of stimuli, such as protein–protein interaction, detergents, pH shift, and heat lower the Bax activation energy and initiate conformational changes, which are followed by Bax translocation and oligomerization [41, 42, 45, 49–53]. Associated with the Bax conformational change are the exposure of an antibody epitope in the N-terminal helix α1, the mobilization of the C-terminal α9 helix that is considered to anchor the protein to the outer mitochondrial membrane as well as the exposure of the Bax BH3 that mediates the oligomerization process [17, 54–57].

In contrast to the anti-apoptotic BCL-2 proteins having a BH3 site at the C-terminal surface, cytosolic full-length Bax also has a binding pocket at its N-terminal surface, by the convergence of helices α1 and α6 [49]. This unexpected binding site was revealed after NMR and biochemical studies with a stapled BH3 peptide of Bim led to a structural model of the Bax and BH3 helix interaction [49, 56]. This BH3 site of inactive Bax regulates Bax activation by a hit-and-run mechanism;

therefore, it was termed the Bax activation or trigger site [49, 56]. The N-terminal BH3 binding pocket of Bax is formed by a narrow hydrophobic crevice at the juxtaposition of helices α1 and α6. Polar, positively charged and negatively charged residues surround hydrophobic residues of the activation site. The Bax activation site topologically is very similar to the canonical BH3 interaction site of anti-apoptotic BCL-2 proteins. Other BH3 helices of pro-apoptotic proteins Bid and Puma have since been demonstrated to bind to the N-terminal helices α1 and α6 and induce Bax activation [58, 59]. Recently, the crystal structure of the C-terminal-truncated BaxΔC21 determined in domain-swapped conformation and revealed an additional BH3 binding site for Bax with similar features that resemble the BH3 groove of pro-survival BCL-2 proteins [60].

The structure of Bak without the C-terminal helix and residues of the flexible N-terminus was determined by X-ray crystallography. Two crystal structures of Bak showed a homodimer structure; however, in one case the homodimer interface was mediated by zinc ion coordinated with two pairs of aspartate and histidine residues and in the other case by a cysteine disulfide linkage of symmetry-related monomers [39, 61]. Zinc binding to Bak inhibits the pro-apoptotic function of Bak, suggesting that the homodimer structure of Bak is an inhibited conformation [39]. In contrast to Bax, Bak has its BH3 binding site at the C-terminal surface similar to the anti-apoptotic counterparts [32, 61]. The structures demonstrated that the Bak BH3 groove is significantly occluded and lack the deep hydrophobic crevice compared to the anti-apoptotic BH3 grooves. This may explain a relatively weaker binding for activating BH3 peptides [32, 62–64]. Bak is considered constitutively attached to the outer mitochondrial membrane in a conformation, presumably with its a9 anchored into the membrane, which can be activated by BH3-only proteins. Therefore, the interaction of Bak with BH3-only proteins should be dynamic and transiently formed to enable the process of Bak oligomerization [64–66].

Similar to anti-apoptotic BCL-2 structures, Bax and Bak contain an unstructured loop between helices α1 and α2 [31, 32]. The structures of inactive pro-apoptotic Bax and Bak show that the unstructured loop interacts with helices α1 and α6 and they are relatively rigid. Interestingly, the unstructured loop masks the exposure of select residues that comprise the binding epitope of specific monoclonal antibodies, that recognize the active conformations of Bax and Bak, suggesting that activation of Bax and Bak requires the displacement of the unstructured loop to expose their active conformation [55, 65]. Indeed, NMR analysis and biochemical studies with the stapled Bim BH3 helix and Bax demonstrated that the unstructured α1–α2 loop displacement from the BH3 α1/α6 trigger site coincides with the exposure of the 6A7 antibody epitope residues that become exposed upon Bax activation. Conversely, the α1–α2 loop displacement is essential for further Bax conformational changes and oligomerization [49, 56].

BH3 helices of Bax and Bak are essential to mediate homo-oligomerization of Bax and Bak, but they are also involved in mediating interactions with the pro-survival counterparts [67–69]. The structures of both Bax and Bak revealed that their BH3 helices in the monomeric form bury their hydrophobic residues into the hydrophobic core. The same residues form the main interactions in heterodimeric complexes with the pro-survival proteins. This suggests that heterodimeric

interaction requires Bax and Bak to undergo a conformation change to expose the hydrophobic surface of their BH3 helices for interaction with the pro-survival proteins. This conformational change would require mobilization and dislocation of the C-terminal α9 helix from its hydrophobic groove and destabilization of interactions between the BH3 helix and helices α1, α5, α8 and the α1-α2 loop. Evidence of mobilization of the C-terminal α9 helix from its hydrophobic pocket and exposure of the Bax BH3 helix was suggested by NMR analysis and biochemical studies [56].

Several biochemical and biophysical studies of pro-apoptotics Bax and Bak suggest that the hydrophobic helices α5, α6, and α9 are involved in the membrane insertion process and pore formation at the mitochondrial outer membrane [57, 70–72]. Two modes of oligomerization have been proposed based on these studies: (a) the asymmetric oligomerization mode suggests that active monomers inserted in the outer mitochondrial membrane using α5, α6, and α9 helices oligomerize in a sequential mode by interacting through the membrane-inserted helices and other solvent exposed regions of different monomers and (b) the symmetric oligomerization mode suggests that active dimers are formed initially through interaction involving the BH3 domain of one active molecule and the BH3 groove of another active molecule, and dimers oligomerize by an interaction with a different surface that includes helix a6 [57, 70–75]. These modes of oligomerization require further structural validation; however, recent EPR and X-ray studies suggest that a BH3 helix to BH3 groove intermolecular interaction for Bax is necessary for the dimerization and oligomerization process [57, 60]. Studies with isolated peptides from helices a5 and a6 of Bax have also supported the notion that these hydrophobic helices have the propensity and ability to form pores in a lipid environment [76, 77]. Surprisingly an X-ray diffraction study of an isolated peptide of helix α5 from Bax demonstrated evidence of pores with significant lipidic content as a mechanism of Bax-mediated pore formation [78].

Several studies of Bax and Bak activation suggest that membrane insertion, oligomerization, and pore formation is a highly dynamic process that requires extensive unfolding of the inactive monomeric structures to expose the hydrophobic segments for insertion and oligomerization. In addition, there is evidence that a purely proteinaceous pore model, as originally proposed, may require further evaluation and consideration of the lipid's contribution to membrane permeabilization. The lack of structural insights for the processes of Bax and Bak activation requires further structural studies of full-length constructs and the use of combination of biochemical, biophysical, and structural methods capable of capturing and monitoring dynamic conformational states along the Bax/Bak activation pathway.

11.5 Structural Insights of Pro-apoptotic BH3-Only Proteins

BH3-only proteins comprise a large number of variable sequences that commonly possess a BH3 helix capable of interacting with the multidomain BCL-2 proteins [16, 79–80]. Almost all BH3-only proteins are predicted to have long unstructured

regions, which presumably are regulatory elements of function through posttranslational modifications and may adopt a secondary structure upon binding. Sequence alignments and secondary structure predictions suggest that the BH3-only proteins, except Bik, its structure is yet to be determined, have intrinsic disordered regions and most secondary structure is found around the BH3 sequence region [81]. The BH3 domain of BH-3 only proteins is constitutively available or becomes available upon interaction or posttranslational modification to interact with the pro and anti-apoptotic BCL-2 proteins [81, 82]. Structural investigations of BH3-only proteins have been scarce due to the challenges with their inherent instability and insolubility. Nevertheless, the structure of Bid was one of the first BCL-2 family protein structures determined by two independent NMR studies, Fig. 11.3 [83, 84].

The structure of human and mouse Bid surprisingly revealed that despite the low sequence homology with the multi-BH proteins, the structure resembles a very similar BCL-2 fold, Fig. 11.3c. Bid has distinctively similar structure to Bax and Bak and requires a conformational change to expose its BH3 helix and become active for binding. The main difference observed for Bid is the structural topology of the pocket formed between helices α3 and α6 that appears to be a shallow hydrophobic pocket. This feature resembles the hydrophobic pocket for pro-apoptotic Bax and Bak, although a biological function to this pocket still remains unknown. Similar to multi-BH proteins, Bid has a long disordered loop between helix α2 and α3, which is about 40 residues. This α2–α3 loop in Bid is mainly a cellular substrate for caspase-8 but other proteases can also cleave at this site [85–87]. Cleavage of this loop by proteases generates p7 and p15 fragments which remain in a noncovalent complex after cleavage due to strong hydrophobic interactions between helices α1 and α3 [85, 86]. However, cleavage of Bid by caspase 8 leaves a conserved myristolation site at the n-terminus of the p15 fragment [87]. N-myristolation of the C-terminal p15 fragment upon interaction with the mitochondrial membrane promotes better association of the p15 fragment with the membrane and causes the dissociation of the p7 fragment [87].

The C-terminal p15 fragment, also known as tBid (truncated Bid) was studied by solution and solid state NMR in lipid micelle and lipid bilayer [88, 89]. In aqueous solution, tBid adopts an alpha-helical but dynamically disordered conformation, which is stabilized when tBid engages its BH3 domain in a stable heterodimeric interaction with the hydrophobic groove of Bcl-x_L [88]. Interestingly tBid adopts a unique helical conformation in micelles and in lipid bilayers tBid helices are parallel to the membrane surface suggesting that there is no trans-membrane helix insertion of tBid [89]. Using fluorescence techniques, tBId was univocally shown to bind the membrane and interact with Bax causing Bax membrane insertion and oligomerization that culminates in membrane permeabilization [45]. Therefore despite the similarities of inactive Bid and Bax structure, Bid does not form oligomeric structure in the membrane to induce MOMP [90].

Further structural investigations are required to understand the function of additional BH3-only proteins beyond Bid and explore their mechanisms of activation and inhibition by different BCL-2 binding partners. Our current understanding of the mechanisms of Bax/Bak activation is based on tBid and BH3 helices from Bid, Bim and Puma, however additional mechanisms that induce MOMP through Bax/Bak or independent of Bax/Bak may be possible [91].

11.6 Structural Studies of BH3 Domains with Anti-apoptotic BCL-2 Members

The expression levels and binding interactions of the three subgroups of BCL-2 protein family dictate the decision for MOMP induction and thus cellular survival or death (Figs. 11.1 and 11.2). The BH3 domain of BH3-only proteins plays the key role in promoting apoptosis by binding and neutralizing the anti-apoptotic BCL-2 members [16, 80–82]. Multiple structural, binding and mutagenesis studies have characterized the interactions of the BH3 domains with pro-survival BCL-2 proteins [29, 67–69, 92–99] (Fig. 11.4a, b, e). In general, BH3-only proteins bind the BH3 grooves of anti-apoptotic Bcl-2 proteins with very high affinity, with dissociation constant reaching the low nanomolar range. However, while some BH3 domains bind to all anti-apoptotic members with similar affinity, others demonstrate selectivity for their anti-apoptotic heterodimerization partner. Activator BH3-only proteins bind strongly to all anti-apoptotic proteins whereas sensitizer BH3-only proteins demonstrate specificity for particular anti-apoptotic members. More specifically, BH3 domains from pro-apoptotic Bim, Bid and Puma bind strongly to the BH3 pocket of all anti-apoptotic proteins, Bad BH3 and Bmf BH3 binds strongly to the BH3 pockets of Bcl-2, Bcl-x_L and BCl-w, while Noxa BH3 is capable of binding to Mcl-1 and Bfl-1 only. Therefore, sequence variability among the BH3 domains confers specificity for binding and functional effect related to MOMP.

A wealth of structures has been determined using only the BH3 domains of pro-apoptotic proteins, including Bid, Bim, Puma, Bad, Noxa and Bmf, bound to pro-survival BCL-2 proteins (Fig. 11.4a, b, e) [29, 92–99]. The structures of these complexes recapitulate biological function and demonstrate a structural paradigm, in which BCL-2 family members interact in heterodimerization mode. The BH3 domains interact along the conserved, hydrophobic groove formed by helices α2–α5 and α8. Despite the amino acid differences of the various amphipathic BH3 helices and the exact interactions that form along the hydrophobic groove, the structure of the complexes shares some similarities. BH3 domains use a 13-residue motif Φ1SXXXΦ2XXΦ3SDZΦ4B that is defined by four conserved hydrophobic residues (Φ) at one face of the amphipathic helix that interacts with the hydrophobic residues of the groove, an aspartic acid (D) residue that form an important salt bridge interaction with a conserved arginine found in the BH1 domain of all anti-apoptotic proteins [97]. Other nonconserved but common residues in the binding motif are small residues like glycine, serine, or alanine (S); a polar residue (Z); and a residue with a hydrogen bond donor (B). These nonconserved BH3 residues form hydrogen bonds and salt bridges with residues at the perimeter of the hydrophobic groove. Other residues of the binding motif can be variable (X). Thus, hydrophobic interactions are important for heterodimerization with BH3 domain, and polar and charged interactions also play an important role.

Structures of anti-apoptotic proteins with their BH3 domain complexes show also evidence of a degree of flexibility from the hydrophobic grooves in order to accommodate the BH3 helices. For example, structures of Bcl-x_L suggest that helix α4 and

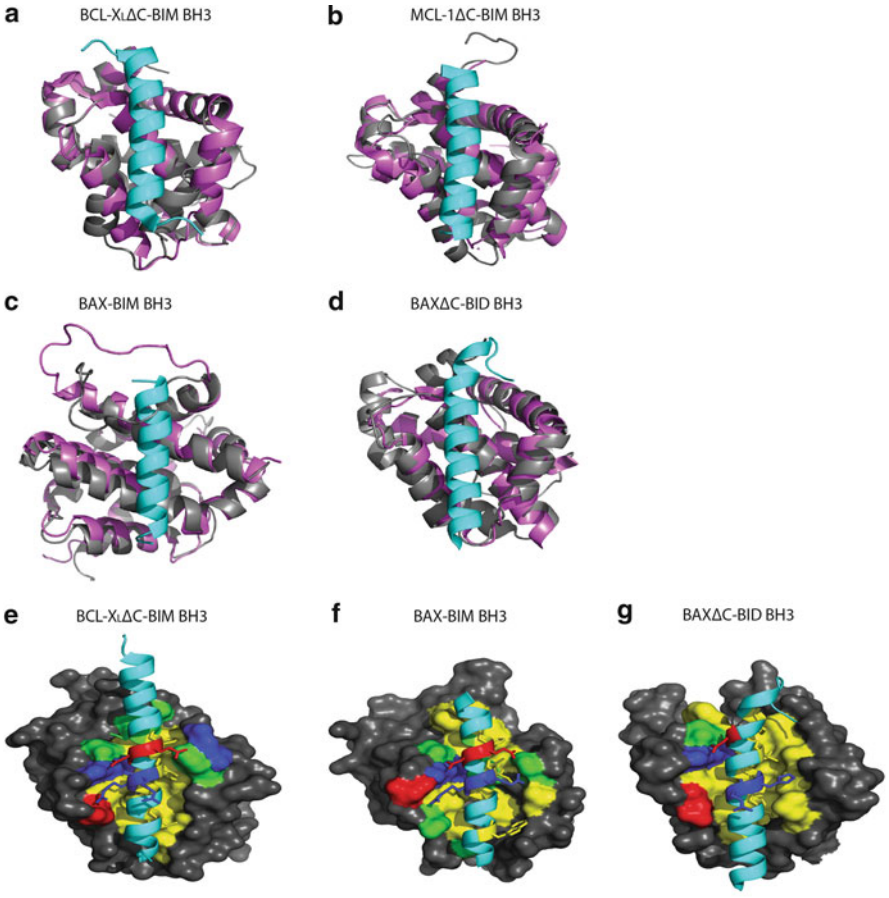

Fig. 11.4 Representative structures of BCL-2 proteins in unbound and BH3 domain bound conformations. (**a**) Overlay of Bcl-xLΔC unbound structure in *gray* and Bim BH3 bound structure in *purple*. (**b**) Overlay of Mcl-1ΔC unbound structure in *gray* and Bim BH3 bound structure in *purple*. (**c**) Overlay of Bax unbound structure in *gray* and Bim BH3 bound structure in *purple*. (**d**) Overlay of BaxΔC unbound structure in *gray* and Bid BH3 bound structure in *purple*. Surface representation of (**e**) Bcl-xLΔC (**f**) Bax and (**g**) Bax ΔC conformation highlighting the key hydrophobic interactions in *yellow*, positive (*blue*) and negative (*red*) charged pairs between the protein and the BH3 bound peptide (cyan)

α3 rotate to accommodate the tight binding of the BH3 helix and in some cases partial or complete unfolding of these helices has been observed [24, 67, 93, 99]. In addition, structural studies of Mcl-1 complexes with Bim BH3 and mutants show a degree of flexibility, particularly within helix α3, and tolerance to accommodate nonconserved mutants of the Bim BH3 helix [95, 97]. An interesting NMR study with Bcl-w and Bid BH3 interaction demonstrated that the Bid BH3 appears to undergo a transition from

unfolded to folded structure upon binding to the groove and this process is coupled with the displacement and unfolding of the C-terminal α9 helix of Bcl-w from the groove [94]. Similarly, induced helical formation of the tBid BH3 domain was also concluded in NMR binding studies to Bcl-x_L groove [88]. Such mechanism, that couples folding and binding, suggests that the high degree of conformational plasticity in interactions of BH3 domains with the anti-apoptotic grooves may play a role in optimizing the specificity for the various BCL-2 grooves.

Structural plasticity is also evident from additional studies that explored the idea of incorporating nonnatural amino acids, β-amino acids and hydrocarbon staples, which stabilize the helical conformation and offer stability against proteolysis, to the BH3 sequences [100–102]. Suprisingly even the structures determined with these modified peptides and anti-apoptotic grooves preserve many binding features of the natural BH3 helices. The structural study of Mcl-1 with a stapled peptide from the BH3 sequence of Mcl-1 demostrated additional hydrophobic interactions from the hydrocarbon staple to the hydrophobic residues of the groove, resulting in gain of high binding affinity [102].

Binding studies also demonstrated that the BH3 domains of pro-apoptotic Bax and Bak, bind strongly to the anti-apoptotic grooves. The BH3 domain of Bax has a preference for Bcl-2, Bcl-w and A1/Bfl-1 while the BH3 of Bak binds better to Bcl-xL, Mcl-1, and A1/Bfl-1 [68]. Structures of anti-apoptotic proteins with the BH3 domain of Bax and Bak have also been determined [67–69]. In fact, the first structure of BH3 domain in complex with an anti-apoptotic groove was determined between Bak BH3 and Bcl-x_L [67]. This complex established the mechanism of how anti-apoptotic proteins associated with the mitochondrial membrane heterodimerize using their hydrophobic groove to sequester the BH3 domain and therefore inhibit Bax and Bak activation. Similarly to the BH3-only proteins, BH3s of Bax and Bak using the four conserved hydrophobic residues (Φ1–Φ4) to interact with the hydrophobic residues lining on the groove of Bcl-x_L. Moreover the conserved salt bridge of the aspartic acid in BH3 and arginine in BH2 of Bcl-x_L is also important for the interaction and neutralization of Bax and Bak. Additional structures of Bax or Bak BH3 domains to Bcl-x_L, Bcl-2, and Mcl-1 have been determined showing similar interaction characteristics. One interesting structural feature of the Bax BH3 complexes with Bcl-x_L, Bcl-2, and Mcl-1 [68, 69] is that interaction with the groove extends beyond the canonical BH3 domain (α2) including nonconserved residues of helix α3 that become part of continuous longer helix.

Structural biology of BH3 helical peptides with anti-apoptotic proteins has provided tremendous insights about the mechanism of heterodimerization between BCL-2 family proteins. Defining the structural basis of these interactions and determining their specificity has been paramount to the understanding of the role of each BH3-only protein to regulate multi-BH proteins within the BL-2 family's interaction network. Importantly, the knowledge that Bax/Bax BH3s use the same hydrophobic face that is buried into the hydrophobic core of the inactive Bax and Bak structures, to interact with the anti-apoptotic groove, led to the hypothesis that Bax and Bak need to undergo a conformational change to expose the hydrophobic BH3 domain upon their activation. Structural

investigations of these complexes have also led to the development of small molecules targeting specifically the anti-apoptotic grooves and development of BH3 mimetics as potential cancer therapeutics [13].

11.7 Structural Studies of BH3 Domains with Pro-poptotic Bax and Bak

The role of the pro-apoptotic proteins Bax and Bak is essential in activating apoptosis and is mediated by direct and indirect mechanisms. Although the indirect are well understood, through various structural investigations of isolated BH3 domains with pro-survival BCL-2 proteins, the events involved in the direct activation mechanisms have been somewhat contentious because of the lack of structural information. One fundamental difference that challenged structural studies is that, although pro-survival protein interactions are stable and amenable to structural studies, the interactions of BH3 with Bax and Bak are more dynamic. BH3-only proteins such as Bim and Bid, need to induce global conformational changes and transform the inactive structures of Bax and Bak into active forms, capable of oligomerizing in the mitochondrial membrane to form putative oligomeric pores [17]. Due to the transient nature of the molecular species involved in the activation and oligomerization steps, this process is less amenable to structural analysis using conventional approaches. Nevertheless, using a stapled peptide form of Bim BH3, which induce more helical structure to the peptide and increase the binding affinity to the inactive form of Bax in solution, the first structural model of direct BH3 interaction with the pro-apoptotic proteins was elucidated [49].

Using NMR analysis and biochemical validation, a distinct binding site for Bim BH3 was discovered at the N-terminus of Bax (Fig. 11.4c, f) [49]. The binding site of Bim BH3 on Bax is defined by the two helices α1 and α6, with the interhelical junction forming a hydrophobic cleft surrounded by a perimeter of hydrophilic and positively and negatively charged residues. The structural model suggests that the amphipathic Bim BH3 helix uses the three hydrophobic residues, L152, I155, and F159, to engage with the hydrophobic crevice of the Bax trigger site. The specificity of the interaction is also mediated by key salt bridges between charged residues of Bim BH3 E158, R153, D157 and charged residues of the Bax trigger site K21, E131, R134, respectively. The location of the Bax trigger site is a geographically distinct protein surface, from the canonical BH3 binding site found at the C-terminal surface. Nevertheless, Bim BH3 engages in topographically similar interactions with the Bax trigger site. However, the Bax trigger site has less hydrophobic content and is shallower than the canonical BH3 site of anti-apoptotics. This is compatible with the requirement of a BH3 peptide in a pre-organized helical structure to bind with a detectable interaction to BAX, whereas the anti-apoptotic BH3 site has a deep hydrophobic groove that allows folding of the BH3 peptide upon binding [49].

Complementary NMR analysis and biochemical studies suggested that binding of the Bim BH3 to the Bax trigger site induces a number of structural changes

that lead to Bax activation and oligomerization [56]. Bim BH3 engagement to the Bax trigger site induces the displacement of an unstructured loop between helices α1 and α2 from a closed loop conformation to an open-loop conformation. The conformational change of the α1-α2 loop is essential for the BAX activation process. Through allosteric sensing from BH3 binding and displacement of the α1-α2 loop, the C-terminal α9 helix is mobilized from its pocket to interact with the mitochondrial membrane, and the Bax's own BH3 domain is exposed from the the hydrophobic core of BAX. The conformational active BAX with its BH3 domain exposing its hydrophobic residues becomes available to propagate the activation of other inactive Bax molecules with the same mechanism as Bim BH3 triggers BAX activation. This auto-activation mechanism amplifies the kinetics of the Bax translocation process and leads to a critical mass of Bax molecules in the outer mitochondrial membrane to enable the formation of the putative pore capable of inducing MOMP.

A recent study determined the crystal structures of the C-terminal truncated BaxΔC21 in a domain-swapped conformation [60]. The observed domain-swapped dimer was induced in solution from monomeric BaxΔC21 by BH3 peptides of Bid and Bax as well as nonionic detergents (Fig. 11.4d, g) [60]. The BaxΔC21 domain-swapped conformation is analogous to the domain-swap dimer structure of Bcl-xLΔC [47]. Although the dimer-swapped conformation may not be biological, there are interesting findings from these structures. The BH3 peptides in these structures occupy the C-terminal BH3 pocket described for pro-survival proteins. Thus, similar to the BH3 peptides engaging the pro-survival proteins, Bid BH3 uses the four hydrophobic residues at the conserved positions of the BH3 motif (I86, L90, V93, and M97) and a key salt bridge between Bid D95 and Bax R109 in helix α5. Additionally, Bid has an extra hydrophobic interaction with the Bax α3 and α4 helices mediated by Bid I82 and I83. Analogous interactions are also observed with the Bax BH3 bound to the Bax-swapped dimer. Interstingly, the Bim BH3 peptide induces the same domain-swapped conformation with BaxΔC21 in the presence of CHAPS detergent; however, the BH3 peptide is not present in the crystal structure. Notably, the interactions found to be important for binding to the trigger site of Bax by Bim BH3 overlap with the key residues that are required for binding to the C-terminal pocket of BaxΔC21. The existence of the two BH3 sites on Bax suggests that triggering and membrane translocation of cytosolic Bax is mediated by the N-terminal BH3 site and when Bax is in a membrane-attached conformation, with the α9 helix released from its native position, it can be additionally activated by BH3 interactions at the C-terminal BH3 groove [49, 60].

The domain-swapped dimer conformation of BaxΔC21 is structurally equivalent to the monomers connected at the bottom of the hairpin of α5–α6 helices, with the first five helices of one monomer representing the «core domain» paired with the α6–α8 helices, representing the «latch domain» of the second molecule. A model of Bax activation that includes the unlatching of the α6–α8 helices from the core domain and exposure of the Bax BH3 helix to mediate the dimerization process was conceived based on the domain-swapped dimer structure. This led to solution of the crystal strucure of the N-terminal core alone, α2–α5 helices, forming another

dimer conformation in which the BH3 helix (α2) of one molecule interacts with the second molecule in the same orientation as the monomer [60]. The α2–α5 core dimer suprisignly leaves a large and planar hydrophobic surface exposed suggesting a potential surface that engages the membrane and allows the integration of certain helices into the membrane. This model of activation and oligomerization is consistent with a number of other biophysical and biochemical studies but contradicts other studies which suggested the requirement of helices α5 and α6 inserted into the membrane to mediate oligomerization [60, 70–72].

The NMR solution structure of BakΔC in complex with the stapled helical peptide of Bid BH3 (SHABa) sequence revealed the mechanism of Bak activation [66]. SHABa peptide has increased affinity for the occluded canonical pocket of Bak and enabled the NMR structure determination of the complex [32, 66]. Structural and biochemical data confirmed that BH3 binding to the canonical pocket of Bak at the C-terminal surface leads to Bak activation. SHABa uses the four conserved hydrophobic residues of Bid BH3 (I86, L90, V93, and M97) to position in the groove in similar mode that Bid BH3 binds to the BaxΔC21 C-terminal BH3 groove. The complex interaction is also mediated by key hydrogen bonds of Bak R127 and N124 with Bid D95 and D98 residues. The structure suggests that Bid binding to the occluded BH3 groove results in subtle conformation changes in Bak that may represent the initial event in the Bak activation process. Bak activation undergoes a "hit-and-run" mechanism. Therefore, when Bid BH3 binds to the canonical BH3 pocket, it induces opening of the occluded BH3 pocket and subsequently a conformational change that enables the exposure of the Bak BH3 helix from the hydrophobic core. A neighboring Bak BH3 is able to dissociate Bid BH3 helix from the BH3 groove and ultimately this leads to dimerization and oligomerization of Bak through the canonical pocket. This model of Bak activation is consistent with the membrane-tethered form of Bak and follows the symmetric oligomerization model [73].

Despite challenges involved with studying the process of Bax and Bak activation, recent structural and functional studies provided important missing insights into the nature of initial events that occur during Bax and Bak activation. These studies should initiate further investigations of the activation and oligomerization process that results to MOMP with the inclusion of native proteins and membranes. Meanwhile, such structural insights on Bax and Bak activation already establish a path to designing molecules that will be able to modulate the activating BH3 interactions and regulate Bax- and/or Bak-mediated apoptosis for therapeutic benefit [103].

11.8 Summary

Structural studies of the BCL-2 family proteins have significantly contributed to our understanding of their function and regulatory interactions and ultimately how apoptosis is initiated and controlled at the mitochondrial level. A number of

complexities underlying the function of BCL-2 family proteins, such as conformational changes upon activation, their cytosolic or mitochondrial membrane conformations, and the interplay of interactions with different mechanisms that lead to different apoptotic outcomes have become more apparent from structural studies. Importantly, these structural studies have also lead the way to the discovery of small molecule probes that mimic the protein–protein interactions mediated by the BH3 domains. This has inspired the development of BCL-2 family inhibitors that are currently evaluated in clinical trials as cancer therapeutics. Despite this success, we still lack important information about the structural conformations of many BCL-2 family proteins and how they regulate apoptosis signaling under homeostatic and pathologic conditions. It is certain that further structural studies of the BCL-2 proteins and their protein–protein complexes, particularly those within membrane environments, will lead to novel insights into possible mechanisms of apoptosis regulation and ultimately development of effective therapeutics.

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Chapter 12
Structural Basis of Death Receptor Signaling

Jixi Li and Hao Wu

Abstract Death receptors (DRs) initiate formation of intracellular signaling complexes upon stimulation by extracellular ligands to induce apoptosis and necrosis. Here we review the structural information on DR signaling accumulated in the past decade and more. These studies highlight the assembly of oligomeric death domain superfamily complexes in caspase activation in apoptosis and the assembly of a functional amyloid complex in kinase activation in necrosis. They open a new vista for our understanding of intracellular signaling in the DR pathway and other innate and adaptive immune pathways.

Keywords Death receptor • Fas • TNFR1 • Apoptosis • Necrosis • Necroptosis • Death domain • Caspase • Higher-order assemblies • Amyloid • Functional amyloid

12.1 Introduction

Death receptors (DRs) are mediators of extrinsic cell death and form a subfamily of the TNF receptor superfamily for which the intracellular regions contain a protein-interaction module known as the death domain (DD) [1]. When stimulated, these receptors have the capability to recruit and activate caspases, leading to apoptosis

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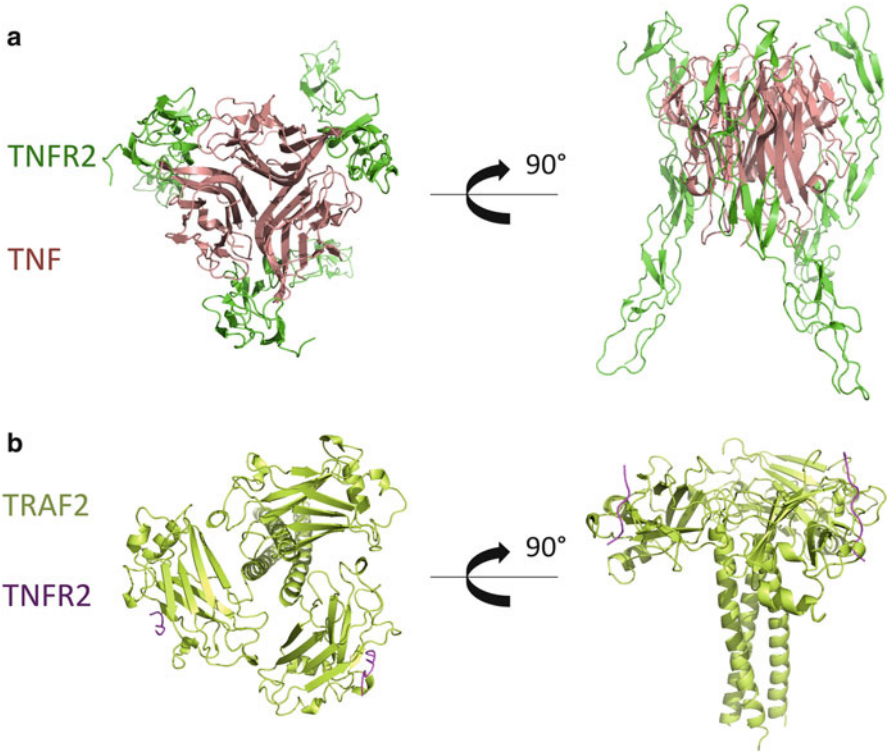

Fig. 12.1 TNF receptors form a 3:3 complex with their extracellular ligands and the C-terminal TRAF domain of TRAF proteins. (**a**) The 3:3 complex structure of a TNF family ligand with TNFR2. (**b**) Structure of the C-terminal TRAF domain of TRAF2 with bound TNFR2 peptide

induction. More recently, DRs are shown to induce programmed necrosis in addition to apoptosis, in a highly regulated manner. The DR family currently has eight members: Fas, TNF-R1, DR3, DR4, DR5, DR6, EDAR, and p75 NGFR. TNFR superfamily members that do not contain an intracellular DD are often survival receptors, which recruit TNF receptor associated factors (TRAFs) to activate the IκB kinase (IKK) to turn on the NF-κB transcription factors. Canonical ligands for DRs belong to the TNF superfamily; however, noncanonical ligands also exist such as APP for DR6 and NGF for the p75 NGFR.

It has been known for a long time through structural studies that soluble TNF-like ligands interact with the extracellular regions of their receptors through a 3:3 interaction [2]. TNF-like ligands are trimeric; receptor chains are bound at the interfaces between two protomers of a TNF trimer and become trimerized [3] (Fig. 12.1a). Until fairly recently, there has been limited information on the intracellular interactions of DRs. However, for non-DD-containing TNF receptors, earlier structural studies have shown that the C-terminal domains of TRAFs interact with receptors in

a 3:3 stoichiometry (Fig. 12.1b). Therefore, it seems that there might be a perfect correspondence of symmetrical 3:3 interaction between the extracellular and the intracellular interactions for both DRs and non-DD-containing TNF receptors.

However, recent structural, biochemical, and cell biological studies have begun to paint a very different picture for the structural mechanism of DR signaling, revealing a new concept for these pathways. The key finding is that 3:3 interactions are not the full scenario nor sufficient for DR signaling. Instead, higher-order oligomerization of both the intracellular and the extracellular interactions is crucial for formation of the receptor signaling complexes in apoptosis and necrosis. In this scenario, the organization of the signaling complexes could also accommodate non-canonical ligand/DR pairs such as NGF/NGFR and APP/DR6. In this chapter, we will focus on the structural mechanisms of apoptosis and necrosis induction in DR pathways.

12.2 Fas- and TNFR1-Induced Apoptosis

Fas and TNFR1 are prototypical members of the DR family. Upon interaction with its ligand FasL at the extracellular region, Fas recruits the adapter protein Fas-associated death domain (FADD) through an interaction between their DDs. FADD also contains a death effector domain (DED), which in turn recruits caspase-8 and -10 through an interaction with the tandem DED in the prodomain of these caspases [4]. The ternary complex of Fas, FADD, and caspase-8 or -10 has been traditionally named the death inducing signaling complex (DISC) [5], which brings the catalytic domains of the caspases into proximity for dimerization and auto-processing (Fig. 12.2a). Both DDs and DEDs are members of the DD fold superfamily, which also comprises the caspase recruitment domains (CARDs) and the Pyrin domains (PYDs) [6]. Proteins containing these domains are known for their ability of homotypic interactions, which play central roles in many apoptotic and inflammatory pathways [7].

In contrast to Fas, which readily induces cell death, TNFR1 induces pleiotropic effects including NF-κB activation, apoptosis, and programmed necrosis [8]. Upon interaction with TNF, the intracellular DD of TNFR1 recruits TNF receptor-associated DD protein (TRADD), which in turn recruits receptor-interacting protein 1 (RIP1). Depending on the cellular context, RIP1 controls the outcome of TNF signaling. If the E3 ligases, including the TRAF2/cIAP and LUBAC complexes, ubiquitinate RIP1 in the TNFR1 signaling complex, polyubiquitinated RIP1 engages downstream adaptors such as NEMO to activate IKK to promote NF-κB transcriptional activity, leading to cell survival, proliferation, and differentiation [9]. When RIP1 ubiquitination is blocked by removal of the E3 ligases cIAP1 and cIAP2 through genetic ablation, RNAi knock down, or IAP antagonists, RIP1 forms a secondary complex in the cytosol with FADD and caspase-8 termed the Ripoptosome to initiate apoptotic cell death [10–12]. TRADD can also form a secondary cytolic complex with FADD and caspase-8 known as complex II to induce apoptotic cell death [13].

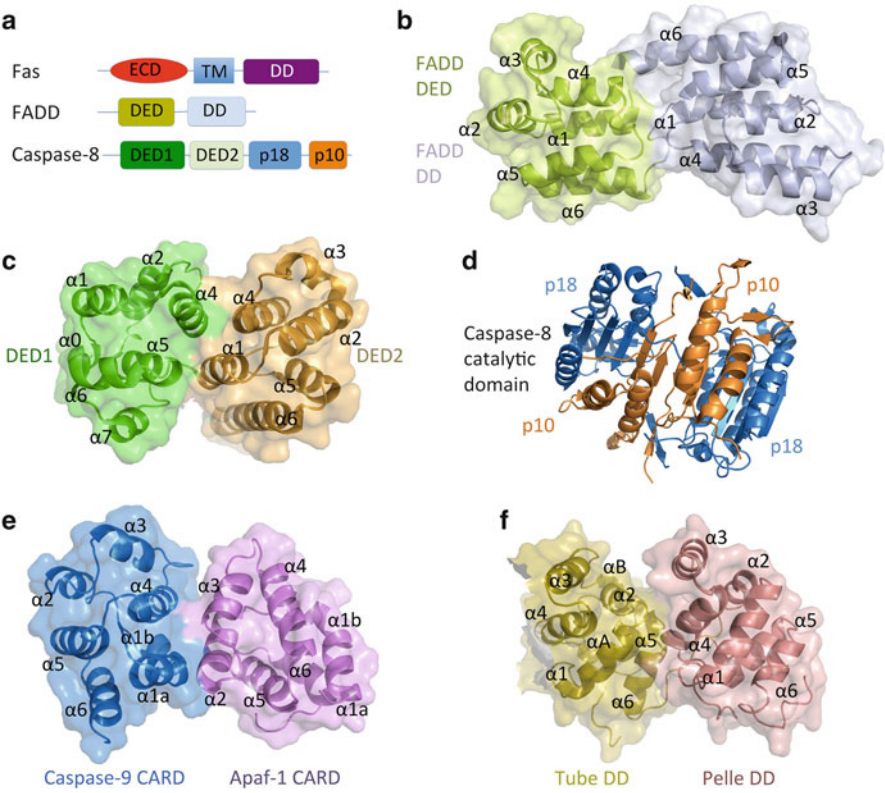

Fig. 12.2 Structures of component proteins in the DISC and binary complexes in the DD superfamily. (**a**) Domain schematics of Fas, FADD, and caspase-8. Fas consists of an extracellular domain (ECD), a transmembrane domain (TM), and an intracellular death domain (DD). FADD consists of a DED and a DD. Caspase-8 consists of two DEDs (tandem DED) and a p18 and a p10 for the catalytic domain of the caspase. (**b**) NMR structure of full-length FADD. Six helices in each domain are denoted α1–α6 and labeled. (**c**) The tandem DED structure of a viral Caspase-8/10 inhibitory protein vFLIP that may represent a model for caspase-8 tandem DED. (**d**) The crystal structure of Caspase-8 catalytic domain (p18 and p10). (**e**) Crystal structure of the heterodimer between the CARDs of Apaf-1 and Caspase-9. (**f**) Crystal structure of the heterodimer between the DDs of Tube and Pelle

12.3 Structures of Individual Proteins and Binary Complexes in the DISC and Other DD Fold Signaling Complexes

Because of the biological importance of the DISC, its component proteins have been the subjects of intensive structural investigations. The structure of the Fas DD was determined by the NMR method [14]. As the first structure of the DD fold superfamily, the structure revealed an antiparallel six-helical bundle architecture

now known to be common for the entire superfamily. Structures of both the DD and the DED of FADD have been determined followed by elucidation of the full-length FADD structure containing both domains, all by the NMR method (Fig. 12.2b). The structure of the tandem DED domain of caspase-8 or caspase-10 has not been resolved. However the structure of the tandem DED domain of a viral Caspase-8/10 and FLICE/caspase-8 inhibitory protein (FLIP) from poxvirus Molluscum contagiosum virus (MCV) has been determined, revealing a dumbbell-shaped arrangement that should be common to all tandem DEDs [15, 16] (Fig. 12.2c). The structure of the catalytic domain of caspase-8 has been determined [17, 18], revealing a dimeric structure common to all caspases (Fig. 12.2d). The first structures of DD superfamily complexes are the heterodimer of the Apaf-1 and Caspase-9 CARDs [19] (Fig. 12.2e) and the heterodimer of drosophila Pelle and Tube DDs [20] (Fig. 12.2f).

12.4 DD Interactions in the PIDDosome and Myddosome as Principles of Oligomeric Assembly in the DISC

We began our studies on the DD interaction between Fas and FADD in the DISC but failed to obtain high-resolution crystals of the Fas DD: FADD DD complex. Instead, we were successful in obtaining the DD complex between p53-induced protein with a death domain (PIDD) and RIP-associated Ich-1/CED homologous protein with death domain (RAIDD). PIDD and RAIDD are component proteins in the PIDDosome for caspase-2 activation [21]. PIDD is a multidomain protein, which undergoes autolytic processing upon sensing genotoxic stress [22]. This cleavage first occurs at the junction between the two ZU5 domains, generating a C-terminal fragment with a ZU5 and a DD domain for interacting with RIP1, leading to activation of NF-κB and promotion of DNA damage repair [23]. A second cleavage occurs after the second ZU5 domain, producing a C-terminal fragment with just a DD that is capable of interacting with RAIDD through a DD/DD interaction. RAIDD also contains a CARD, which recruits caspase-2.

We reconstituted the DD interaction between PIDD and RAIDD and found that the molecular mass of the complex is consistent with a total of 12 DD subunits in the complex. We had assumed that this must mean a 6:6 stoichiometry with a symmetrical arrangement. However, when we solved the structure, we discovered that the structure contains five PIDD DD and seven RAIDD DD [24] (Fig. 12.3a). All the pairwise interactions within the complex are asymmetric in that each subunit interacts with a different surface of the partner subunit, yet all DDs in the complex are in quasi-equivalent environments with up to six neighboring molecules. The interactions in the PIDD DD/RAIDD DD complex can be divided into three types of asymmetric interactions that define 6 unique interfaces: type Ia that contacts type Ib, type IIa that contacts type IIb, and type IIIa that contacts type IIIb [24] (Fig. 12.3b).

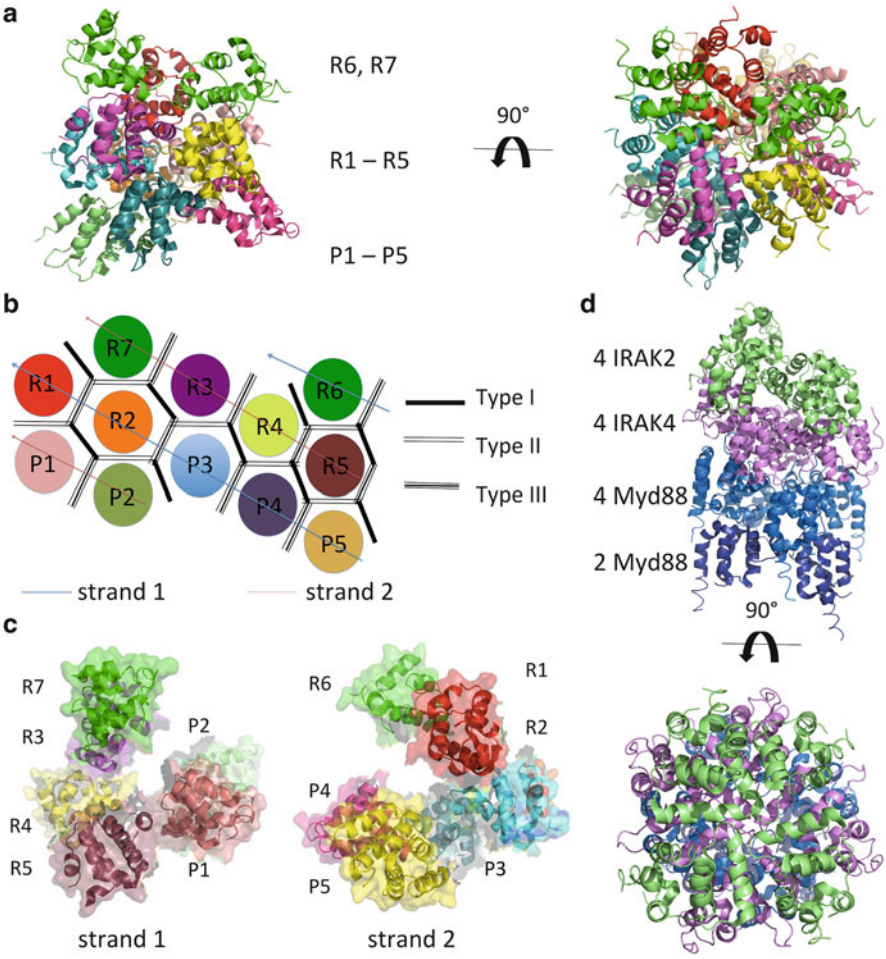

Fig. 12.3 Overview of the PIDD DD: RAIDD DD and MyD88/IRAK4/IRAK2 complexes. (**a**) *Side* and *top* views of the PIDD DD: RAIDD DD complex. The *top* layer contains two RAIDD DD molecules. The *middle* layer contains five RAIDD DD molecules. The *bottom* layer contains five PIDD DD molecules. (**b**) A schematic planar diagram for the PIDD DD: RAIDD DD complex. The PIDD DD: RAIDD DD complex is a double-stranded left-handed helix of DDs. Each strand contains six DDs connected via type III interactions. (**c**) The two helical strands are shown with surface representations. (**d**) *Side* and *top* views of the crystal structure of the MyD88 DD: IRAK4 DD: IRAK2 DD complex

The structure may be divided into a layer of five PIDD DD, a layer of five RAIDD DD and a partial layer of two RAIDD DD. The incomplete partial layer of two RAIDD DD may be due to a low affinity between the RAIDD DD layers, in contrast to the high affinity between the RAIDD DD and PIDD DD layers. In the crystal, electron densities for these two RAIDD DD molecules are weaker, suggesting that they are loosely bound to the core 5:5 complex [24]. In this oligomeric context, when caspase-2 is recruited to the complex by RAIDD, 5–7 molecules of caspase-2

will then be brought into proximity to allow dimerization and activation. Retrospectively, another way to look at the structure shows that this layered arrangement possesses double-stranded helical symmetry, with three PIDD DD and three RAIDD DD in one helical strand and two PIDD DD and four RAIDD DD in the other helical strand (Fig. 12.3b, c). We reached this understanding when the structure of the MyD88/IRAK4/IRAK2 complex in Toll-like receptor signaling was solved and shown to possess a single-stranded helical symmetry with six MyD88, four IRAK4, and four IRAK2 molecules [25] (Fig. 12.3d). Remarkably, this helical symmetry has become a unifying feature of interactions in the DD fold superfamily with the two known 1:1 interactions, Apaf-1 CARD: caspase-9 CARD in cell death signaling in mammals, and Pelle DD: Tube DD in Toll signaling in drosophila, utilizing the type I and type II interactions, respectively, of the helical assembly elements [7].

12.5 Structure of the Fas DD: FADD DD Complex in the DISC

When we reconstituted the Fas DD: FADD DD interaction in vitro using coexpression in *E. coli*, we obtained a complex in the molecular mass range of 115–130 kDa, much larger than a 3:3 complex. Crystals of the complex were obtained but they only diffracted to about 6–7 Å resolution. The low resolution of the Fas DD: FADD DD crystals prompted us to try alternative structural methods. We were surprised to find that class projection averages of negatively stained samples showed similarity to the class averages of the PIDD DD: RAIDD DD complex, both in size and appearance [26]. We then performed nanoflow electrospray ionization and tandem mass spectrometry, which showed that the Fas DD: FADD DD complex contains a mixture of five Fas: five FADD, six Fas: five FADD, and seven Fas: five FADD complexes. These data agreed well with the similarity between these two complexes. We built a layer of five Fas DD and a layer of five FADD DD based on the layered structure of the PIDD DD: RAIDD DD complex. We then performed molecular replacement calculations with the layers, which showed that the Fas DD layer is the upper layer and the FADD DD layer is the lower layer, forming a 5:5 core Fas DD: FADD DD complex (Fig. 12.4a). Independent studies of the Fas DD: FADD DD complex using NMR titration implicated a structure with asymmetric interactions as in the PIDDosome [27].

Fas mutations in humans are associated with autoimmune lymphoproliferative syndrome (ALPS). The structure of the complex immediately explained the disease phenotype of Fas mutations because they reside on the interfaces in the complex (Fig. 12.4b) and fail to form a complex with FADD DD [26]. Structure-based mutations also disrupt Fas–FADD interaction in living cells. Almost all Fas DD mutations in ALPS are dominant and from a heterozygous background. This is because ALPS-associated Fas mutations that cannot bind FADD interfere with Fas-induced apoptosis by dominantly disrupting the wild-type Fas from assembling the DISC. Assuming the complete loss of function of a Fas mutant and a 1:1 mix of wild-type:

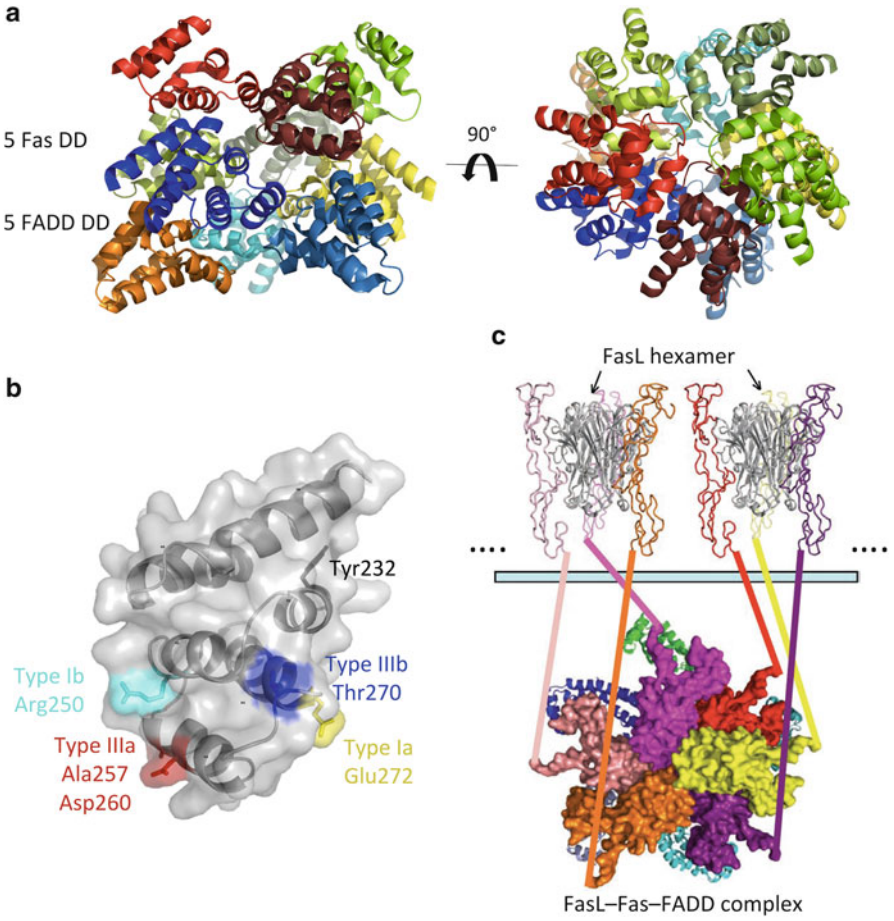

Fig. 12.4 Overview of the DISC complex. (**a**) Crystal structure of the Fas DD: FADD DD complex, shown in two orthogonal orientations. (**b**) Mapping of ALPS-associated mutation residues onto the surface of Fas DD. Tyr232 does not map to the interface while all others do. In the family from which Tyr232 was isolated, the Y232C Fas mutation inhibited apoptosis and caused ALPS only when coexpressed with an extracellular R137W mutation on the other allele, likely by reducing Fas surface expression [63]. (**c**) A model of the hexameric FasL: Fas: FADD complex. "…." denotes additional receptor and signaling complex aggregation on the cell surface

mutant Fas in a heterozygous patient, the amount of the wild-type signaling complex would be $<1/2^5$, which is ~3 %, explaining the strong dominant negative phenotype of ALPS-associated Fas DD mutations [26].

It is well established that the membrane-bound form of FasL and cross-linked anti-Fas antibodies, but not the proteolytically processed, trimeric soluble form of FasL, trigger apoptosis [28–30]. When soluble trimeric FasL is dimerized into an engineered hexameric molecule, it is highly competent to signal apoptosis, suggesting that the minimal signaling competent form of FasL is hexameric [31].

Interestingly, a dimer of FasL trimers would bring six Fas intracellular DDs into proximity, which is ideal in inducing the formation of the oligomeric complex comprised of 5–7 Fas molecules (Fig. 12.4c). Notably, ligand-independent association of Fas and TNF receptors has been shown to be critical for signaling [32, 33], and at least for the TNF/TNF-R2 complex, aggregates are formed on the cell surface [34], suggesting additional association beyond the hexameric complex. These data are consistent with formation of higher-order receptor/signaling protein complexes in the DISC [35].

12.6 Implications for Other DD Complexes in Apoptosis-Inducing Complexes

TNFR1 induces apoptosis through secondary TRADD/FADD/caspase-8 and RIP1/FADD/caspase-8 complexes [10–13]. We propose that TRADD DD and RIP1 DD associate with FADD DD in a manner that is similar to the Fas DD/FADD DD interaction [26].

12.7 DED Interactions in DISCs

DR-mediated apoptosis relies on formation of the DISCs. Recent studies identified the stoichiometry of component proteins in the DISCs of Fas and other DRs [36, 37]. Combined with different methods, including quantitative western blots, mass spectrometry, and mathematical modeling, the amount of DED proteins procaspase-8/10 and c-FLIP was shown to exceed that of FADD by seven- to ninefold within the DISCs [36, 37]. One proposed model showed that procaspase-8/10 and c-FLIP could form a caspase-activating chain via their DED domains. Mutations of some key interacting residues in procaspase-8 DED2 abrogate DED chain formation in cells [36, 37]. Interestingly, the DED1 of procaspase-8 does not form filament nor induce apoptosis, whereas the DED of FADD and the DED2 of procaspase-8 form filaments in Hela and Jurkat Tag cells and induce apoptosis independently of Fas signaling upon overexpression [38]. The filaments formation can be blocked by coexpression of viral anti-apoptotic DED-containing proteins, MC159 and E8, but not by Bcl-2 family protein Bcl-X [38]. The DED interactions in DISC are important for the chain assembly and may drive caspase-8 dimerization and activation, leading to apoptosis.

12.8 TNFR1-Induced Programmed Necrosis

Apoptosis and necrosis are two major ways of cell death [39]. Recent studies showed that programmed necrosis is an alternative way for cell apoptosis in the immune system [40–46]. In the TNFR1 signaling pathway, active caspase-8 within

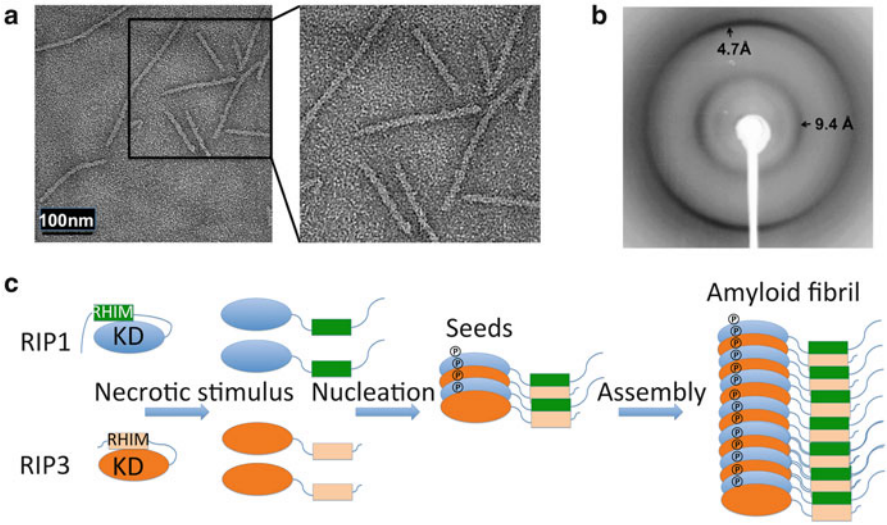

Fig. 12.5 RIP1 and RIP3 form an amyloidal filamentous complex. (**a**) Negative stain electron microscopy images of the RHIM complex of RIP1 and RIP3. (**b**) An X-ray diffraction image of partially aligned RIP1/RIP3 fibrils. The diffractions at 4.7 Å and 9.4 Å resolutions represent β-strand spacing and β-sheet separation, respectively. (**c**) A nucleation/polymerization model for RIP1/RIP3 complex assembly and kinase activation

the Ripoptosome cleaves and inactivates RIP1 [47, 48] and RIP3 [49]. When caspases activities are inhibited, RIP1 and RIP3 form the necrosome to initiate cell programmed necrosis or necroptosis [50–52].

Both RIP1 and RIP3 have an N-terminal kinase domain (KD) and a RIP homotypic interaction motif (RHIM), whereas RIP1 has one more death domain (DD) at its C-terminal end. RIP1 and RIP3 form a necrotic signaling complex via the RHIM domain [53, 54]. The RIP1/RIP3 complex is the core of the necrosome; however, no structural information was available for the necrosome until recently when we found that the RIP1/RIP3 complex forms filamentous structures [55] (Fig. 12.5a).

12.9 Structure of the RIP1/RIP3 Complex in Programmed Necrosis

The RHIM domain that mediates the interaction of RIP1/RIP3 is less than 100 residue long and contains a highly conserved (I/V/L)Q(I/V/L)G motif. When we purified the RIP1/RIP3-RHIM and full-length RIP1/RIP3 (RIP1/RIP3-FL) complexes in vitro, they eluted around the void position from a gel filtration column, much larger than the expected heterodimeric molecular mass [56]. Based on secondary structure prediction, we found that only the short segments of sequence around the I(V)QI(V)G motif have propensities for β strands, whereas the other regions besides the KD and the DD appear to contain random coil structures. We used electron

microscopy (EM) to visualize the RIP1/RIP3 complex, which clearly showed filamentous structures. Moreover, the endogenous RIP1/RIP3-FL complex purified from HT-29 cells had the similar filamentous structures after treatment with the protease subtilisin to remove associated proteins and flanking domains. The fibrils vary in length but exhibit an 8 nm ordered core with flexible extensions.

Amyloids are fibrous protein aggregates composed of cross-β cores [57]. The RIP1/RIP3 complex showed classical properties of β-amyloid fibrils with specific binding to dyes Thioflavin T and Congo red, and characteristic Fourier transform infrared spectra [55]. X-ray diffraction of partially aligned RIP1/RIP3-RHIM fibrils clearly showed two orthogonal diffractions at 4.7 Å and 9.4 Å, corresponding to the inter-β strand spacing along the meridional axis and the inter-β sheet stacking distance along the equatorial direction, respectively (Fig. 12.5b).

Both recombinant and endogenous RIP1/RIP3 complexes are ultrastable in harsh conditions (4 M urea or 150 mM NaOH), consistent with the generally recognized stability of amyloidal structures [58]. When RIP3 deficient Hela cells were reconstituted with RIP3-mCherry and stimulated with TNF, zVAD-fmk, and LBW242 to induce necrosis, ThT staining perfectly overlapped with the RIP1/RIP3 puncta, demonstrating the amyloidal nature of the necrosome. Both the RHIM domains and kinase activity of RIP1/RIP3 are required for death receptor-induced programmed necrosis [50]. Amyloid-β amyloidogenesis occurs via a nucleated polymerization mechanism [59]. Based on the filamentous amyloidal structure of the RIP1/RIP3 complex, we propose a nucleation/polymerization model to offer the feed-forward, gain-of-function mechanism in which RIP1/RIP3 kinase activation and amyloid scaffold formation are mutually reinforcing (Fig. 12.5c). The amyloid scaffold may function as a crucial platform for recruiting other components, such as MLKL or PGAM5L [60–62], and triggering the downstream execution mechanisms of necroptosis.

12.10 Summary

In this chapter, we reviewed higher-order signaling complexes in death receptor pathways including the oligomeric death domain scaffold for caspase activation in apoptosis and the functional amyloid scaffold for kinase activation in necrosis. While the three types of asymmetric interactions dominate assembly of caspase activating complexes, the β-sheet core underlies assembly of amyloid complexes. Why do cells use highly oligomeric supramolecular assemblies in death receptor pathways? The biophysical principles for the formation of these complexes dictate a cooperative process for threshold control of the pathway [35]. In addition, the requirement for nucleation in the assembly of death domain and amyloid complexes leads to a delay in cellular responses that may be crucial in protecting cells from accidental cell death [35]. The higher-order complexes also act as scaffolds to drive proximity, leading to caspase dimerization and kinase auto-activation, and execution of the respective pathways.

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