Essentials of Apoptosis

Second Edition

Xiao-Ming Yin · Zheng Dong
Editors

Essentials of Apoptosis

A Guide for Basic and Clinical Research

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Humana Press

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Preface to Second Edition

The first edition of this book was published in 2003. It was intended to provide the basic and essential information of apoptosis for those who were new to the field and who wanted to apply the knowledge to their own research. The book therefore focused on the concepts, the basic molecular architecture, and the pathophysiological significance of apoptosis. Since the first edition, there have been tremendous new developments in the field of apoptosis and cell death in general. The concept of various types of cell death has been further developed. The studies in both basic and clinical disciplines have been greatly expanded. In particular, notable progress has been made in extending the work into the therapeutic arena. As a result, the field has matured considerably and developed extensive cross-talk with works in other fields.

We strive to incorporate and reflect these new developments in the second edition of this book. Our goal is to provide readers with the most updated and advanced knowledge in the field, while maintaining the fundamental information as presented in the first edition. To this end, the book has been significant expanded, not only in the page and chapter numbers but more importantly in the depth of coverage. Most of the chapters have been revised and/or rewritten, and 15 new chapters have been added to give rise to a total of 31 chapters. In Part I, in addition to the discussion of the major apoptosis molecules, the activation and regulatory pathways, and the clearance of apoptotic cells, we also present important issues that examine apoptosis from more integrated points of view. Thus, the roles of reactive oxygen species, metabolism, and transcription control in apoptosis activation and regulation are explored. A new systems biology approach to studying apoptosis is also introduced. In Part II, we discuss apoptosis and cell death in four model systems, including plant, yeast, *C. elegans,* and *Drosophila*, which together have contributed greatly to our current understanding of cell death. In Part III, we focus on mammalian cell death under various pathophysiological situations in all major systems, including the hematopoietic and immune system, the brain, the heart, the liver, the lung, and the kidney. Two integrated chapters discussing cell death in normal development and in cancer biology have also been included. Furthermore, a separate Part IV has been added to discuss the alternative mechanisms and pathways of cell death. Finally, Part V discusses the technical aspect of apoptosis

research. This new edition should be valuable for both novice and seasoned investigators as a comprehensive reference as well as a practical guide.

As broad as the content to which they have contributed, our more than 80 contributors come from across the world, representing institutes from 15 countries and regions. We would like to acknowledge the hard work by all the authors, who are recognized experts and leaders in the field of apoptosis research. Without their dedicated contributions, this book would not have been possible. We are also especially grateful to our families for their wholehearted and enduring support, which makes the edition of this book very rewarding and enjoyable. Finally, we wish to dedicate this edition to the memory of Dr. Stanley J. Korsmeyer (1950–2005), a beloved mentor, colleague, and friend. Dr. Korsmeyer was a pioneer and a visionary in the field of apoptosis. His seminal works on the Bcl-2 family proteins, on the mitochondria and endoplasmic reticulum pathways of apoptosis activation and regulation, and on the pathophysiological significance of apoptosis in embryonic development, in immune response, and in cancer biology and cancer therapy tremendously changed and advanced the field. He will be remembered by all of us.

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Preface to First Edition

Life and death are topics that no one takes lightly. In the cell, death by apoptosis is just as fundamental as proliferation for the maintenance of normal tissue homeostasis. Too much or too little apoptosis can lead to developmental abnormality, degenerative diseases, or cancers. Although apoptosis, or programmed cell death (PCD), has been recognized for more than 100 years, its significance and its molecular mechanisms were not revealed until recently.

We have witnessed rapid progress in apoptosis research in the last decade. Apoptosis can now be defined not only by morphology, but also by molecular and biochemical mechanisms. As a result, there has been an information explosion in the field. On one hand, this has dramatically expanded our understanding of the role of apoptosis in both biology and medicine; on the other hand, it has made the study of apoptosis quite complicated, and sometimes confusing. One often wonders whether findings from other laboratories can be generalized or wheather the methods used can be made applicable to other systems.

Studies of apoptosis are unusual in that the common focus on a basic process that is driven by specific sets of biochemical machinery is studied in an array of very diverse research areas. Investigators from different fields have documented their views of apoptosis in numerous review articles. These reviews, published in various scientific journals, are aimed at either summarizing the latest findings or providing brief introductions to apoptosis. However, essential information about apoptosis, such as its mechanisms and pathophysiological roles, has yet to be presented in a systematic and concise way. This has posed a great hurdle to many investigators who want to enter this field or to apply the knowledge to their own research, and are not sure where and how to begin.

Essentials of Apoptosis: A Guide for Basic and Clinical Research serves as a starting point for those investigators who are relatively new to apoptosis research. Therefore, instead of describing detailed findings in one specific field, we present the concepts, the molecular architecture (the molecules and the pathways), and the pathophysiological significance of apoptosis. Controversial results are presented only if they are related to the essential process. In addition, standard biochemical and cellular approaches to apoptosis research are described as a guideline for bench work. *Essentials of Apoptosis: A Guide for Basic and Clinical Research* is intended to provide

readers with the basics of apoptosis in order to stimulate their interests and to prepare them for the commencement of apoptosis-related research in their chosen areas. We hope that *Essentials of Apoptosis: A Guide for Basic and Clinical Research* will prove useful reading for all those interested in apoptosis research.

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Color Plates

Color Plate1 **Schematic representation of the amplificatory loop at the mitochondrial level in response to an apoptotic stimulus.** Three main interconnected mitochondrial steps are represented: (1) oligomerization of Bax and Bak, which generates a physical pathway for the efflux of proteins across the outer mitochondrial membrane; (2) Opa1-controlled remodeling of the cristae, leading to the redistribution of cytochrome c in the intermembrane space; (3) activation of mechanisms that cause mitochondrial fragmentation, following calcineurin (CnA)-dependent dephosphorylation of Drp1, or interaction of the latter with TIMMP8a, a component of the import machinery of mitochondria that is released together with cytochrome c. (Chapter 6, Fig. 1; *see* discussion on p. 167)

Color Plate 2 **History of apoptotic systems modeling**. Published systems biology studies were classified into four methodological categories and ordered chronologically. *Arrows* indicate an influence or logical connection between different studies with respect to the adoption of biological or methodological information (Chapter 12, Fig. 2; *see* discussion on p. 287)

Color Plate 3 **The molecular machinery of yeast apoptosis**. Exogenous and endogenous induction of yeast apoptosis leads to the activation of the basic molecular machinery of cell death, which is configured by conserved apoptotic key players such as the yeast caspase Yca1p, the yeast homologue of mammalian HtrA2/OMI (Nma111p), or the apoptosis-inducing factor Aif1p. Furthermore, it involves complex processes like histone modification, mitochondrial fragmentation, cytochrome c release, and cytoskeletal perturbations (Chapter 14, Fig. 1; *see* discussion on p. 334)

Color Plate 4 **Physiological scenarios and yeast apoptosis**. A wild-type yeast population promotes its own long-term survival and spreading of the clone by eliminating unfertile, damaged, or genetically unadapted individuals. Death in the population may also be triggered by toxins from nonclonal enemy strains or higher eukaryotes that hijack the PCD machinery of yeast (Chapter 14, Fig. 2; *see* discussion on p. 340)

Color Plate 5 **The molecular model for the cell corpse engulfment process**. Two partially redundant pathways mediate the engulfment process. CED-1 and CED-7 act on the surface of engulfing cells to mediate cell corpse recognition and to transduce the engulfing signal through CED-6 to the cellular machinery of the engulfing cells for engulfment. CED-7 also acts in dying cells. DYN-1 acts in the CED-1 pathway to promote the delivery of intracellular vesicles to the phagocytic cups and the maturation of phagosomes. RAB-2 and RAB-7 mediate lysosome fusion with phagosomes and are important for the degradation of internalized apoptotic cells. The CED-2/CED-5/CED-12 ternary complex mediates the signaling events from externalized phosphatidylserine (PS)/PSR-1 and other unidentified engulfing signal(s) and receptor(s) to activate CED-10 during phagocytosis (Chapter 15, Fig. 5; *see* discussion on p. 364)

Color Plate 6 **Molecular model of PS externalization during apoptosis**. Phospholipid asymmetry is maintained through the action of three classes of proteins: scramblases, ABC transporters, and aminophospholipid translocases. In a living cell, scramblases are not activated and show little to no activity, ABC transporters are only used to maintain lipid balance between the two bilayers, and the aminophospholipid translocases transport any externalized PS and PE to the inner leaflet. During apoptosis, scramblases are activated, for example, by WAH-1 released from mitochondria, randomly scrambling phospholipids on the membrane, ABC transporters may be activated to transport specific lipids to the outer leaflet, and the aminophospholipid translocase is inactivated, leading to PS externalization on the outer leaflet (Chapter 15, Fig. 6; *see* discussion on p. 366)

Color Plate 7 Live imaging demonstrates that cell death in the pupal eye is temporally and spatially regulated. (**A**–**D**) Pupal retinae of developmental ages as shown with cell boundaries outlined in *white*. For clarity, an ommatidium is shaded (primary pigment cells are *dark gray* and cone cells are *light gray*) in each panel and bristle groups are indicated by *green arrows*. IOCs account for the remainder of cells. In (D), the

remaining IOCs after death are *colored pink*. (**E**) The percentage of cells observed dying graphed relative to specific regions. The shading correlates to the regions in (**F**). Two pink asterisks indicate that no cells were observed to die in these positions. (F) Schematic of the pupal retina, with each *shaded region* corresponding to a position in which cells will either be more likely to live (*pink and green*) or die (*orange and yellow*). Figure adapted from **(**139**)**. (Chapter 16, Fig. 4; *see* discussion on p. 389)

Color Plate 8 **Bcl-2 family proteins regulate multiple aspects of hematopoiesis**. Schematic representation of the non-lymphoid hematopoietic hierarchy. Proposed pro-apoptotic (⊥) and antiapoptotic (↑) functions for Bcl-2 family members are indicated. Hematopoietic stem cell (HSC), common myeloid progenitor (CMP), megakaryocyte/erythroid progenitor (MEP), granulocyte/macrophage progenitor (GMP), burst-forming unit-erythroid (BFU-E), megakaryocyte progenitor (MkP), colony-forming unit granulocyte (CFU-G), colony-forming unit macrophage (CFU-M) (Chapter 19, Fig. 1; *see* discussion on p. 443)

Color Plate 9 **Progression of cell death in multiple models of acute neuronal injury**. The progression of cell death varies across cell death models. Focal ischemia and traumatic brain injury display acute injury within hours of the insult, marked by the presence of necrotic morphology within the core of the injury (*black regions*), and progressing rapidly to inflammation (*dark red regions*). Delayed cell death, with morphological features of apoptosis or mixed morphologies, is found in regions proximal to the core of the injury and occurs over days to weeks (*red regions*). Global ischemia and kainic acid-induced epilepsy affect similar overall regions in both hemispheres of the brain, but in differing subregions. Cell death is more delayed compared to focal ischemia or traumatic brain injury and presents hallmarks of programmed cell death, with limited necrotic phenotypes. The figure represents moderate injury models; the range and severity of cell death are highly dependent on the degree of toxicity and the species or method used to induce injury. (Chapter 20, Fig. 1; *see* discussion on p. 462)

Color Plate 10 **Imaging cardiac muscle apoptosis in vivo**. Confocal image of TUNEL-positive cardiomyocyte labeling in the mouse heart after aortic banding, with TUNEL-positive nucleus (*red*), sarcomeric actin (*green*), and nuclei counterstained with DAPI (*blue*) (Chapter 22, Fig. 1; *see* discussion on p. 506)

Color Plate 11 **Function of Bcl-2 family proteins**. Named after the founding member of the family, which was isolated as a gene involved in B-cell lymphoma (hence the name *bcl*), the Bcl-2 family is comprised of well over a dozen proteins, which have been classified into three functional groups. Members of the first group, such as Bcl-2 and Bcl-xL, are characterized by four short, conserved Bcl-2 homology (BH) domains (BH1–BH4). They also possess a C-terminal hydrophobic tail, which localizes the proteins to the outer surface of mitochondria, with the bulk of the protein facing the cytosol. The key feature of group I members is that they all possess antiapoptotic activity and protect cells from death. In contrast, group II consists of Bcl-2 family members with proapoptotic activity. Members of this group, which includes Bax and Bak, have a similar overall structure to group I proteins, containing the hydrophobic tail and all but the most N-terminal, BH4 domain. Group III consists of a large and diverse collection of proteins whose only common feature is the presence of the ~12- to 16-amino-acid BH3 domain. The Bcl-2 family of proteins function primarily to protect or disrupt the integrity of the mitochondrial membrane and control the mitochondrial release of proapoptotic proteins like cytochrome c, AIF, and Smac/DIABLO. Antiapoptotic Bcl-2 members (Bcl-2, Bcl-xL) protect the mitochondrial membrane. In response to environmental cues, these antiapoptotic proteins engage another set of proapoptotic proteins of the Bax subfamily (which includes Bax, Bak), normally loosely residing on the mitochondrial outer membranes or the cytosol. The interaction between Bak and Bax proteins results in oligomerization and insertion into the mitochondrial membrane of the complete complex (Chapter 22, Fig. 2; *see* discussion on p. 508)

Color Plate 12 **Activation of DISC by cigarette smoke**. MRC-5 cells at 70% confluence were exposed to 20% CSE in serum-free media. Immunofluorescence images of MRC-5 double-labeled with indicated antibodies (anti-FAS in *blue* and anticaspase-8 in *green*) are shown. The cyan pseudocolor (*arrow, top panel*) indicates a co-localization of Fas and caspase-8. The same images with either the green or blue color removed are shown for clarity (*middle and bottom panels*). Data in this figure are representative of 20–49 cells analyzed for each time point. All panels are the same scale **(**97**)**. [Figure reproduced from Park et al. **(**97**)** with permission from the American Association of Immunologists.]

The cartoon illustrates the potential role of protein kinase-c (PKC) isoforms in regulating DISC formation. PKCα displayed an antiapoptotic effect in CSE-treated cells and in chronic cigarette smoke-exposed mice, whereas PKCζ displayed a proapoptotic effect. PKCα potentially inhbits DISC trafficking by activating the PI3K pathway in fibroblasts. PKCζ promoted DISC trafficking by inhibiting the PI3K pathway **(**97**)** (Chapter 23, Fig. 3; *see* discussion on p. 536)

Color Plate 13 **Schematic representation of liver diseases**. Hepatocyte apoptosis is initiated by various stimuli via direct effects and/or inflammatory responses. Massive hepatocyte apoptosis with impairment of hepatocyte regeneration results in acute liver failure. Chronic hepatocyte apoptosis leads to liver cirrhosis and liver cancer. Kupffer cells engulf apoptotic bodies of hepatocytes and release fibrogenic cytokines, which trigger collagen production by hepatic stellate cells. Chronic hepatocyte apoptosis also stimulates hepatocyte regeneration, and dysregulation of the balance between hepatocyte proliferation and cell death causes hepatocarcinogenesis (Chapter 24, Fig. 1; *see* discussion on p. 547)

Color Plate 14 (**a**) A model that describes how chromosome loss or nondisjunction occurs in spindle checkpoint-defective cells (MAD2-depleted cells or complete BUB1-depleted cells). In spindle checkpoint-mutant cells, the spindle checkpoint is not activated even if there are defects in kinetochore–microtubule attachment. No mitotic delay occurs, which results in the premature exit from mitosis. Thus, there is substantial chromosome loss or nondisjunction, and presumably cell death follows. (**b**) A model that describes the same scenario in partial BUB1-depleted cells. Here, defects in kinetochore–microtubule attachment induce lethal DNA fragmentation (CIMD). Because cells are still arrested in mitosis, the mitotic index remains unchanged. Therefore, the spindle checkpoint appears to be active.(Chapter 28, Fig. 2; *see* discussion on p. 637)

Color Plate 15 CIMD occurs in BUB1-depleted cells in the presence of microtubule inhibitors or 17-AAG. HeLa cells that are BUB1-depleted and 17-AAG-treated exhibit DNA fragmentation (TUNEL-positive; *red*) during mitosis (*top row*, prometaphase; *bottom*, metaphase). Forty-eight hours after HeLa cells were transfected with BUB1 siRNA, they were incubated with 17-AAG (+17AAG, 500 nM) for 24 hours at 37 °C. Fixed samples were stained by using an *in situ* cell

death detection system that contained TMR red (TUNEL-signal; *red*), an antiphosphorylated histone H3 mouse monoclonal antibody, and FITC-conjugated secondary antibodies (*green*). DNA was stained with DAPI (*blue*) to visualize (Chapter 28, Fig. 3; *see* discussion on p. 638)

Part I
Molecules and Pathways of Apoptosis

Chapter 1
Caspases: Activation, Regulation, and Function

Stefan J. Riedl and Fiona L. Scott

Abstract The main effectors of apoptosis are proteases belonging to the caspase family. Caspases represent key mediators in the initiation and execution of the apoptotic program. The apoptotic caspases constitute a minimal two-step signaling pathway that culminates in the controlled demise of the affected cell. At the center of intense research for more than a decade and a half, a thorough picture of these regulatory proteases has emerged. A plethora of recent reports shed exciting new and refined light on their activation, regulation, and function. In addition to an advanced understanding of caspases in the apoptotic program, additional functions of these proteases in other pathways and their intriguing regulation by new signaling platforms have surfaced. With caspases affecting biological processes extending from apoptosis to other forms of cell death and inflammation, a closer look at these regulatory proteases is paramount for our understanding of cell signaling.

Keywords Apoptosis · Caspase · Protease · Activation · Inhibition · Signaling platforms · Caspase-substrates · Mechanism · Apoptosome · DISC · Inflammasome

Introduction

As the evolution of multicellular organisms took place, the issue of maintaining both body and organ size conducive with health and viability became a challenge. In addition, constant cell proliferation had to be counterbalanced by a mechanism of cell deletion compatible with the newly developing innate immune system. The evolutionarily derived answer to the problem came in the form of programmed cell death or apoptosis. The term "apoptosis" literally

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means "the dropping of leaves from a tree" in Greek and was coined to describe the morphological features observed during this coordinated deconstruction and packaging of the cell for elimination by phagocytes and neighboring cells **(1)**.

The importance of apoptosis during development and in the adult can be seen in many disease states (discussed in detail in Part III of this book). Ischemic injury, neurodegenerative disorders, and AIDS are the result of excessive apoptosis in the absence of sufficient cell replenishment **(2, 3)**. In contrast, the pathogenesis of autoimmune diseases and cancer results from a deficit in apoptosis **(2, 4–7)**. Recent drug design strategies to combat chronic and acute diseases linked to abnormalities in cell death involve directly targeting the apoptotic cellular machinery **(8–10)**.

The past 15 years have seen a fever of research activity aimed toward understanding the mechanisms of programmed cell death. As a result, many of the genes involved have been well characterized at the genomic, biochemical, and protein structural levels and are conserved across species including nematodes, flies, and mammals **(11–13)**. Apoptosis is triggered by diverse stimuli that can be classified as part of either the extrinsic pathway (discussed further in Chapter 5) or the intrinsic pathway (discussed further in Chapter 6), as depicted in Fig. 1.1. Central to both pathways are the cysteine-dependent aspartate-specific proteases,

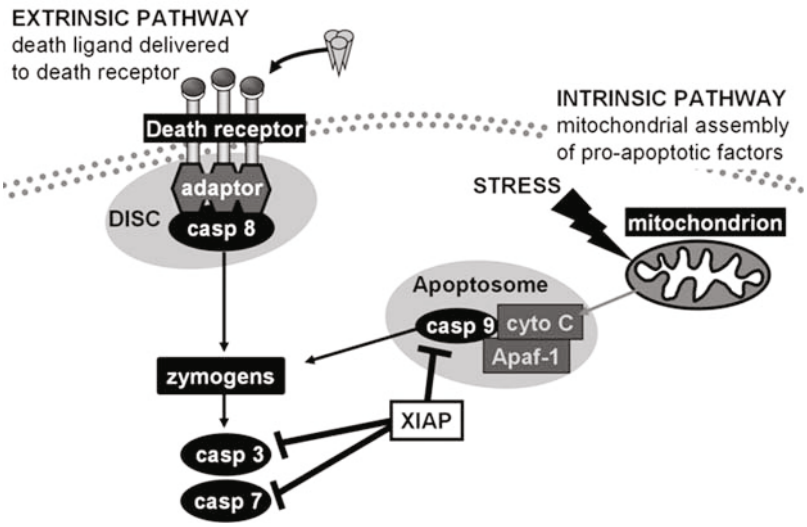

Fig. 1.1 Simplified extrinsic and intrinsic apoptosis pathways. Upon engagement of the cell surface death receptor by a ligand, adaptor molecules are recruited that in turn recruit caspase-8 to form the death-inducing signaling complex (DISC). Caspase-8 is activated by dimerization at the DISC. This is termed the *extrinsic pathway*. In response to cell stress, the mitochondria release cytochrome c from the intermembrane space into the cytosol. This triggers the formation of the apoptosome, which activates caspase-9 by dimerization. Active caspase-8 and -9 can proteolytically activate the executioner caspases-3 and -7. XIAP inhibits caspases-3, -7, and -9

or caspases. These proteolytic enzymes are the suicide weapons of the apoptotic pathway, and their activity directly eradicates the cell. We will discuss recent advances in our understanding of caspase activation, regulation, and their cellular substrates, both in apoptosis and in nonapoptotic biology.

Caspases—A Historical Perspective

In 1992, the cysteine protease responsible for maturation of interleukin-1β was cloned by two independent groups **(14**, **15)**. Initially termed "interleukin-1β-converting enzyme" (ICE), it has since been renamed "caspase-1." Caspase-1 cleaves pro-interleukin-1β at a critical aspartate residue, converting it to the mature form, interleukin-1β. During the same time, through extensive studies of cell deletion during development in *Caenorhabditis elegans,* Horvitz and colleagues identified three central genes critical for normal programmed cell death—*ced-3, ced-4,* and *ced-9* (Fig. 1.2; discussed further in Chapter 13). The *ced-3* gene product was found to share greater than 24% identity with ICE **(16)**. These exciting findings prompted researchers to look for other cysteine proteases with catalytic preference for aspartic acid residues, anticipating that they, too, would have a role in cell death. A huge body of research has led to the classification of 11 human caspases. Only seven of these are involved in apoptosis. The apoptotic caspases are further divided into those involved in the initiation phase of apoptosis (caspases-2, -8, -9, and -10) and those involved in the execution phase (caspases-3, -6, and -7). Interestingly, the functional orthologue of CED3 is not caspase-1 (Fig. 1.2). Caspase-1 and the other remaining caspases are cytokine activators and are important in inflammation

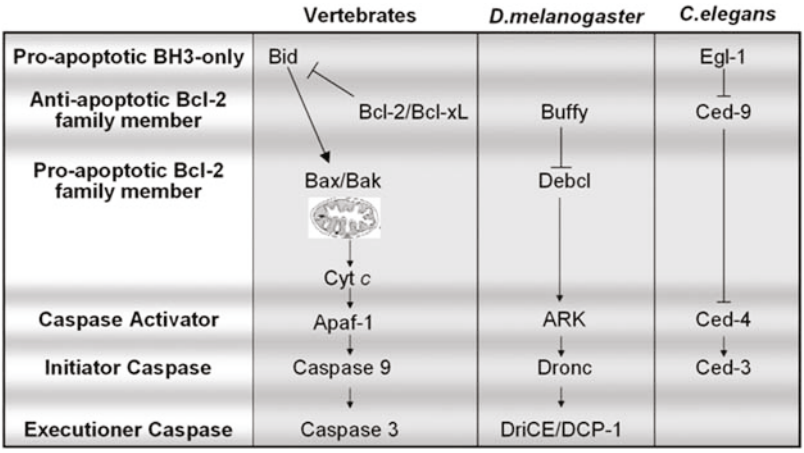

Fig. 1.2 The caspase cascade. Functional alignment of apoptosis regulators in the vertebrates *Drosophila melanogaster* and *Caenorhabditis elegans*

(caspases-1, -4, and -5) or are involved in keratinocyte differentiation and maintenance of barrier function of the skin (caspase-14) **(17)**. The key to caspase involvement in such a diversity of biological processes lies in their mechanism, and more specifically their intriguing routes of activation.

Activation of Caspases

As described above, caspases are highly evolved signaling proteases that participate in a stunning variety of cellular functions and pathways **(18–20)**. At the heart of their extraordinary capabilities lies their flexibility to be activated in different manners **(21–23)**. In the absence of an activating stimulus, caspases exist as latent forms, or zymogens. Activation can occur by two means, namely activation by limited proteolysis or activation through binding to so-called activation platforms, each of which is used by different caspase groups **(20, 24–26)**. The executioner caspases, responsible for the execution phase of the apoptotic program, become activated by limited proteolysis. Since these caspases operate at the bottom of the apoptotic cascade (Fig. 1.2), it is feasible that their zymogen forms are cleaved and activated by caspases higher in the hierarchy or other proteases such as granzyme B **(26–29)**. The existence of inactive zymogens and their activation by limited proteolysis is a feature common to proteases **(30)**. The cleavage-based activation of executioner caspases thus quickly became an accepted paradigm. However, the question soon arose as to how the apical caspases become activated. That is, how do caspases at the apex of the pathways sense a nonproteolytic signal, become activated, and thus translate this signal into a proteolytic signal? The answer lies in the unusual properties and architecture of caspases, which set them apart from most other proteases. This architecture is very simple at first glance [Fig. 1.3(A)]. All caspases possess a catalytic domain, which shows a characteristic caspase fold **(24)**. Additionally, many members of the family possess one or more adaptor domains belonging to the DEATH domain family, namely CARD and DED **(31)**. In fact, it is this architecture, slight differences in the catalytic domain, and the presence or absence of an adaptor domain that facilitate different activation in different caspase groups. While the executioner caspases rely solely on activation by cleavage, the initiator caspases become activated by binding to activation platforms **(26, 32, 33)**. The latter triggers self-association (dimerization) of the initiator caspases leading to their activation as postulated by the so-called induced-proximity model, which is discussed ahead **(34, 35)**.

Activation of Apoptotic Executioner Caspases

The executioner caspase zymogens follow the paradigm of activation by cleavage. The activity of their zymogens is essentially zero, but once cleaved, executioner caspases such as caspase-7 are highly functional legitimate proteases **(36, 37)**. Typically, they are expressed as single-chain proteins, which exist in the cell as

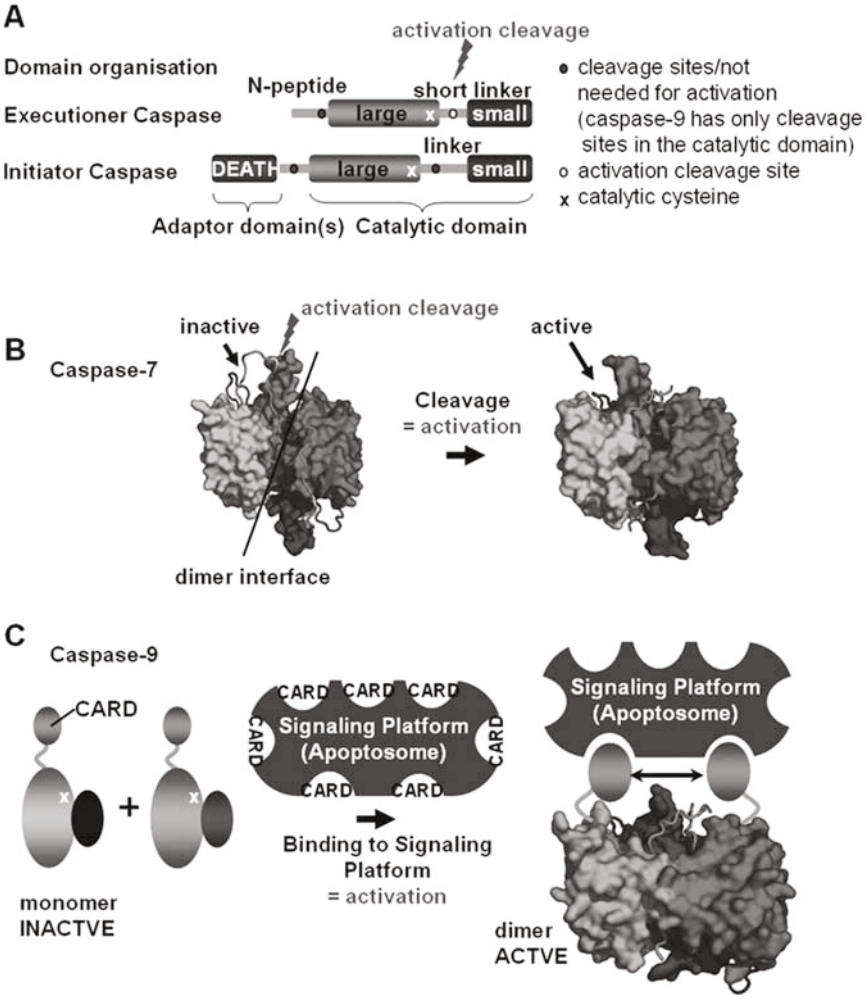

Fig. 1.3 Caspases: domain organization and activation mechanisms. (**A**). Domain organization. All caspases possess a catalytic domain, with a characteristic organization of a large subunit connected to a small subunit by a linker region, which carries caspase/protease cleavage sites. Additionally, caspases exhibit an N-terminal region comprising only a short peptide in the case of executioner caspases or one or two adaptor domains belonging to the DEATH domain family. Cleavage sites are also found between the N-terminal region and catalytic domain with the exception of caspase-9. (**B**). Apoptotic executioner caspase activation. Shown are the zymogen and the active form of caspase-7 in surface representation, with the linker region and loop regions important for active site formation also shown (PDB entries: 1GQF, 1F1J). Executioner caspases are constitutive dimers and become activated by cleavage of the linker region, which allows for the formation of an active site. (**C**). Apoptotic initiator caspase activation. Shown schematically are the inactive monomeric forms of caspase-9, which need to bind to an activation platform, the apoptosome, via their adaptor domains. This leads to dimerization and activation of the caspase according to the induced-proximity model. Cleavage of the linker region can occur but is not necessary for activation

constituent homodimers [Fig. 1.3(B)]. Induction of the apoptotic program leads to cleavage of their short N-terminal regions and, most importantly in terms of their activation, a cleavage within the catalytic domain generating new termini and a large and small subunit **(38, 39)** [Fig. 1.3(A)].

Various studies on the catalytic domain of executioner caspases, among them three-dimensional crystal structures at atomic resolution, now provide a relatively proficient picture of their activation mechanism **(24)**. Their catalytic domains are built by a solid frame consisting of a central beta sheet flanked by alpha helices adopting the characteristic caspase fold. Interestingly, crucial features for the catalytic function of the caspases lie in loop regions between these scaffolding elements. These loop regions also provide the key to caspase activation. As shown in Fig. 1.3(B) for caspase-7, one of the best-studied caspases in terms of activation, both the zymogen and the cleaved/active caspase exist as dimers. Yet distinct loop regions differ between the zymogen and the active caspase, with drastic consequences. One of these regions is known as the linker region. It is in this region that the actual activation cleavage of the executioner caspase occurs [Figure 1.3(A)]. The highly specific cleavage by a more apical caspase or granzyme B results in the generation of a large (~20-kDA) and a small (~10-kDa) subunit. This cleavage has significant mechanistic consequences—in essence, it allows for the formation of the active site in the caspase. The detailed events have been described and reviewed elsewhere **(22–24)** and are also described in Chapter 4, but a general picture of the activation events is outlined in the following (Fig. 1.3). Cleavage in the linker region of the zymogen dimer releases the newly formed termini to move outward. A consequence of this is that another loop switches from a random conformation in the zymogen to a well-defined position in the active caspase. This loop now represents the bottom of the active site cleft and provides central residues that the active caspase utilizes to bind its substrate **(39, 40)**. Cleavage of the linkers followed by these two major rearrangements converts a practically nonexistent active site into a well-defined active site cleft with substrate binding sites and well-ordered catalytic machinery. Again, various studies describe this process in more detail and also discuss the more subtle involvement of additional loop regions using a specifically tailored nomenclature **(41)**. Yet the principles outlined here illustrate the fundamental nature of this process, namely that activation cleavage effectively generates a genuine and well-defined active site in executioner caspases.

Interestingly, in the cleaved active form of the executioner caspases, each newly formed terminus of one caspase interacts with the new terminus of the other caspase of the dimer. Thus, the fact that executioner caspases are constitutive dimers is actually a prerequisite for their activation mechanism by limited proteolysis. This is different in the case of the initiator caspases, which exist as inactive monomers prior to activation and require binding to oligomeric signaling platforms to become activated.

Apoptotic Activation Platforms

The first step in the activation of the apoptotic caspase cascade is the activation of the initiator caspases through the use of specific activation platforms. This is the precise event modulating an apoptotic signal into the activation of a protease cascade, or in other words into a proteolytic signal. The two apoptotic pathways use specific activation platforms (Fig. 1.1). The extrinsic pathway is triggered upon formation of the death-inducing signaling complex (DISC), which ultimately leads to the activation of caspase-8 (or -10) **(33**, **34**, **42**, **43)**. On the other hand, the intrinsic pathway is characterized by the formation of a heptameric soluble platform dubbed the apoptosome that is capable of activating caspase-9 **(26)**.

In DISC signaling, extracellular ligands such as FasL and TRAIL bind in a conventional manner to the extracellular domains of transmembrane receptors, termed *death receptors*. In essence, the signal is then transmitted to the cytosol by clustering of the death receptors followed by recruitment of the initiator caspase-8 (Fig. 1.1) via the adaptor protein FADD. While the details of this process are not yet understood, the essence of DISC formation is to present or arrange adaptor domains, namely the death effector domains (DED) of FADD molecules, in a manner so that caspase-8 can be recruited via its DEDs. The caspase-8 paralogue, caspase-10, is also regarded as an initiator in DISC-mediated cell death in humans (mice apparently lack a caspase-10 gene), yet existing debate in the literature questions the ability of caspase-10 to functionally replace caspase-8 in death receptor signaling **(44)**.

Structural information on the DISC and its target caspase complex is vague. However, recent data on the adaptor protein FADD **(45)** and homologues of caspase-8 suggest that the activation of caspase-8 **(46**, **47)** at the cytosolic face of the cell membrane occurs by an induced-proximity mechanism (see ahead). Additionally, the exact process of ligand binding and receptor oligomerization may require further factors, such as receptor internalization, palmitoylation, and the presence of lipid rafts in addition to clustering **(48–50)**.

The activation platform of the intrinsic pathway is formed by Apaf-1, which represents the main constituent of the apoptosome and acts as a soluble cytosolic receptor **(26)** (Fig. 1.1). Following an apoptotic signal, Apaf-1 senses the release of its ligand, cytochrome c, from the mitochondrion, which, upon binding, triggers a nucleotide-dependent oligomerization of Apaf-1 to form the apoptosome **(51)**. Apaf-1 as a soluble receptor lacks the two-dimensional arrangement of a transmembrane receptor spanning the cell membrane, instead using a different mechanism to generate a two-dimensional surface platform for signaling. This process has been reviewed **(22)** and is characterized by a mechanism-based oligomerization that utilizes the specialized AAA+ domain of Apaf-1 to oligomerize into a heptameric ring **(52**, **53)**. Similar to the DISC, the apoptosome presents adaptor domains to the target caspase, in this case

CARD domains suited for the recruitment of caspase-9 via the CARD domains of the caspase.

In summary, while each of these apoptotic signaling platforms uses a unique mechanism, the goal in both cases is to properly present adaptor domains, namely DEDs in the case of the DISC and CARD domains in the case of the apoptosome, to attract adaptor domains of their target initiator caspases. This leads to recruitment of the initiator caspases and their activation.

Activation of Apical Caspases

Once apoptotic signaling platforms are formed, they are capable of activating their target initiator caspases. One of the most well-understood apical caspases in terms of its activation is caspase-9 and its activation through the apoptosome signaling platform. Two main hypotheses for the activation have been brought forth. The first proposes an allosteric model that postulates the activation of a caspase monomer directly by the apoptosome **(54)**. The second model, the induced-proximity model, postulates that the apoptosome provides a platform for caspase-9 dimerization [as indicated in Fig. **1.3**(C)] **(55)**. The latter also includes allosteric events in capsase-9. Thus, to clarify, the allosteric model proposes the activation of caspase-9 monomers directly by surfaces on the apoptosome, while in the induced-proximity model, the apoptosome causes a dimerization of caspase-9 catalytic domains as the actual activation event. Both hypotheses have been reviewed at length, including recent revisions of the models **(21**, **56)**. Kinetic data and recent results from caspase-9 and caspase-9 CARD/caspase-8 fusions are in favor of an activation by dimerization and thus the induced-proximity model **(35**, **57)**. Therefore, this model is used in the following for the illustration of initiator caspase activation via signaling platforms.

As mentioned earlier, the unique architecture of caspases allows for activation by cleavage, as in the case of the executioner caspases, as well as activation in the absence of cleavage. In the case of the executioner caspases, cleavage of the short linker regions between large and small subunits allows for the formation of a productive active site prerequisite for the enzymatic activity of the caspase. However, investigation of the initiator caspases, such as caspase-9 showed **(58)** that cleavage is not critical for the activity of initiator caspases even though they possess several cleavage sites. Instead, it was found that, unlike executioner caspases, which are constitutive dimers, a typical initiator caspase exists as an inactive monomer under cellular concentrations in the absence of an apoptotic signal **(35**, **57**, **59)** [Fig. 1.3(C)]. Furthermore, although similar to executioner caspases in their basic architecture, initiator caspases differ in two critical features. One is the presence of an adaptor domain, such as the CARD domain in caspase-9, and the second is a significantly longer linker region between the large and small subunits.

With these attributes of initiator caspases in mind, a series of events can be strung together whose orchestration leads to the activation of caspase-9 **(22**, **35**, **57)**. In the absence of an apoptotic stimulus, caspase-9 exists as an inactive monomer lacking a productive active site [Fig. 1.3(C)]. Upon an apoptotic stimulus, the activation platform for capsase-9, the apoptosome, is formed and recruits caspase-9 via homotypical CARD-CARD interactions. The close proximity of caspase-9 molecules—that is, the high local concentration of catalytic domains—now favors dimerization of the caspase. This in turn causes the formation of a productive active site on caspase-9 through means of a similar mechanism as that described for the cleaved executioner caspases. The length of the linker in caspase-9 is believed to provide the necessary freedom to accommodate the active conformation of the caspase even in the absence of cleavage. While the initiator caspases possess putative cleavage sites, both in the linker regions and (with the exception of capsase-9) between the adaptor domain(s) and the catalytic domain, the functions of these sites are not yet fully understood. Importantly, even in the induced-proximity model, it is conceivable that additional contacts and placements from the activation platform further add to the activation of the caspase dimer.

Taken together, the examples of the apoptotic executioner caspases and the initiator caspases illustrate that caspase activation can be achieved by cleavage within the linkers of existing caspase dimers, or that cleavage is dispensable and replaced by the involvement of activation platforms leading to dimerization and activation of the caspases. Central in this nonproteolytic activation of caspases is the interaction of the caspases and the signaling platforms via adaptor domains of both proteins. The combination of different adaptor domains in different caspases and their activation platforms gives caspases the opportunity to selectively participate in a variety of pathways. Thus, it is not surprising that crucial functions in several apoptotic and nonapoptotic pathways were recently linked to a variety of specific new activator platforms targeting a number of caspases.

Other Activation Platforms and Their Caspases

In recent years, one group of activation platforms consisting of receptors belonging to the NLR (nod-like receptor) protein family has become the center of intense research. Bearing similarities to Apaf-1, the apoptotic protein forming the apoptosome, these proteins were implicated in forming signaling platforms activating caspases-1 and -5 (Fig. 1.4). Like Apaf-1, most members of the NLR protein family show the typical domain arrangement of an adaptor domain, usually a CARD or PYRIN domain, an oligomerization domain termed an NACHT domain, and a signal-sensing region consisting in this case of leucine-rich repeats (Fig. 1.4). The latter recognizes pathogen-associated molecular patterns (PAMPs) or other inflammatory stimuli **(60**, **61)** triggering

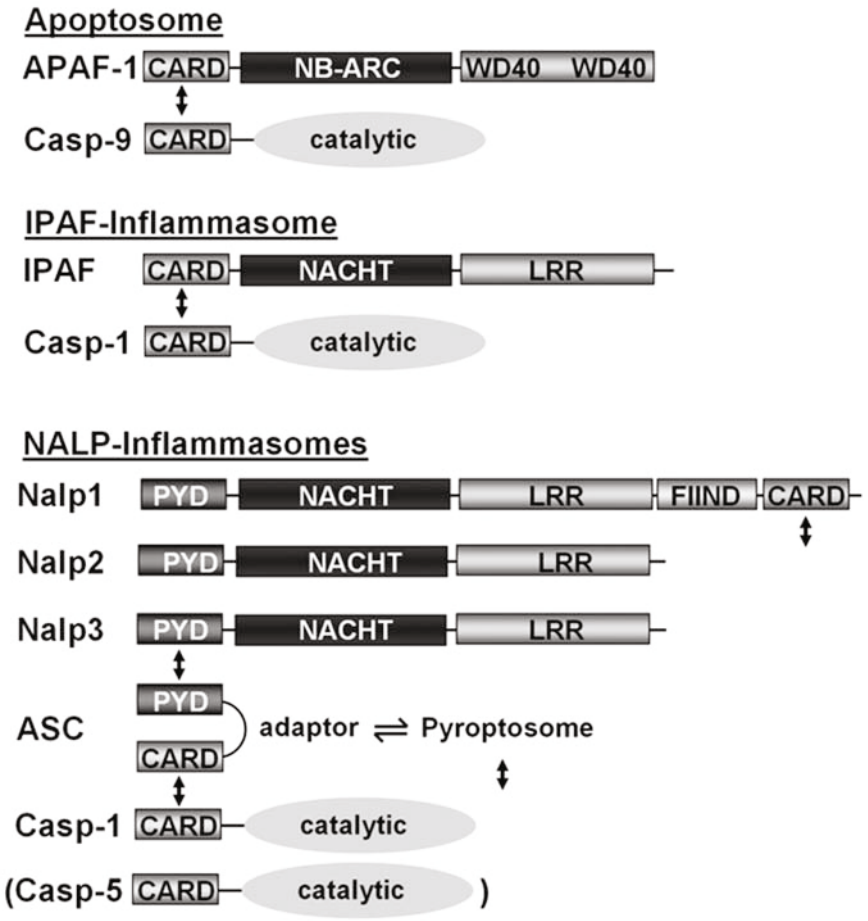

Fig. 1.4 Other signaling platforms, adaptors, and target caspases. In analogy to the apoptosome, other signaling platforms were identified. They typically contain an oligomerization region (NB-ARC, NACHT), a signal-sensing region (WD40, LRR), and adaptor domains to bind to their target caspase (caspase-1 or -5) either directly or via the adaptor protein ASC. ASC was additionally found capable of forming a large caspase activating assembly of its own dubbed pyroptosome

the formation of signaling platforms dubbed inflammasomes, which lead to the activation of the target proteins. As the term "inflammasome" indicates, these NLRs were initially viewed as analogous to Apaf-1, which forms the apoptosome **(62)**. Although overall similar in their functional domain organization (Fig. 1.4), it is not yet clear if the NLR proteins share the same mechanism seen for Apaf-1 in terms of their active signaling platforms and mode of caspase activation.

Several inflammasomes have been described **(20, 60, 61, 63)**. One prominent platform is formed by the NLR protein Ipaf. This protein was found to activate capsase-1 upon sensing bacterial flagellin **(63–67)**. The domain structure of Ipaf indicates that it possesses an N-terminal CARD domain that recruits caspase-1 via homotypical CARD-CARD interactions once the Ipaf inflammasome is formed **(62)**. Other NLR proteins possess an N-terminal pyrin domain. Nalp1, 2, and 3 belong to this group and are known to form so-called NALP-inflammasomes. While all of these receptors are capable of activating caspase-1, only Nalp1 was also reported to activate caspase-5 **(19, 63, 68)**. To accommodate recruitment of their target caspases, they utilize ASC, which consists of a pyrin domain and a CARD domain, thus representing an ideal adaptor linking the pyrin domain of the NLR to the CARD domain of the caspases **(69, 70)**.

Surprisingly, recent results identify a pyroptosome, which represents a large signaling entity solely formed by ASC **(71)**. Proposed to be a supramolecular arrangement of ASC dimers, it would certainly be capable of activating capsase-1 according to the induced-proximity model. Yet in this case, the exact mechanism remains to be elucidated.

Another and perhaps one of the most intriguing putative activating platforms is the PIDDosome and its caspase, caspase-2 **(72, 73)**. In what seems like a fusion between the characteristics of the apoptotic DISC and signaling platforms such as the inflammasome or apoptosome, the heart of the PIDDosome is built by a mixture of the signaling receptor PIDD and the adaptor protein RAIDD. Both proteins fashion a multifaceted interaction complex via their death domains that arranges the CARD domains **(74)** in proper orientation to cause activation of their target caspase-2.

While questions about the exact hierarchy remain, the role of caspase-2 activation on the PIDDosome is linked to the initiation of apoptotic pathways and also inflammation but is not yet fully understood **(73)**. Inflammasomes, on the other hand, have been linked to both the induction of inflammatory responses as well as cell death and even autophagy **(61)**. While apoptotic cell death seems to be triggered by some inflammasomes, the activation of caspase-1 by the pyroptosome has been linked to a specific form of cell death dubbed "pyroptosis" **(71, 75)** characterized by distinct features and mechanisms **(76–78)**. Further regulation of caspase activation by activation platforms is achieved by inactive truncated derivatives of caspases bearing an inactive catalytic domain or only the adaptor domain. Adaptors and variations of adaptors, such as ASC2 lacking the CARD domain of its counterpart ASC, further affect caspase activation, as has been recently reviewed **(79)**. Taken together, the exact biology surrounding platforms such as inflammasomes and various adaptors and regulators is still not fully understood. Further research will undoubtedly reveal stunning new mechanisms and pathways of activation platforms and their target caspases.

Caspase Inhibitors

Equally as pertinent to the regulation of apoptosis as caspase activation is the understanding of caspase inhibition. Caspase inhibitors have evolved in both mammals and viruses to mediate cell death. The first inhibitor to be discovered was the cowpox virus cytokine response modifier A (CrmA). CrmA is a member of the serine protease inhibitor, or serpin, superfamily of pseudosubstrate inhibitors. Like other serpins, the protease inhibitory activity of CrmA is dependent on a mobile reactive center loop (RCL). However, inhibition of aspartate-specific caspases requires that CrmA possess an aspartic acid residue at the P_1 position. The sequence of the CrmA RCL contains LVAD⇓C. Therefore, CrmA has evolved the perfect bait with which to specifically catch aspartic acid-specific proteases. This is unusual, with the only other serpin containing an acidic P1 residue, glutamic acid in this case, being serpinb9 (PI-9). Whether PI-9 can also inhibit caspases is somewhat controversial **(80–84)**. Before the biochemical characterization of CrmA, serpins had only been described as serine protease inhibitors. CrmA inhibition of caspases was the first example of cross-class inhibition, where a serpin inhibited proteases that use either cysteine or serine residues as the catalytic nucleophile.

CrmA most potently inhibits caspases-1, -8, and -10, and to a lesser extent caspases-4 and -5 **(85–87)**. As a consequence, CrmA modulates viral infection at multiple points. Through inhibition of caspases-1, -4, and -5, CrmA inhibits maturation of interleukin-1β and interleukin-18 cytokines. As a result, cells infected with CrmA-expressing viruses have reduced caspase-mediated cytokine processing and a suppressed inflammatory response. This limits the recruitment of immune cells to the site of infection, thereby facilitating viral replication and promoting infection. Through inhibition of caspase-8, CrmA blocks apoptosis induced by tumor necrosis factor α (TNFα) and other extrinsic stimuli **(88, 89)**. CrmA orthologues (SPI-2) from vaccinia and ectromelia virus also inhibit the extrinsic apoptosis pathway **(90–92)**. Therefore, CrmA inhibition of caspase-8 contributes to the etiology of disease by preventing the virally infected cell from being directly killed by immune cells via the extrinsic apoptosis pathway.

Regardless of its potent inhibitory activity toward caspase-1 and -8, CrmA is a very poor inhibitor of executioner caspase-3, in spite of their structural and functional similarity **(85, 86)**. This poor inhibition is due to suboptimal specificity of the RCL sequence (caspase-3 prefers DEVD) and predicted structural clashes between the specificity determining loop of caspase-3 with surface residues of CrmA **(93)**.

The second caspase inhibitor discovered was also a viral protein. The p35 gene product from baculovirus inhibits the activity of most caspases from multiple species with high potency **(94–98)**. Because of its broad caspase inhibitory profile, p35 has become a useful tool in apoptosis research. A close relative of p35, sharing 50% identity, is p49 from another baculovirus. p49

also potently inhibits most caspases, including DRONC from *Drosophila melanogaster* (Fig. 1.2), which is relatively poorly inhibited by p35 **(99, 100)**.

The method of caspase inhibition employed by p35 is novel in biology and has been termed the "bowstring mechanism" **(101)**. Through structural determinations and mutagenesis studies, it was shown that a protruding loop containing the caspase recognition sequence DQMD⇓G is engaged by the caspase, with the formation of a covalent thiol linkage resulting between the catalytic cysteine and the P_1-aspartate residue **(39, 102)**. Subsequent cleavage of the scissile bond repositions the amino-terminus of p35 such that it is close to the active site of the caspase and prevents deacelyation of the thioester bond by blocking the access of hydrolytic water molecules.

Considering the potentially lethal consequence of caspase activation, there are surprisingly few mammalian gene products that directly inhibit the proteolytic activity of caspases. Viral systems again provided the basis for the discovery of the inhibitor-of-apoptosis (IAP) family.

Complementation studies showed that baculoviral IAP can substitute for p35 in protecting insect cells from virus-induced apoptosis **(103)**. Baculoviral IAPs contain at least one 70-80 amino acid zinc-binding fold termed the "baculoviral IAP repeat" (BIRs). The discovery of baculoviral IAP provided the impetus to search for mammalian IAP family members. There are currently eight members characterized in humans [reviewed in **(104)** and described in detail in Chapter 3 of this book].

With regard to caspase inhibition, X-linked IAP (XIAP) is the only IAP protein that directly inhibits the catalytic activity of caspases [reviewed in **(105)**], including the original baculoviral IAPs **(106)**. As a consequence of its caspase inhibition function, XIAP is the most thoroughly studied of the mammalian IAPs. XIAP contains three BIR domains in tandem, and a carboxy-terminal really interesting new gene (RING) finger. Structural and biochemical studies have precisely mapped the regions in XIAP required for caspase inhibition. Sequences encompassing the second BIR domain (BIR2) inhibit caspases-3 and -7, while the third BIR domain (BIR3) inhibits caspase-9 **(107)**.

Despite sharing close to 40% identity, studies at the atomic level revealed that the BIR domains in XIAP utilize strikingly diverse modes for inhibiting proteases of the same mechanistic family. Potent caspase inhibition is achieved via a two-site binding mechanism. The first binding site is a conserved surface groove shared by many BIR domains that bind IAP-binding motif (IBM)-containing proteins **(108)**. Upon activation, caspases are processed and the newly generated amino-terminus constitutes an IBM. For inhibition of caspase-9 by BIR3, this interaction was observed in a crystal structure of the complex **(109)**. For inhibition of caspase-3 by BIR2, the interaction was seen as a result of crystal contacts, and later confirmed through solution biochemistry **(110, 111)**. The IBM interacting groove of the BIR domain functions as an exosite, acting as an anchor to strengthen the inhibitor-caspase complex. Although the anchoring interface is conserved between the caspase/BIR pairs, the mechanism of enzyme inhibition is not.

All structures of a BIR2/caspase complex reveal that the peptide strand flanking the second BIR domain at its amino-terminus (residues 140–156) stretches across the active site of the protease **(110**, **112**, **113)**. Therefore, to efficiently inhibit caspases-3 and -7, XIAP requires two surfaces: an active site-directed interaction and an exosite-anchoring motif. At first glance, this targeting of the active site would appear to be similar to a standard "lock and key" inhibitor mechanism. However, this is not the case. Compared with manmade peptidyl inhibitors, there are considerable differences. Peptidyl inhibitors such as DEVD-CHO mimic a natural substrate, occupying the specificity recognition sites of the caspase active site. They are covalent modifiers that exploit the catalytic function of the protease and trap the bound state. In contrast, the BIR2 amino-terminal peptide strand stretches across the substrate-binding cleft and (1) most of the specificity pockets remain unoccupied, (2) all possible carbonyls are too far from the catalytic cysteine for nucleophilic attack, and (3) it binds in a "back-to-front" manner.

In contrast with most natural protease inhibitors, including BIR2 of XIAP, the BIR3 domain does not directly target the active site of caspase-9, but abolishes activity using an entirely different mechanism. The inhibitory surface encompasses a helix found immediately following the BIR3 domain that packs against the dimer interface of caspase-9, converting it to the inactive monomer conformation with a collapsed active site **(109)**. This essentially reverses the mechanism of caspase-9 activation, which is an obligate dimer. XIAP is the first example of a natural protease inhibitor that uses this type of allosteric mechanism.

Despite the promiscuous nature with which caspases interact with inhibitors discussed here, their differing mechanisms of activation, and their involvement in diverse biological processes, the commonality among these complex proteases is their stringent specificity for aspartic acid in the P_1 positions of their physiological substrates.

Caspase Substrates

Substrate specificities of the caspases have been exhaustively studied using multiple *in vitro* approaches **(114–117)**. Substrate specificities for individual caspases can be generally defined as listed in Table 1.1. It should be emphasized that this classification is not absolute, as there are many examples of natural substrates in the literature that do not fit the generalized criteria. This reflects the fact that these studies use small synthetic substrates to define the specificity. However, the presence of the consensus sequence alone does not necessarily make a protein a caspase substrate; additional criteria need to be met. First, the recognition sequence must be in a region that has a certain degree of flexibility or disorder. For instance, the X-ray crystal structure or NMR-derived solution structures of procaspase-7 [a caspase-8 and -9 substrate **(38**, **39)**], Bid

Table 1.1 Synthetic caspase substrate specificity

Substrate	Caspase
DEVD	3, 6, 7, CED-3
IETD	8, 9, 10
LEHD	9
WEHD	1, 4, 5
YVAD	1, 4, 5
VDVAD	2

[a casapse-8 substrate **(118**, **119)**], and DFF45/inhibitor of caspase-activated DNAse (ICAD) [a caspase-3 substrate **(120**, **121)**] reveal that the cleavage sites are in mobile loops that do not have a defined structure. This is a common feature of protease substrates. Second, there may be a requirement for exosite interactions, in areas that are distant from the substrate-binding site, that serve to orient the substrate for docking into the active site and facilitate catalysis. Proteases within the blood coagulation pathway are well known for their use of exosites in the cleavage and activation of clotting factors **(122)**. Although this area of caspase biology is relatively understudied and there are no clear examples in the literature, there are some observations that suggest exosites may contribute to substrate recognition. For instance, although caspases-3 and -7 have identical specificities on small peptides (DEVD⇓A/G/S), their specificity on natural native substrates is different. Human ICAD is a better substrate for human caspase-3 than human caspase-7, whereas automodified PARP and pro-endothelial monocyte-activating polypeptide II are better substrates for caspase-7 than caspase-3 **(123–126)**. The significance of this difference is more palpable when considering that caspase-3 is at least 10-fold more catalytically active than caspase-7 on small synthetic substrates. Also, Bid is cleaved by caspsae-8 at $LQTD^{60}\Downarrow G$ but not at $IQAD^{75}\Downarrow S$, an equally accessible site, and preferentially cleaved by granzyme B in the same extended flexible loop **(127**, **128)**. Taken together, this implies the involvement of further inherent features in large native protein substrates, in addition to the recognition sequence, that contribute to catalysis.

Although we will not discuss specific substrates in detail, it is useful to highlight a few that demonstrate that the end result of caspase-mediated proteolysis in the apoptotic program can be classified as either "gain-of-function" or "loss-of-function" events. Cleavage of Bid at $LQTD^{60}\Downarrow G$ by caspase-8 results in translocation of truncated Bid to the mitochondria and induction of mitochondrial outer membrane permeability, or MOMP **(128**, **129)**. This is considered a gain-of-function event. On the other hand, cleavage of ICAD at $DETD^{117}\Downarrow S$ and $DAVD^{224}\Downarrow T$ by caspase-3 can be considered a loss-of-function event as ICAD loses its ability to bind and inhibit caspase activated DNase (CAD/DFF40) **(130–132)**.

There are over 400 caspase substrates identified to date. We refer readers to a recent review and the searchable online database The CASBAH (http://bioinf.gen.tcd.ie/casbah) **(133)**. Note that not all substrates identified

to date are critical for apoptosis; in fact, very few have been definitively proven essential for apoptosis. This is likely because no single proteolytic event is enough to kill the cell, but a critical mass of cleavage events is necessary to tip the balance toward death.

Conclusion

Taken together, caspases represent a unique and intriguing family of proteases that play a vital and highly regulated role in the apoptotic program. Yet their exceptional properties in regard to activation, inhibition, and substrate specificity allow them to act as distinct signaling proteases not only in programmed cell death, but also in an arising variety of additional vital pathways. We will undoubtedly see more exciting findings concerning these key regulatory proteases of the cell in the years to come.

Abbreviations

ced, *C. elegans* death proteins; CARD, caspase recruitment domain; DED, death effector domain; FasL, Fas ligand; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; FADD, Fas-associated protein with death domain; Apaf-1, apoptotic protease activating factor 1; AAA+, superfamily of ATPases associated with diverse cellular activities; NACHT, nucleotide binding oligomerization domain; Ipaf, ICE-protease activating factor; ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; PIDD, p53-induced protein with a death domain; RAIDD, receptor-interacting protein associated ICH-1/CED-3-homologous protein with death domain; DRONC, *Drosophila* Nedd2-like caspase; Bid, BH3-interacting domain death agonist.

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Chapter 2
The Bcl-2 Family Proteins

Wen-Xing Ding and Xiao-Ming Yin

Abstract The Bcl-2 family proteins are a group of evolutionarily conserved molecules that regulate apoptosis mainly at the site of mitochondria. This family of proteins consists of both antideath and prodeath molecules. The latter are also composed of multidomain prodeath molecules and the BH3-only prodeath molecules. While the BH3-only molecules act at the distal, receiving the death signals, the multidomain prodeath and antideath molecules regulate the mitochondrial outer membrane's permeability to control apoptosis. Protein interactions among the family members are important for their functions and have been explored for therapeutic purposes, as illustrated by the development of the BH3-only mimetics. Recent studies have also indicated that these molecules can act in other subcellular locations and their functions are beyond apoptosis regulation. Thus, the Bcl-2 family proteins also play important roles in autophagy, cell proliferation, and many other cellular functions.

Keywords Bcl-2 family proteins · Multidomain prodeath molecule · BH3-only molecule · BH3 mimetics · Mitochondria

Introduction

Apoptosis is an active process of cellular destruction with distinctive morphological and biochemical features. Two major apoptotic pathways have been defined in the mammalian cells: the death receptor pathway and the mitochondrial pathway. The Bcl-2 family proteins are the most important regulators of the mitochondrial pathway. Signals from the death receptor pathway could be also bridged to the mitochondrial pathway via the Bcl-2 family proteins. This family of proteins consists of both antiapoptosis and proapoptosis members.

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While the proapoptosis members serve as sensors to death signals and executors of the death program, the antiapoptosis members inhibit the initiation of the death program. The Bcl-2 family proteins are evolutionarily conserved but may accomplish these tasks by different mechanisms. In addition, multiple cellular signals can modify the activities and locations of these proteins, thus forming an intracellular signaling network that sets the delicate balance between cell death and survival.

Evolutionary Conservation of Bcl-2 Family Proteins

Bcl-2, the prototype of the Bcl-2 family proteins, was the first defined molecule involved in apoptosis. It was initially cloned from the t(14;18) breakpoint in human follicular lymphoma **(1–3)**. Although its role as a proto-oncogene was quickly realized, its biological function as an antiapoptosis gene was not realized until some years later **(4, 5)**. A number of proteins were soon discovered, which share sequence homology with Bcl-2, but only some of those possess the antiapoptosis activities. Others actually promote apoptosis (Table 2.1).

Notably, this family of proteins is evolutionarily conserved. A number of viruses encode Bcl-2 homologues, including most, if not all, gamma herpes viruses **(6)**. Most of these viral homologues are antiapoptotic, probably because viruses need to keep the infected cells alive for the latent and persistent infection **(6)**. Bcl-2-related genes can also be found in the sponge, sea urchin, and zebra fish **(7)**. The nematode *C. elegans* has its sequence and functional homologues for a death antagonist, CED-9 **(8)**, and a BH3-only death agonist, EGL-1 **(9)**. On the other hand, both prodeath homologue (dBorg-1/Drob-1/Debcl/dBok) and antideath homologue (dBorg-2/Buffy) have been described in *Drosophila* **(10)**. These homologues are discussed in details in Chapters 13 and 14, respectively.

One of the key features of the Bcl-2 family proteins is that members share sequence homology in four domains, the BH1, 2, 3, and 4 domains, although not all members have all the domains **(11–14)**, (Table 2.1, Fig. 2.1). Mutagenesis studies have revealed that these domains are important for function as well as for protein interactions among the family members. The BH1, BH2, and BH4 domains are necessary for the death repression function of antideath molecules, whereas the BH3 domain is required for the death promotion function of prodeath molecules **(15, 16)**.

Thus, the antideath members contain all four BH domains, whereas the prodeath molecules can be further divided into those with the BH3 domain only and those with BH1, BH2, and BH3 domains. It seems that the so-called BH3-only molecules, such as Bid, Bim, and Bad, are sensors for the peripheral death signals and are able to activate the "multidomain" prodeath molecules, Bax or Bak, either directly or indirectly, which in turn activate the mitochondria apoptosis program.

Table 2.1 Major functions of the mammalian Bcl-2 family proteins

Category	Molecule	Function	Refs.
Antideath: Multidomain (BH1, BH2, BH3, BH4)	Bcl-2 (1G5M)	Inhibits apoptosis, autophagy, and proliferation. Genetic deletion causes major phenotypes in the lymphoid system, the kidney, the melanocytes, and other cells.	**(7, 16, 146)**
	Bcl-xL (1R2D)	Inhibits apoptosis, autophagy, and proliferation. Genetic deletion causes embryonic lethality, abnormalities in fetal erythoid, and neuronal development.	**(7, 16, 143)**
	Bcl-w (1MK3)	Inhibits apoptosis. Genetic deletion causes male sterility.	**(150)**
	Mcl-1 (1WSX)	Important for the development of trophectoderm and hematopoietic stem cells.	**(144, 145)**
	Bfl-1/A1	Important for the survival of granulocytes and mast cells.	**(148, 149)**
	Bcl-B/Bcl-2L10/Nrh	Interacts with Bax, but not Bak, and suppresses only Bax-mediated cell death.	**(34, 36)**
Prodeath: Multidomain (BH1, BH2, BH3)	Bax (1F16)	Genetic deletion causes male sterility and abnormal neuronal death. Together with Bak, controls all mitochondria-mediated apoptosis. Also involved in ER-related function and cell proliferation.	**(125, 151)**
	Bak (2IMS)	No obvious defects in knockout mice, but combined deletion with Bax blocks all mitochondrial apoptosis.	**(125)**
	Bok/Mtd	Apoptotic effects could be suppressed by Mcl-1, BHRF-1, and Bfl-1, but not Bcl-2 or Bcl-xL, consistent with the heterodimerization pattern.	**(37, 190)**
Prodeath: BH3-only (classical)	Bad	A potential target in growth factor-mediated suppression of apoptosis. Involved in glucose-stimulated insulin resistance in pancreatic beta cells.	**(160)**
	Bid (2BID)	Is cleaved by proteases and links the death signals mediated by these proteases, such as those during death receptor activation, to the mitochondria.	**(101, 158, 159)**

Table 2.1 (continued)

Category	Molecule	Function	Refs.
		Genetic deletion causes resistance of hepatocyte to Fas-mediated apoptosis and disturbed myeloid homeostasis.	
	Bik/Nbk/Blk	Single genetic deletion does not cause any obvious defect. Concomitant loss of Bik and Bim in mice causes defective spermatogenesis.	**(7)**
	Bim/Bod	Important for apoptosis in the immune system; important for apoptosis induced by ER stress and many other agents.	**(140, 156)**
	Bmf	Not required for apoptosis induced by developmental process and UV irradiation, or for anoikis. Participates in glucocorticoid or HDAC inhibitor-induced apoptosis in lymphocytes.	**(161)**
	Hrk/DP5	May participate in neuronal death caused by NGF deprivation, but genetic deletion does not result in obvious phenotypes.	**(7)**
	Noxa/APR	Participate in cell death induced by irradiation, particularly by UV irradiation.	**(152, 154, 155)**
	PUMA/BBC3	Important for apoptosis induced by DNA damage, cytokine deprivation, and several other agents.	**(152–155)**
BH3-only (nonclassical)	Bnip3/Nip3	Mainly induces autophagic death, which requires its TM domain, not the BH3 domain. Participates in mitophagy during erythrocyte maturation.	**(167)**
	Nix/Bnip3L	Required for mitophagy during erythrocyte maturation.	**(165, 166)**
	Beclin1	Participates in autophagy induction, which may be suppressed by its interaction with Bcl-2, Bcl-xL, and Mcl-1. Does not induce apoptosis.	**(18)**
	Apolipoprotein L1 (ApoL1)	Induces autophagic death that requires the BH3 domain.	**(169)**
	Spike	Does not interact with any other Bcl-2 family proteins but the BH3 domain required for its	**(191)**

Table 2.1 (continued)

Category	Molecule	Function	Refs.
		killing activity. Resides at ER and interacts with Bap31 to suppress the interaction of the latter with Bcl-xL.	
	MULE/ARF-BP1	An E3 ligase that can bind to and ubiquitinate Mcl-1 and p53 for degradation.	**(177, 178)**

Notes: Protein Data Bank (PDB) identifiers are included in the parentheses for those molecules whose structures have been resolved. Other much less characterized Bcl-2 homologues include Boo/Diva, Mil-1/Bcl-RAMBO, Bcl-G, Bfk, and MOAP-1/MAP-1 **(7, 43, 192, 193)**. These molecules have atypical BH domains, a nonconventional combination of different BH domains, or a controversial function in regulating cell death. For discussion of the Bcl-2 family proteins in *C. elegans* and *Drosophila*, see Chapters 13 and 14. The nonclassical BH3-only molecules differ from the classical ones in that the former either do not induce apoptosis or do not interact with the antideath members.

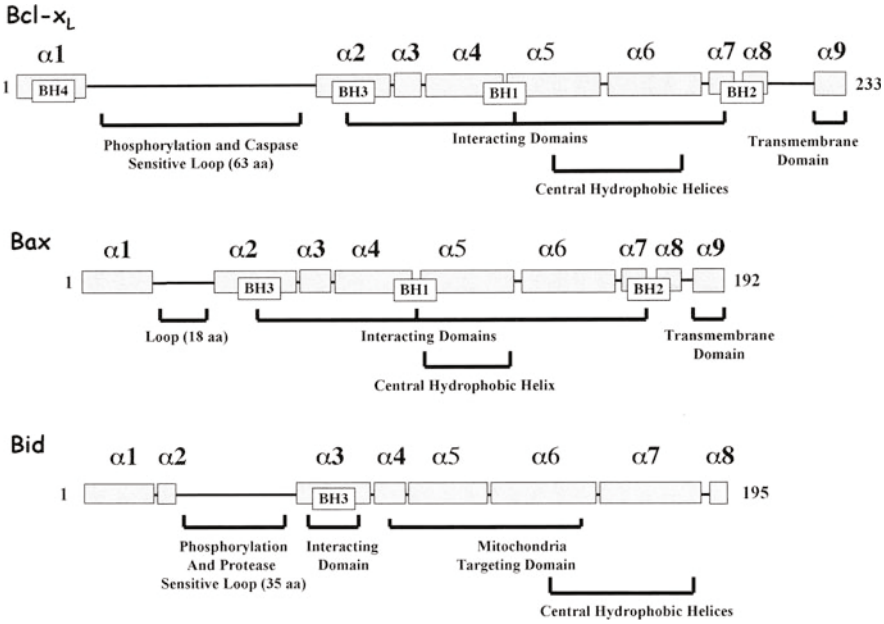

Fig. 2.1 Structural features of representative Bcl-2 family proteins. Bcl-xL, Bax, and Bid represent the three subgroups of the Bcl-2 family proteins: the multidomain antideath molecules, the multidomain prodeath molecules, and the BH3-only prodeath molecules (see Table 2.1). Bid is the only BH3-only molecule that has a structure similar to that of a multidomain protein. Other BH3-only molecules, such as Bim, Bad, and Bmf, are intrinsically unstructured. The structures of Bcl-2, Bax, and Bid are composed of multiple alpha helices with one or two central hydrophobic helices surrounded by six to eight amphipathic helices. The α7 helix of Bcl-xL **(194)** is also called the α6' helix **(78)**. A large, flexible, unstructured loop is present between the α1 and α2 helices. The loop is known to be a regulatory domain, sensitive to protease or kinase effects. The Bcl-2 homology domains 1-4 are distributed

The definition of BH3 domain was initially based on the homology to a nine-amino-acid domain present in Bax, Bak, and Bik/NBK/Bip1 **(12)**. Functionally, this domain is required for the interaction of the prodeath members with the antideath members and for their activity. Structural analysis indicated that it is contained within an amphipathic alpha helix **(17)** (Fig. 2.1). Thus, the definition of the BH3 domain has been broadly defined as a sequence that could form a four-turn amphipathic alpha helix containing the sequences motif A-X-X-X-A-X-X-A-B-C-X-A, where A represents hydrophobic residues, B represents a small residual, typically glycine, and C is Asp or Glu **(18-22)** (Fig. 2.1). The A and/or B residues form the hydrophobic side of the amphipathic helix. Detailed mutagenesis studies have indicated that residuals at the hydrophobic face have the greatest effects on heterodimerization and prodeath activity **(17)**. This loose definition may contribute to the identification of several nonclassical BH3-only molecules because of the lack of invariant residues. As the result, some of these BH3 molecules cannot interact with other Bcl-2 family proteins, such as Spike. Some of them may not promote apoptosis, such as Beclin1 and Bnip3, while others may promote cell death via completely different biochemical mechanisms, such as via ubiquitination-mediated degradation, as in the case of MULE/ARF-BP1 (Table 2.1). Thus, it seems that a distinctly new category of BH3-only molecules is emerging that have diverse functions.

Bcl-2 Family Protein Interactions and Their Functional Significance

Protein Interactions Among Bcl-2 Family Members

The Bcl-2 family proteins can interact with each other and also with other proteins. In fact, the first proapoptosis Bcl-2 family protein, Bax, was cloned based on its interaction with Bcl-2 **(23)**. Many other Bcl-2 family proteins were also cloned based on this type of interaction. A number of systems have been utilized to determine such interactions, including yeast two-hybrid, co-immunoprecipitation, phage expression library screening, and GST pull-down assay. More recently, peptides corresponding to the BH3 domain of various BH3-only molecules have been used to assess the variations in their interactions with the antideath members **(19–22)**. Interpretation of the protein-protein interactions based on *in vitro* systems can sometimes be complicated. For example, it was found that the *in vitro* interaction of Bax with Bcl-2 occurred only in the presence of certain detergents, which caused a conformational change in Bax

Fig. 2.1 (continued) over one or two alpha helices. They are involved in protein-protein interactions. Other alpha helices are involved in membrane binding (the TM domain in Bcl-xL and Bax, and the alpha helices 4-6 in Bid) or pore forming (the central hydrophobic alpha helices). The TM domain of Bax, but not that of Bcl-xL, was included in the structural analysis, which was shown to bind to the hydrophobic pocket formed by the BH1, BH2, and BH3 domains. This feature is confirmed with Bcl-w, for which the TM domain was also included in the structural analysis

(24). However, the authenticity of such interactions may be verified by an *in vivo* interaction system, such as the yeast two-hybrids, that does not involve the use of detergent **(25**, **26)**. In addition, interactions among family members, such as Bcl-2 and Bax, likely occur on membranes *in vivo*, where a proper conformation may be assumed as a result of activation by death signals **(24**, **27**–**30)**.

Based on these analyses, several interaction patterns can be defined [Fig. 2.2(B)]. The most common is the interaction between the antideath and prodeath members, such as Bcl-2 versus Bax **(23)** or Bid **(31)**. This can result in antagonistic action of the two types of molecules and thus can set a rheostat

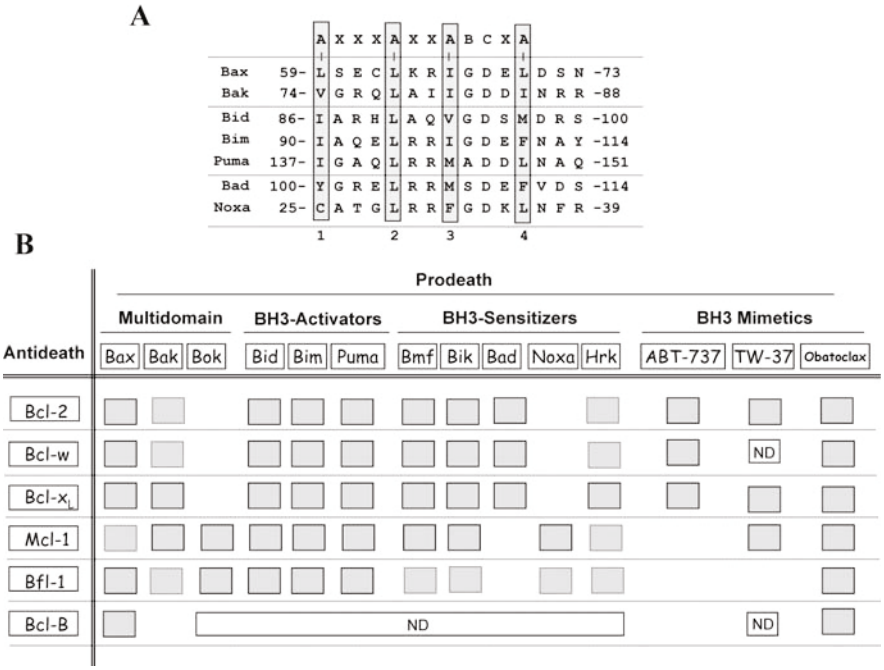

Fig. 2.2 Interactions between the antideath and prodeath Bcl-2 family proteins. (**A**) Sequence homology of the BH3 domain of representative prodeath molecules (human origin). Structurally, this domain forms an amphipathic α helix that has four turns, **(17–22)**. The four hydrophobic residues are important for the interactions and are represented by A, whereas B denotes a small residue and C denotes Asp or Glu. These seem to be the only signature of the BH3 domain. (**B**) Illustrated are the interaction patterns of six antideath molecules with three multidomain antideath molecules and eight classical BH3-only molecules **(19**, **21**, **22**, **25**, **33–35**, **37**, **132)**, which can be further divided into activators and sensitizers based on their ability to directly permeabilize mitochondria outer membranes. *Gray squares* indicate the interaction occurs between the selected members, such as that between Bak and Bcl-xL or Mcl-1. Squares with a *dotted line* indicate interactions observed in certain studies but not in others, or with some methods but not with other methods, thus suggesting the interactions could be weak. ND indicates that no data are publicly available at the moment. Small molecule BH3 mimetics have been developed, which can bind to different antideath members to competitively disrupt the endogenous interactions with the prodeath molecules to induce apoptosis **(132**, **180**, **186–189)**. See the text for a discussion of the relative strength of interactions

control of the death program **(32)**. Interestingly, not all antideath molecules can interact with all prodeath molecules. It seems that some members of one group will preferentially bind to some members of the other group. For the interaction between the multidomain prodeath molecules and the antideath molecules, Bax seems to be able to interact with all of them except Mcl-1 **(33, 34)**. Previously, Bax had been found to interact with Mcl-1 in yeast two-hybrid system **(25)**. On the other hand, Bak preferentially binds to Mcl-1 and Bcl-xL as a full-length molecule, although its BH3 peptide can bind to all antideath molecules except Bcl-B, in fluorescence polarization assay (FPA) **(34, 35)**. Functional studies support the FPA result; thus, Bak-overexpression-induced apoptosis can be suppressed by overexpressed Bcl-2, Bcl-xL, Mcl-1, or Bfl-1/A1, but not Bcl-B **(36)**. Finally, it has also been shown that Bok/Mtd binds to Mcl-1, BHRF-1, and A1, but not to Bcl-2 or Bcl-xL **(37)**. Correspondingly, these molecules may only antagonize the function of those molecules to which they bind.

The BH3-only molecules also show different binding capability to the different prosurvival members **(19–22)** [Fig. 2.2(B)]. In general, Bim and PUMA bind avidly to all the prosurvival proteins, whereas the other BH3-only molecules demonstrate a range of selectivity. Noxa may engage only Mcl-1, whereas Bad and Bmf bind mainly to Bcl-2, Bcl-xL, and Bcl-w. Bid, Bik, and HRK prefer Bcl-xL and Bcl-w over Bcl-2 or Mcl-1. Bad only binds to Bcl-2, Bcl-xL, and Bcl-w. Bim, PUMA, and Bid are consistently potent killers, as they are able to neutralize all prosurvival molecules, whereas a combination of Noxa and Bad is required to achieve maximal prodeath effects. These findings indicate that efficient apoptosis requires the neutralization of multiple prosurvival proteins.

The second type of interaction occurs between two members of the same functional group. In the prodeath group, one "BH3-only" molecule can interact with one "multidomain" molecule, such as Bid to Bax **(31)** or Bak **(38)**, and Bim_S or Bim_{AS} to Bax **(39)**. This can be important for the activation of the multidomain executioner molecules Bax or Bak by the "BH3-only" molecules **(20–22, 29, 38)** (see the section entitled "Activation of the Multidomain Bax and Bak at the Mitochondria"). Interactions between two members of the antideath group have also been reported, such as between Bcl-B and Bcl-2 or Bcl-xL **(36)**, although the significance is not clear. The third type of interaction is dimerization or oligomerization of the same molecule. This has been observed in both antideath molecules, such as Bcl-2 or Bcl-xL **(11, 25, 26, 40)**, and prodeath molecules, such as Bax and Bak **(38, 41)**. Oligomerization of Bax or Bak has been considered important for releasing mitochondrial apoptotic factors, such as cytochrome c **(38, 41)**.

The Importance of BH Domains in Bcl-2 Family Protein Interactions

BH domains are critically involved in protein interactions among family members, whereas the structures involved in homotypic interactions are less clear. In general, although the hydrophobic pocket formed by the BH1, BH2, and BH3

domains of the antideath molecules interact with the BH3 domain of the prodeath molecules, with the latter serving as the donor for the former's "pocket site," as suggested by structural studies **(7**, **11**, **12**, **31**, **42)**. Similarly, the domains involved in the interactions of two prodeath proteins, such as Bid and Bax, are the BH3 domain of the BH3-only molecule and likely the hydrophobic pocket formed by the BH1, BH2, and BH3 domains of the multidomain molecules **(31)**. Mutations at one of the domains can usually disrupt such interactions. Critical amino acids have been defined in each BH domain, such as Gly^{145} in the BH1 domain of Bcl-2, Trp^{188} in the BH2 domain of Bcl-2, and Gly^{94} in the BH3 domain of Bid **(11**, **31)**. However, it may be necessary to introduce mutations in all BH domains of a particular antideath molecule to disrupt its interaction with a prodeath molecule **(43)**. At other times, regions outside the BH domain may be required for interactions, such as the N-terminal region and the transmembrane domain in the case of Bnip3 **(44)**.

Interestingly, there is selective use of certain amino acids in one molecule when it interacts with different partners, which is of functional significance. For example, while Bcl-xL can bind to both BH3-only molecules, such as Bid and Bad, and multidomain molecules, such as Bax and Bak, certain amino acids (Phe^{131} and Asp^{133}) seem to be important for binding to the BH3-only molecules, but not to Bax **(45**, **46)**. The mutant (F131V, D133A) will bind to Bid, Bad, or Bim_L, but not to Bax. However, it retains the antideath function and indicates that it may block the activity of the BH3-only molecules **(46)**. In addition, variations in certain key amino acids may result in different affinities in binding to the same molecule, as indicated in two Bcl-2 isoforms for the binding to Bak- or Bad-derived BH3 peptides **(47)**.

The binding of antideath Bcl-2 family members to the prodeath death members may require the latter's BH3 domain to be exposed. This may not be an issue for binding to BH3-only molecules, such as Bid, which exposes its BH3 domain following protease cleavage **(48**, **49)**, or Bim, which does not seem to have a structural confinement **(30)**. However, this could be an issue for binding to the multidomain prodeath molecules Bax and Bak. The nonactivated conformer does not seem to bind to the antideath molecules **(22)**. The BH3 domain may need to be exposed in an activated conformation for the binding **(50)** (see ahead).

Interactions Between Bcl-2 Family Proteins and Other Molecules

Regulation of the Bcl-2 Family Proteins by Nonfamily Molecules

The Bcl-2 family proteins can also interact with many other proteins. While some of the interactions relate to their functions in cell death, others lead to different physiological activities. A number of proteins can sequester the prodeath Bcl-2 molecules to prevent them from activation. For example, 14-3-3ε binds to phosphorylated Bad and prevents it from translocation to the

mitochondria **(51)**. Similarly, Bax can be sequestered by 14-3-3ζ in the cytosol, which is liberated when the latter is phosphorylated by JNK **(52)**, or sequestered by Ku-70 **(53)**, which is liberated when the latter is acetylated **(54)**, a process that could be inhibited by SIRT1 deacetylase and stimulated by histone deacetylase inhibitors (HDAC). Other proteins that have similar functions include humanin, ARC, HSP70, and crystallins **(50)**. On the other hand, the dynein light chain complex 1 or 2 retains Bim_{EL}/Bim_L or Bmf in the microtubular dynein motor complex or actin filamentous myosin V motor complex, respectively **(55, 56)**. The mechanism of liberation is less clear in these cases.

Bak can be sequestered on the mitochondria by the antideath Bcl-2 members Mcl-1 and Bcl-xL **(35)** or by the nonfamily member VDAC2 **(22, 57)**. The displacement of these molecules by BH3-only molecules allows Bak to be activated. Similarly, Bak interaction with insulin-like growth factor-binding protein 1 (IGFBP1) may prevent its interaction and activation by p53 **(58)**.

Interactions with some of the other proteins can lead to direct activation of the prodeath Bcl-2 members. One example is the interaction of Bax and Bak with Bif-1, which seems to promote their oligomerization upon apoptotic stimulation **(59)**. Bax can also be activated by MOAP-1/MAP-1, which has a BH3-like domain that binds to Bax **(43)**. p53 is another well-studied activator. The tumor repressor p53 is able to promote apoptosis through its transcriptional activity to upregulate a number of prodeath molecules, such as PUMA, Noxa, Bid, Bad, and Bax (see Chapter 9). However, cytoplasmic p53 can also directly participate in the mitochondrial apoptosis pathway by binding to Bax **(60)** or Bak **(61)** to dissociate their interactions with the prosurvival molecules Bcl-xL and Mcl-1, respectively, leading to their oligomerization and cytochrome c release. In this capacity, p53 acts like an "activator" BH3-only molecule (see ahead). Interestingly, like Bax or Bak, p53 may also be "sequestered" and thus suppressed by the prosurvival Bcl-2 family molecules, such as Bcl-xL, and liberated by a BH3-only molecule, such as PUMA, via competitive binding to Bcl-xL **(62)**. Interestingly, PUMA is a transcriptional target of p53 and also promotes apoptosis by engaging in dissociating Bax/Bcl-xL interaction, thus allowing p53/Bax interaction.

As mentioned above, the binding of p53 with Bak can be blocked by a non-Bcl-2 family protein, IGFBP1, in hepatocytes due to competitive binding **(58)**. It is thought that this mechanism may explain why hepatocytes are, in general, insensitive to p53-mediated apoptosis following genotoxic stress. Remarkably, IGFBP1 is also a transcriptional target of p53, thus constituting a differential mechanism to selectively block the apoptosis function of p53 without affecting its other functions. The coordinated actions of the nuclear and cytoplasmic p53 are also well illustrated in the case of Bad, which is a transcription target of p53 **(63)**. But Bad can also bind to cytoplasmic p53 to prevent it from entering the nucleus. Instead, the complex is redirected to the mitochondria, where Bak is then activated by the complex under genotoxic stress.

One dramatic effect caused by the nonfamily binding partner was the functional conversion of the family member. An orphan nuclear receptor,

Nur77/TR3, can convert Bcl-2 or Bcl-B into a prodeath molecule upon interaction, perhaps by inducing conformational change that leads to exposure of the BH3 domain **(64, 65)**.

Participation in Multiple Functions via Binding to Different Molecules

Bcl-2 family proteins may bind to other proteins to regulate their activity, thus indirectly regulating cell death, or to affect cell death. For example, CED-9 binds to CED-4 to prevent it from activating the caspase CED-3 **(66)**. Bcl-xL may bind to BAR to regulate the activity of caspase-8 **(67)** and to Aven to regulate the activity of Apaf-1 **(68)**. Bcl-2 or Bcl-xL may also bind to Bap 31 at the site of the endoplasmic reticulum to regulate the activity of caspase-8 **(69)**. Recently, Bcl-2 family proteins have been found to affect the mitochondrial morphology associated with apoptosis, which can be related to protein-protein interactions, such as that between Bak and Mfn1 or Mfn2 **(70)**, or between protein-lipid interactions, such as that between Bid and cardiolipin **(71)**.

Interaction of Bcl-2 with calcineurin may be responsible for Bcl-2-mediated inhibition of cell cycle progression **(72)**. In addition, some Bcl-2 molecules can reside in the nucleus, which can be increased following ionizing radiation treatment. Nuclear Bcl-2 can interact with both Ku70 and Ku86 via its BH1 and BH4 domains to inhibit the nonhomologous end-joining pathway, which may lead to an accumulation of DNA damage and genetic instability **(73)**. Bcl-2 can interact with IP3 receptor 1 to affect calcium homeostasis in the ER **(74)**, and Bax and Bak can interact with IRE-1α to regulate the unfolded protein response (UPR) following ER stress **(75)**. Finally, Bcl-2 and Bcl-xL can interact with NALP1 to suppress NALP-1-mediated caspase-1 activation, which is required for the generation of the inflammatory cytokine interleukin-1 **(76)**. Thus, Bcl-2 and Bcl-xL can regulate the inflammation process. In addition, this relationship among Bcl-2/NALP-1/casaspe-1 is analogous to that of CED-9/CED-4/CED-3 in which the Bcl-2 family proteins (Bcl-2, CED-9) are connected to the activation of caspases (caspase-1, CED-3) via the link of a molecule that has a CARD domain and a nucleotide-binding oligomerization domain (NALP-1, CED-4), indicating an evolutionary conservation in the mechanism.

The association of the BH3-only molecule Bad with a non-Bcl-2 family protein, glukokinase (hexokinase IV), on the mitochondria actually promotes a survival function by enhancing its activity. Notably, this activity is associated with glucose-mediated survival signaling that leads to Bad phosphorylation and inactivation of its prodeath function **(77)**. This activity seems important for normal glycolysis and glucose homeostasis. Another BH3-only molecule, Beclin1, while not participating in apoptosis, is important for autophagy induction through its interactions with a large number of proteins, including the Class III PI-3 kinases VPS34, UVRAG, and Ambra-1, in addition to its interaction with the prosurvival Bcl-2 and Bcl-xL (see Chapter 29).

The Crystal and Solution Structures of the Bcl-2 Family Proteins

The crystal and solution structures of several Bcl-2 family proteins (i.e., Bcl-xL, Bcl-2, Bcl-w, Mcl-1, Bax, Bak, and Bid) have been defined [see the review **(7)** and also Chapter 4]. One of the common structural features is that these proteins are all composed of alpha helices and assume an overall similar conformation. These alpha helices consist of two central hydrophobic helices surrounded by multiple amphipathic ones. Such an arrangement of alpha helices is similar to that of the membrane translocation domain of bacterial toxins, in particular diphtheria toxin and the colicins, and suggests that the Bcl-2 family proteins may be capable of forming pores. Indeed, Bcl-xL, Bcl-2, Bax, and Bid have been shown to possess ion channel activities *in vitro* on lipid bilayers or liposomes. This activity may relate to the function of these molecules on regulating mitochondrial permeability. In addition, the BH1, BH2, and BH3 domains of the multidomain proteins form a hydrophobic pocket that is the binding site for the BH3 domain of another molecule. The hydrophobic pocket may be further stabilized by the BH4 domain.

Some viral proteins, such as Ks-Bcl-2, M11L, and N1L, have a helical fold similar to that of Bcl-xL **(78, 79)**. Interestingly, only Ks-Bcl-2 has some sequence homology to Bcl-2; neither M11L nor N1L has sequence similarity to Bcl-2 family proteins. However, they can all inhibit apoptosis by binding to Bax/Bak. These studies indicate that structural homology may be the most important feature conserved in evolution.

Despite the similarities, differences do exist. For example, for the solution structures of Bcl-2 and Bcl-xL, differences in the structural topology and electrostatic potential of the hydrophobic pocket can be detected, consistent with the finding that the two molecules have different affinities to various interacting molecules **(42, 47, 78)**. Such a difference even exists between two different isoforms of human Bcl-2 **(47)**, and between the human Bcl-2 and its viral homologue, KSHV Bcl-2 **(78)**. These variations within an overall conserved structure are compatible with the conserved antiapoptosis function but are also indicative of an emphasis on different strategies to achieve this function.

The multidomain prodeath molecules Bax and Bak have an overall structure very similar to that of the prosurvival molecules Bcl-2, Bcl-xL, Bcl-w, and Mcl-1. As of this publication, it is not clear whether and how their opposite function is affected by their structural similarity. Alternatively, these two groups of proteins may not differ in their fundamental activity in engaging the membranes, instead differing in the consequence depending on the activity of other molecules, such as the BH3-only molecules, the exposure of the BH3 domain, or the status of oligomerization during the membrane-dependent conformational change **(27)**.

Structures of Bax and Bcl-w include the C-terminal transmembrane domain. Interestingly, they adopt a conformation similar to that of C-terminal truncated

Bcl-xL binding to a Bak BH3 peptide **(7, 42)** (see also Chapter 4). Here the transmembrane domain of these molecules has actually occupied its own hydrophobic pocket formed by the BH1, BH2, and BH3 domains.Bax needs to be activated for its proapoptotic function through conformational change **(24, 29)**. It is likely that the solution structure of an activated Bax would be quite different from that of a quiescent Bax. The transmembrane domain of Bax may be released when Bax changes its conformation, thus freeing the hydrophobic pocket for interaction with other Bcl-2 family proteins, such as Bid, and/or exposing the BH3 domain to exercise the prodeath function. Similarly, the interaction of a BH3-only molecule with the prosurvival molecule may allow the displacement of the transmembrane domain, which becomes exposed and targets the molecule to the membrane location. The reverse process may also be possible, in which conformational changes related to membrane translocation trigger the release of the transmembrane domain, making the hydrophobic pocket available for binding to the BH3-only molecules. Interestingly, cytosol to mitochondria translocation occurs for Bcl-xL, Bcl-w, Mcl-1, and Bax upon apoptosis induction **(7)**.

Among the BH3-only molecules, only Bid retains a conserved structure similar to that of the multidomain molecules **(48, 49)**. This feature may confer to Bid some activities that may not be shared with other BH3-only molecules, such as the pore-forming activity **(80)**, which may in turn contribute to its apoptotic function. However, one major structural difference between the multidomain proteins and Bid is that the hydrophobic pocket formed by the BH1, BH2, and BH3 domains is not present in Bid. This may permit a quick exposure of the BH3 domain upon activation, such as by proteolysis to remove the N-terminus **(15, 48)**. On the other hand, Bad, Bmf, and Bim are intrinsically unstructured but are subjected to localized conformation upon interaction with Bcl-2 **(30)**. For example, when Bim binds to the prosurvival Bcl-2 members, only the BH3 element becomes structured, while most residues remain disordered. Together, these observations indicate that the BH3 domain of Bid or any other BH3-only protein may function as a "donor" in its interaction with the multidomain proteins, whose hydrophobic pocket can serve as an "acceptor." Structural studies of antideath members interacting with a BH3 domain peptide support this notion **(7, 42)** (see also Chapter 4).

It is not clear how the multidomain prodeath molecules Bax and Bak will change their conformation to allow their BH3 domain to be exposed, which seems to be "locked" with their BH1 and BH2 domains in the pocket site. The conformational change may be achieved upon membrane insertion **(27)**. It is interesting to note that if the BH3 domain is made freely accessible, the molecules may acquire active killing ability via the engagement of this domain. It may very likely be the case that when Bcl-2 or Bcl-xL is cleaved by caspase-3 to remove the N-terminal region **(81, 82)**, or when Bcl-2 or Bcl-B undergoes conformational change upon interaction with Nur77/TR3 **(64, 65)**, they are endowed with apoptotic activity.

The differences in the structure as well as sequence of the various BH3-only molecules may suggest a diverse origin and evolution of these molecules and indicate that they may be further divided into the core group (Bcl-2, Bcl-xL, Bcl-2, Mcl-1, Bax, Bak, and Bid) that shares both the sequence and structure homology, and the group that differs either in the structure, such as Bim and Bad, or in the sequence, such as the viral proteins M11L and N1L. This may further imply that nonconserved functions may be expected among these molecules (see the upcoming section entitled "The Physiological Roles of the Bcl-2 Family Proteins").

Regulation of the Bcl-2 Family Proteins

Regulation of Expression

Because of the potent effects of Bcl-2 family proteins on the balance between life and death, cells impose strict regulations on the expression and activity of these molecules. While certain antideath or prodeath molecules are expressed constitutively in cells, others are expressed only following death stimuli. This is particularly true for a number of proapoptosis molecules. For example, DNA damage can induce the expression of PUMA, Noxa, Bid, and Bad in a p53-dependent manner **(63**, **83–86)** (see also Chapter 9). The upregulation of prodeath molecules can also be developmentally regulated, such as EGL-1, which is required for the death of the HSN neurons in the male *C. elegans* **(9)** (see Chapter 13). Similarly, deprivation of nutritional factors can also induce the expression of proapoptosis molecules. For example, Hrk/DP5 or Bim_{EL} can be induced in cultured sympathetic neurons following NGF withdrawal **(87)**. While the death signals for the upregulation of the BH3-only molecules can be specific, those that can upregulate the multidomain molecule Bax are often more diverse, indicating the central position of this prodeath molecule (see the upcoming section entitled "Molecular Mechanisms of the Control of Apoptosis by the Bcl-2 Family Proteins").

The expression of antiapoptosis molecules can be induced by survival signals or inflammatory signals, which may occur in a cell-specific or time-specific manner. For example, Mcl-1 can be upregulated by GM-CSF in myeloid cells **(88**, **89)** and Bfl-1/A1 can be induced in endothelial cells or neutrophils in response to the phorbol esters LPS, TNFα, IL-1, or G-CSF **(90–92)**. The expression of Bcl-xL and Bcl-2 in thymocytes and matured T cells is a good example of how homeostasis can be maintained by differential expression of these genes in a temporal-specific manner **(93)**. Thus, Bcl-xL, but not Bcl-2, is preferentially expressed in the immature CD4/CD8 double positive cells. On the other hand, Bcl-2, but not Bcl-xL, is expressed in the matured CD4 or CD8 single positive cells. However, the expression of Bcl-xL is upregulated in the activated matured T cells. This probably allows the activated cells to survive for their immune functions **(94)**.

Regulation Through Alternative Splicing

A puzzling fact of the regulation of the Bcl-2 family proteins is the alternative splicing. A number of these proteins, including pro- and antideath members, can be expressed in different forms. For some the differentially spliced forms can have opposite functions, such as Bcl-xL versus Bcl-xS **(95)** and Mcl-1L versus Mcl-1S **(96)**. For others, alternative splicing does not alter the prodeath or antideath nature of the product, but their potency. The longer form of Bcl-2, Bcl-2α, is more potent than the short form of Bcl-2β **(97)**, whereas the short form of Bim, Bim_S, is much more potent than the long form, Bim_L, or the extra-long form, Bim_{EL} **(98)**. Bim_S and another newly defined Bim splicing variant, BimAD, may also activate the mitochondria by different mechanisms (see the upcoming ection "Activation of the Multidomain Bax and Bak at the Mitochondria"). It is not clear how the alternative splicing is regulated. Tissue-specific or signal-specific mechanisms may be involved. For example, Bcl-GL is widely expressed, but Bcl-GS is only found in the testis **(99)**. Thus, it is possible that splicing variants could regulate apoptosis in a temporally and spatially specific way.

Regulation Through Posttranslational Modifications

Posttranslational modification is probably the most significant mechanism to regulate the activities of the Bcl-2 family proteins. This is particularly important for those prodeath molecules that are normally expressed in healthy cells. These modifications often occur in response to death or survival signals and mainly include proteolytic cleavage and phosphorylation. In addition, protein conformational changes or degradation could be also induced as a posttranslational event.

Change of Subcellular Locations Resulting from Posttranslational Modifications

One of the main outcomes of the posttranslational modifications is the translocation of the modified death agonists to the mitochondria, as in the case of Bax, Bid, Bim, and Bad. In these cases, the Bcl-2 family proteins serve as sensors to the external death signals and transmit those signals to the mitochondria.

The first type of posttranslational modifications is conformational change, which, for Bax, is the first step in response to death signals **(24**, **28)**. This change may be due to an elevated cytosolic pH **(100)**. Cellular alkalinization may alter the ionization of key amino acid residues at the N- and C-termini, thus breaking the intramolecular interactions maintained by the ionic force. The conformational change allows the exposure of the two termini, and the availability of the hydrophobic C-terminus now gives the molecule the ability to target the mitochondrial membrane **(100)**. Translocation of Bax can also be

regulated by several Bax binding partners, such as 14-3-3ζ **(52)** and Ku70 **(53, 54)**, upon phosphorylation or acetylation (see the earlier section on interactions between Bcl-2 family members and other molecules). The insertion of Bax seems to be also greatly facilitated by the presence of another prodeath molecule, Bid, which may induce further conformational change of the molecule **(101)**.

Translocation of Bid is dependent on caspase cleavage, which is the second type of posttranslational modification **(101)**. Bid can be activated by multiple proteases in various apoptosis scenarios **(101)**. The cleavage occurs at the so-called loop region (aa 43-77) (Fig. 2.1), which is also susceptible to cleavage by granzyme B (Asp^{75}) and lysosomal enzymes (Arg^{65}). The 15-Kd carboxy-terminal cleaved fragment of Bid (aa 60-195), called tBid, can be further myristoylated at Gly^{60} near the N-terminus **(102)**. The modified Bid can now efficiently target mitochondria. This newly acquired ability may be due to the appearance of a large hydrophobic surface, which was previously buried, but revealed by the protease cleavage **(15, 48)**. The changes in hydrophobic exposure and the related surface charges, together with the myristoylation, contribute to the translocation and integration of tBid into the mitochondrial membranes.

The third type of posttranslational modification that results in subcellular translocation is phosphorylation and dephosphorylation. For example, phosphorylated Bcl-2 is largely present in the ER and correlates with the change in Bcl-2's ability to regulate the calcium balance in the ER **(103)** (see the upcoming section entitled "Regulation of Apoptosis at the Endoplasmic Reticulum"). However, Bad is the best studied for this regulatory event. In the presence of growth factor, such as IL-3, Bad can be phosphorylated **(51)**. Bad has several phosphorylation sites, but phosphorylation at Ser^{136} and Ser^{112} regulates its subcellular location. While Ser^{136} seems to be mainly phosphorylated by Akt/PKB **(104, 105)**, Ser^{112} is phosphorylated by a cAMP-dependent kinase **(106)**. When phosphorylated, Bad binds to a cytosolic protein, 14-3-3, and is trapped in the cytosol. Subsequent to a death stimulus, such as IL-3 deprivation or calcium influx, dephosphorylation occurs through certain phosphatases, such as calcineurin **(107)**. Dephosphorylated Bad disassociates from 14-3-3 and translocates to the mitochondria, contributing to cell death **(51)**. Phosphorylation of Bad at other sites, Ser^{155} and Thr^{201}, can affect other aspects of Bad's function (see the next section).

There are other mechanisms to induce translocation of Bcl-2 family proteins. For example, translocation of Bim_{EL}/Bim_L from the microtubule-associated dynein motor complex to the mitochondria can be induced by cytokine withdrawal, taxol, or UV irradiation **(56)**. Another BH3-only molecule, Bmf, can be activated by anoikis or UV irradiation. It is released from the myosin V motor complex and translocated to the mitochondria **(55)**. In both cases, it seems that some noncaspase proteases are involved to release these molecules from their normal location in cells.

Change of Functions Resulting from Posttranslational Modifications

Both Bcl-2 and Bcl-xL can be phosphorylated by death stimuli. The chemotherapeutic drug taxol is well known for its ability to inactivate Bcl-2 by inducing its phosphorylation **(108**, **109)**. The phosphorylation occurs on serine residuals in the loop region between the first and second alpha helices **(108–110)** (Fig. 1.1). The phosphorylation results in decreased antiapoptotic activities of Bcl-2 and Bcl-xL **(109)**, because their ability to interact with Bax **(103**, **111)** and/or to regulate ER calcium balance is suppressed **(103)**. Phosphorylation of Bcl-2 also inactivates its antiautophagy function. Bcl-2 phosphorylation by JNK dissociates its interaction with the proautophagy molecule Beclin1 and reduces its ability to inhibit Beclin1-mediated autophagy **(112)**. Consistent with other findings, phosphorylation happens to Bcl-2 at the endoplasmic reticulum (ER), where its ability to regulate apoptosis **(103)** or autophagy **(113)** is affected by this modification.

Both Bcl-2 and Bcl-xL may also be subject to caspase cleavage **(81**, **82)**. Caspase-3 is the main caspase that cleaves these molecules, again at the loop region. The cleavage does more than just inactivate the function of these molecules; it actually bestows apoptotic activity to them. Thus, the C-terminal fragment of Bcl-2 or Bcl-xL (tBcl-2 or tBcl-xL) is able to induce apoptosis. Furthermore, cleavage-resistant molecules, which are engineered through site-directed mutagenesis to delete the caspase-3 recognition site, possess stronger antiapoptotic activity **(81**, **82)**. It seems that the altered tBcl-2 or tBcl-xL may not contribute to cell death at the early initiation stage, since the modification occurs only after caspase activation. However, these truncated molecules can further accelerate the death process.

In another case, phosphorylation of Bad can cause the molecule not only to be trapped in the cytosol by 14-3-3 (see the preceding section), but also to be inactivated. The latter was accomplished through phosphorylation at Serine^{155}, which is in the middle of the BH3 domain, by protein kinase A **(114)**. This event is simulated by growth factors and requires the prior phosphorylation at Ser^{136}, which leads to Bad being trapped by 14-3-3 (see above). However, Ser^{155} phosphorylation suppresses Bad-Bcl-xL interaction, thus further restricting it from engaging in apoptosis **(114**, **115)**. Interestingly, growth factor deprivation, while causing Bad dephosphorylation at the three serine sites (Ser^{112}, Ser^{136}, Ser^{155}), can also activate JNK, which in turn phosphorylates Bad at Thr^{201} **(116)**. This phosphorylation event has the same effect as Ser^{155} phosphorylation to suppress Bad-Bcl-xL interaction, providing another way to inhibit apoptosis. Notably, phosphorylation at Ser^{155} or Thr^{201} can engage Bad in nonapoptotic function related to glycolysis **(77**, **117)** (see the upcoming section entitled "Role in Other Physiological Functions"), thus serving as a functional switcher.

Bid is another BH3-only molecule that can be regulated by phosphorylation. It can be phosphorylated by Casein Kinase I or II, which results in the Bid's resistance being cleaved by caspase-8 **(118)**. Phosphorylation-resistant mutant (S61A, S64A) was more cytotoxic than the wild-type molecule, reflecting that

this type of phosphorylation could probably be physiologically relevant. Phosphorylation of Bid at the same sites by ATM during DNA damage or replication stress could allow Bid to perform a function at the intra-S phase checkpoint and cause S phase arrest **(119**, **120)**. This function of Bid's has yet to be more firmly established because disputes have arisen **(121)**.

Molecular Mechanisms of the Control of Apoptosis by the Bcl-2 Family Proteins

The Bcl-2 family proteins regulate apoptosis mainly via their effects on mitochondria. They can also be found at the endoplasmic reticulum and thus can regulate the contributions of these organelles to apoptosis. The activation of the mitochondrial pathway is signified by the release of mitochondrial apoptotic proteins and by mitochondrial dysfunction **(7**, **15**, **16**, **122–124)**. Both processes are inhibited by the death antagonists (Bcl-2, Bcl-xL, etc.) but are promoted by the death agonists (Bax, Bak, Bid, Bim, etc.). A detailed discussion of the mitochondria's activation and mechanisms can be found in Chapter 6. Briefly, the release of the mitochondrial apoptotic proteins results from an increase in the outer membrane's permeability, which may be due to opening of the pore formed by Bax or Bak, the mitochondrial permeability transition pore, or a pore made from components of the two. The mitochondrial dysfunction is often characterized by the mitochondrial depolarization and ROS generation, which are in part contributed by the loss of cytochrome c. Recent studies have also defined the significant impact of the Bcl-2 family proteins on mitochondrial morphology by regulating the fission and fusion processes, therefore affecting the mitochondrial apoptosis process, such as cytochrome c release (see Chapter 6).

Activation of the Multidomain Bax and Bak at the Mitochondria

The multidomain prodeath proteins Bax and Bak are responsible for the induction of the mitochondrial outer membrane permeabilization. Deletion of both Bax and Bak, but not one of them, renders the cell completely resistant to all major mitochondrial death signals, including DNA damage, growth factor deprivation, and endoplasmic reticulum stress, and to the extrinsic pathway signals mediated by Bid **(46**, **125–127)**. We must point out, however, that Bax and Bak may not completely overlap in their functions. In many cases, it seems that Bax is more sensitive to apoptotic stimuli than Bak. In the human colon carcinoma cell line HCT116, which has mismatch repair deficiency, apoptosis induced by nonsteroidal antiinflammatory drugs or TRAIL is dependent on Bax, but not Bak **(128**, **129)**. In addition, the genetic deletion of Bax alone is sufficient to render sympathetic neurons resistant to NGF-deprivation-induced

apoptosis **(130)**. In other cases, Bak, but not Bax, is required for apoptosis induced by conditions such as protein synthesis inhibition **(131)**. One may keep in mind that Bax is usually localized in the cytosol in healthy cells and translocated to the mitochondria in response to death stimuli. But Bak constitutively resides in the mitochondria. This distinction may contribute to the differential stimulation of Bax and Bak in certain cases. Another implication is that since Bax and Bak have a different interaction profile with antideath molecules (Fig. 2.2), induction of Bax- or Bak-dependent apoptosis would have to be regulated by a distinct set of molecules. For example, Bcl-xL, Mcl-1, Noxa, and Bik would be particularly important for modulating Bak-mediated apoptosis **(35, 131)**.

The BH3-only proteins clearly act upstream of Bax and Bak, because they cannot induce apoptosis in cells lacking both Bax and Bak **(46, 126, 127)**. The different BH3 molecules serve as sentinels to different apoptosis signals **(16)**. For example, PUMA and Noxa are mainly responsible for the DNA damage-induced apoptosis, Bid is responsible for protease signals, and Bad and Bim are engaged in apoptosis induced by growth factor deprivation and cytokine deprivation. How Bax and Bak are activated by the BH3-only molecules and how the antideath molecules inhibit the activation are not entirely clear. Currently, two models, a direct activation model and an indirect activation model, have been proposed to explain how the BH3-only molecules can activate Bax or Bak, based on whether or not they interact with each other **(7, 22, 33, 50, 132, 133)** (Fig. 2.3).

In the direct model, the BH3-only molecules can directly activate the multidomain molecules Bax and Bak to initiate the mitochondrial events **(20–22, 38, 41)**. They are thus termed *activators*, which includes Bid, Bim, and PUMA **(20–22)**. Bid can interact with Bax, Bak, and Bcl-2 **(29, 31, 38)**. But its apoptosis-inducing capability is dependent on its ability to interact with the prodeath multidomain molecules, but not the antideath Bcl-2 or Bcl-xL **(22, 31)**. Although Bcl-xL can interact with Bid, Bax, and Bak, its effects consistently seem to be more dependent on its binding to Bid rather than to Bax, based on the use of Bcl-xL mutants that can differentially bind to Bid and Bax **(45, 46)**. Conversely, Bax or Bak mutants that could not bind to Bcl-2, Bcl-xL, or Mcl-1 are still susceptible to suppression by these molecules, indicating that these antideath molecules function by not directly antagonizing Bax or Bak **(22)**. Instead, they bind to Bid, Bim, or PUMA to prevent them from activating Bax and Bak. Other BH3-only molecules, such as Bad, Noxa, and Bmf, can bind to the antideath molecules to liberate the bound Bim, Bid, or PUMA so that the latter can activate Bax or Bak **(22)**. The latter group of BH3-only molecules is called *inactivators* **(22)** or *sensitizers* **(132)**. Notably, the ability of the inactivator to dissociate the activator BH3-only molecules from the binding with the antideath Bcl-2, Bcl-xL, or Mcl-1 is dependent on its affinity to interact with the latter. Thus, while Bad could liberate Bid, Bim, and PUMA from interaction with Bcl-2 or Bcl-xL, it cannot liberate them from interaction with Mcl-1. On the other hand, Noxa works just the opposite. Bik and BMF can only liberate

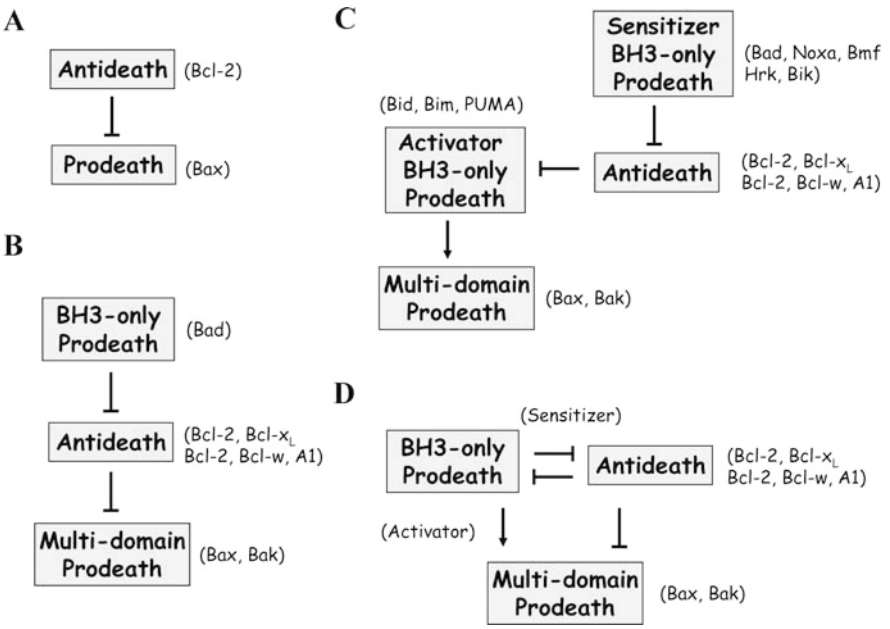

Fig. 2.3 Models for the activation of multidomain prodeath Bcl-2 family proteins. Genetic studies indicate that the multidomain prodeath Bcl-2 family proteins Bax and Bak are required for the mitochondria-mediated apoptosis, which is regulated by other family members. Since there are extensive and diverse interactions among the family members (see Fig. 2.2 and the text), how these molecules activate or suppress each other in response to different death signals is not entirely clear. The first proposed model (**A**) was based on two molecules, Bcl-2 and Bax **(32)**. This rheostat model indicates that Bcl-2 suppresses apoptosis by heterodimerizing with Bax, whereas Bax homodimers kill the cell. Thus, the relative expression levels of Bcl-2 versus Bax dictate the cell's fate. With the discovery of more family members and in particular, the BH3-only molecules, the interaction pattern becomes convoluted and so is the mechanism of activation. Essentially, two models, the indirect model (**B**) and the direct model (**C**), have been proposed, which differ mainly in how Bax and Bak are activated and whether the multidomain or BH3-only prodeath molecules are sequestered by the antideath molecules **(7**, **22**, **33**, **50**, **132**, **133)**. There are experimental observations that could not be explained by either model alone. Therefore, it is possible to propose a model that combines the elements of the two (**D**). See the text for a detailed discussion

Bid from the interaction with Bcl-2 or Bcl-xL. These types of relationships, together with the different ability of the antideath molecules in suppressing apoptosis induced by different signals, signify the importance of understanding the specific relationship of the Bcl-2 family proteins in individual cases. A further distinction of the antideath molecules that are bound with the activator BH3-only molecules (called "primed") from those without the binding partners (called "empty") may have clinical significance in that only inactivation of the "primed" but not the "empty" antideath molecules, e.g., with the corresponding inactivator/sensitizer BH3-only molecules, may be expected to cause subsequent death due to the release of the activator BH3-only molecules **(132**, **134)**.

The direct model may not explain all the phenomena. Thus, the indirect model argues that once the BH3-only molecules have been translocated to the mitochondria, they may not affect Bax or Bak directly but rather bind to the antideath Bcl-2 family proteins to antagonize their function or to convert them to Bax- or Bak-like molecules for oligomerization with Bax or Bak **(7, 33, 50)** [Fig. 2.2(b)]. In this case, Bax or Bak is in check by the antideath molecules, which are deactivated by the BH3-only molecules. BH3-only molecules work as sensitizers only in this model. Perhaps the best evidence to support the activation of Bax or Bak by dissociation from the antideath molecules without the involvement of the activator BH3-only molecules is that cells deficient in Bid and Bim, and with reduced PUMA, are still susceptible to apoptosis **(33)**. Another piece of evidence is that in *C. elegans*, the BH3-only molecule EGL-1 binds competitively to the antideath molecule CED-9 so that CED-4 is released from the binding with CED-9 to activate the caspase homologue CED-3 **(66)**.

The major difference between the two models is whether Bax or Bak is directly activated by a subset of BH3-only molecules (Bid, Bim, and PUMA) (direct model) or indirectly activated due to the dissociation from the antideath molecules (indirect model). Conversely, they differ in whether the antideath Bcl-2, Bcl-xL, or Mcl-1 is sequestering/inactivating Bax and Bak (indirect model) or a subset of BH3-only molecules (Bid, Bim, and PUMA) (direct model). Finally, the sensitizer BH3-only molecules deactivate the antideath Bcl-2, Bcl-xL, and Mcl-1 in both models, but the resulting mechanisms are not considered the same in activating Bax and Bak. In reality, it is likely that events described in both models can occur. Direct activation of Bax and Bak by the activator BH3-only molecules and direct association of antideath molecules with Bax and Bak are both concrete findings. In addition, recent studies using BH3 mimetics demonstrate that both types of prodeath molecules can be desequestered from the complex with the antideath molecules from cancer cells. For example, ABT737 and GX15-070 (Obatoclax) (see the upcoming section entitled "Role of the Bcl-2 Family Proteins in Cancer Biology and the Development of BH3-Based Therapeutic Strategies") could cause the release of Bim from the complex with Bcl-2 or Bcl-xL **(134-136)** and the release of Bak from Bcl-xL or Mcl-1 **(135, 136)**. These results suggest that the antideath molecules are able to sequester both types of prodeath molecules and that activation of Bax or Bak can be caused either by the activator BH3-only molecules, such as Bim, or by the derepressed "spontaneous" oligomerization, depending on individual cellular context [Fig. 2.3(d)].

The indirect activation model implies that Bax and Bak may be capable of "spontaneous" activation or may be preactivated by other molecules to convert into "primed conformation," which is, however, suppressed by the antideath members until the BH3-only molecules inactivate the latter. This model suggests that Bax or Bak may have two conformers in cells, primed with their BH3 domain exposed and unprimed with their BH3 domain hidden. Antiapoptotic Bcl-2 members such as Bcl-2, Bcl-xL, and Mcl-1 bind the primed conformer, as

the BH3 domain of Bax or Bak is required for this interaction **(50)**. Although the signal for priming is not known yet, it is possible that the membrane environment where these molecules reside could provide the condition required for the conformational change and interactions **(27)**.

Regulation of Apoptosis at the Endoplasmic Reticulum

The Bcl-2 family proteins have a distinct role in regulating ER functions, which in turn affect cell death and other cellular functions. Only a brief discussion is given here, as detailed information can be found in Chapter 7. Bcl-2 family proteins, such as Bcl-2 and Bax, can be found in the ER **(75, 103, 137–139)**. The most well-studied function of Bcl-2 family proteins related to ER physiology is regulation of the ER's calcium level. The balance between anti- and proapoptotic proteins at the ER may affect the steady-state ER calcium content and directly impacts the amount of calcium released after stimulation. Thus, cells deficient in both Bax and Bak exhibit decreased ER calcium content **(138, 139)**, similar to the phenotype of the Bcl-2 overexpressing cells **(103)**. This regulation may be mediated by the interaction of Bcl-2 with the IP3 receptor, which is antagonized by Bax and Bak **(74)**. Calcium released from the ER can participate in cell death by activating the mitochondria's permeability transition pore. Thus, Bcl-2 family proteins could regulate the cross-talk between the ER and mitochondria to affect cell death.

A major cause of cell death is ER stress. A complicated cellular response called the *unfolded protein response* can be initiated in response to ER stress, which is mediated by the ATF6, PERK/eIF-2α, and IRE-1 pathways, resulting in both protective and apoptotic effects. Bcl-2 family proteins can participate in this process on two different levels. First, Bim has recently been shown to be a transcriptional target of CHOP, downstream of the PERK/eIF-2α pathway **(140)**. This upregulation of Bim seems to be important for the ER stress-induced apoptosis, which is consistent with the general consensus that persistent activation of the PERK/eIF-2α pathway can lead to cell death. Second, Bax or Bak could bind to IRE-1α to promote stronger IRE-1α-mediated signaling **(75)**. Since this pathway is largely responsible for the protective effects of UPR, this finding suggests that Bax or Bak could have some prosurvival function in this setting. However, these findings have yet to be reconciled with other observations regarding the prodeath function of Bax and Bak at the ER site (via the regulation of calcium).

The Physiological Roles of the Bcl-2 Family Proteins

Because of the critical roles played by the Bcl-2 family proteins in the regulation of apoptosis, these proteins are important to organisms during embryonic development and in adult life. Bcl-2 family proteins also participate in functions not related to the classical apoptosis pathways.

Role in the Regulation of Apoptosis

Programmed cell death was initially defined by developmental biologists as describing the temporally and spatially controlled death of cells during development **(141)**. In fact, the genetic pathway of programmed cell death was first characterized in the nematode *C. elegans* (see Chapter 13). The antideath molecule *ced-9* is essential to the normal development of the worm, so that the loss-of-function mutation of this molecule causes normally survived cells to die, which results in embryonic lethality. Such an essential role of antideath molecules has also been observed in mammals **(7, 142)** (Table 1) (also see Chapter 15). Inactivation of the mammalian antiapoptosis genes *bcl-xL* or *mcl-1* leads to embryonic lethality. While Bcl-xL seems to be important for the development of the neuronal and hematopoietic systems **(143)**, Mcl-1 is critical to the development of trophectoderm, which is important for the implantation of embryos to the uterus **(144)** and to the development of hematopoietic stem cells **(145)**. Although the deletion of Bcl-2 from the mouse genome only results in partial lethality, the survived mice nevertheless have significant developmental defects, including thymic atrophy, polycystic kidney disease, and melanocyte maturation arrest that leads to hypopigmentation **(146)**. These deficiencies could be rescued by the concomitant deletion of Bim **(147)**.

While Bak-deficient mice have no observable phenotype, a large fraction of *Bax* and *Bak* double-knockout mice die during embryogenesis or soon after birth **(125)**. These mice have signficant developmental defects that correlate with the deficiency in cell death. *Bax* and *Bak* doubly deficient cells are resistant to most forms of stress-induced apoptosis **(126)** or death caused by overexpression of the BH3-only molecules **(127)**. These results demonstrate that Bax and Bak have a functional overlap but are essential for mitochondria-mediated apoptosis.

A number of nonlethal defects have also been observed in genetic models where other Bcl-2 family genes have been deleted. For example, mice efficient in one of the four A1 genes *A1A* are normal in development, but an abnormally rapid apoptosis occurs to their granulocytes and mast cells in culture **(148, 149)**. Male mice deficient in *Bcl-w* or *Bax*, although alive, are infertile due to abnormal spermatogenesis **(150, 151)**.

Mice deficient in different BH3-only molecules have various phenotypes, as these molecules serve as sentinels to different death signals. While not affected developmentally, mice deficient in *PUMA* or *Noxa* are resistatnt to p53-dependent DNA damage-induced apoptosis **(152, 153)**. Interestingly, PUMA seems essential for γ radiation-induced apoptosis, while Noxa seems essential for UV radiation-induced apoptosis in embryonic fibroblasts (MEFs) **(154)**. The Noxa and PUMA doubly deficient thymocytes cells are highly resistant to whole body γ-irradiation, equivalent to the loss of p53 **(155)**. PUMA is also important in p53-independent apoptosis induced by cytokine deprivation, glucocorticoids, or phorbol ester **(152, 153)**.

Bim-deficient mice mainly present a phenotype in the immune system, demonstrating a deficiency in the elimination of autoreactive and activated lymphocytes **(156)**. These mice also have a deficiency in responding to other death stimuli. Together, Bim and PUMA are likely activated by the most apoptotic stimuli in multiple cell types, as deletion of both *Bim* and *PUMA* in mice seems to provide the same level of protection as the overexpression of Bcl-2 or the combined loss of Bax and Bak **(157)**.

Bid is activated by protease, particularly by caspase-8 following death receptor activation. Thus, *Bid*-deficient mice are resistant to Fas-mediated apoptosis in hepatocytes, where mitochondrial activation is required for the death receptor-initiated apoptosis **(158)**. In addition, old mice could develop abnormalities in myeloid cells **(159)**. While Bad deletion does not seem to lead to any major deficiency in apoptosis, it causes a significant defect in glucose-stimulated insulin secretion, suggesting that its normal function in cells is more closely associated with glycolysis regulation **(160)**. Mice lacking Bik, Hrk, and Bmf are essentially normal in development and in cellular response to apoptosis **(7)**. Bmf-deficient thymocytes are resistant to glucocorticoids and histone deacetylase inhibitor-induced apoptosis and the mice developed B-cell restricted lymphoadenopathy **(161)**. Finally, double deletion of Bim and Bik leads to male sterility, which was not observed in the single-knockout mice **(7)**, suggesting that these BH3-only molecues have overlapped or compensatory functions.

Role in the Regulation of Autophagy

Autophagy and apoptosis are intimately linked (see Chapter 29). Bcl-2 family proteins can be important for the regulation of autophagy. Several BH3-only molecules directly participate in autophagy activation. The best-studied molecule in this regard is Beclin1, which was originally identified as a Bcl-2-interacting protein **(162)**, and it possesses a conserved BH3 domain that could also interact with Bcl-xL and Mcl-1 **(163)**. Beclin1 is also the homologue of yeast Atg6 and can form a complex with multiple other molecules to promote autophagy. On the other hand, Beclin1 does not seem to cause apoptosis. Its interaction with the antideath Bcl-2 family protein leads to suppression of its proautophagy function. Thus, Bcl-2, Bcl-xL, and Mcl-1 also possess this antiautophagy function **(163, 164)**. Interestingly, only the expression of Bcl-2 at the ER membrane could specifically interact and inhibit Beclin1, suggesting that signaling events originating from the ER are crucial for autophagy. The formation of a Bcl-2-Beclin1 or Bcl-xL–Beclin1 complex could be disrupted and thus suppressed by BH3-only proteins, like Bad, or BH3 mimetics, like ABT373, suggesting that extensive crosstalk can occur between apoptosis and autophagy **(163)**.

Bnip3 and Nix/Bnip3L are two other well-defined BH3-only molecules that seem to be important for autophagy. Bnip3 and Nix share 53–56% amino acid sequence identity. They are important for the autophagic clearance of

mitochondria during the maturation of red cells **(165–167)**. Bnip3-mediated autophagy in a nondevelopmental context could be either detrimental or protective, depending on the context **(167)**. Unlike other BH3-only molecules, the TM domain of Bnip3 but not its BH3 domain is required for its function **(44)**. Bnip3 is not ubiquitously expressed under normal conditions. Its expression is markedly increased in response to hypoxia and appears to be regulated by hypoxia-inducible factor 1 (HIF-1) **(167)**.

Other BH3-only molecules that have been implicated in autophagy include Bik **(168)**, EGL-1 **(113)**, and ApoL1, a recently described lipid binding protein that possesses a BH3 domain **(169)**. The expression of ApoL1 can be induced by p53. When overexpressed, it can cause autophagic death, which requires the BH3 domain and the classical autophagy machinery.

Role in the Regulation of Cell Proliferation

It has been long known that the Bcl-2 family protein could regulate cell cycle progression from G_0/G_1 to the S phase, which can be inhibited by the antideath molecules Bcl-2, Bcl-xL, Bcl-w, and Mcl-1 and the adenoviral Bcl-2 homologue, E1B19K **(16)**, but promoted by the prodeath molecules Bax **(170)**, Bad **(171)**, and Bid **(172)**. This has been demonstrated in different cell types, including lymphocytes, hepatocytes, and fibroblasts.

The mechanism is not completely clear although several possibilities are present **(16, 173)**. One possibility is the regulation of the classical cell cycle machinery, such as $p27^{Kip1}$, cyclin E, and the Rb family member p130. In Bcl-2-overexpressing quiescent T cells, the levels of $p27^{Kip1}$ and the Rb family member p130 were increased following stimulation by growth factors or mitogens. In hepatocytes, while the deletion of Bid **(172)** or the overexpression of Bcl-2 **(174)** does not seem to affect the expression of $p27^{Kip1}$, they do change the kinetics of the expression of cyclin E following partial hepatectomy, which is delayed in both cases. Another possibility is the regulation of proliferation signaling along the calcium-calmodulin-calcineurin-NFAT pathway or the Raf-ERK pathway **(173)**.

Notably, through a series of mutagenesis analyses of Bcl-2 and Bcl-xL, it was found that no mutant could segregate the function on cell death and the function on cell proliferation **(175, 176)**, suggesting that Bcl-2 family proteins could operate through comparable mechanisms to regulate these two functions. In addition, it is possible that like their regulations on cell death, the prodeath and antideath Bcl-2 family proteins could act on common targets in an opposite way to regulate cell proliferation.

Role in Other Physiological Functions

As mentioned earlier (see the section entitled "Participation in Multiple Functions Through Binding to Different Molecules"), by interacting with nonfamily proteins, Bcl-2 family molecules could exert diverse functions, some of which are

further summarized here with additional perspectives. Bcl-2 family proteins could potentially affect the repair of DNA damage. For example, nuclear Bcl-2 could interact with both Ku70 and Ku86 via its BH1 and BH4 domains to inhibit the nonhomologous end-joining pathway, which may lead to an accumulation of DNA damage and genetic instability **(73)**. Although still controversial, Bid may be phosphorylated by ATM to play a role in the intra-S phase checkpoint **(119–121)**. This ability of Bid is not dependent on its BH3 domain **(120)**.

The BH3-only molecule Bad has a unique role in reuglating glycolysis via interaction with the mitochondrial glucokinase in a complex that also contains protein kinase A (PKA), protein phosphatase 1, and a PKA-anchoring protein (WAVE-1) **(77)**. The glucokinase is responsible for phosphorylating glucose to produce glucose 6-phosphate, the first step in several pathways of glucose metabolism, including glycolysis and the storage of excess glucose as glycogen. A recent study further finds that BAD plays a physiological role in glucose-stimulated insulin secretion by beta cells **(160)**. This function is also specifically dependent on the phosphorylation of the Ser^{155} at the BH3 domain, which is important for Bad to interact with glucokinase. Interestingly, Bad can also interact with another key glycolysis enzyme, phosphofructokinase-1 (PFK-1), and phosphorylation of Bad at Thr^{201} by JNK is required for the activation of PFK-1 by Bad **(117)**.

Finally, MULE/ARF-BP1 is an E3 ligase that can ubiquitinate Mcl-1 for degradation during DNA damage-induced apoptosis **(177)**. This E3 ligase has a BH3-only domain that is required for the binding with Mcl-1. Interestingly, MULE/ARF-BP1 can also ubiquitinate p53 for degradation, which is suppressed by ARF **(178)**. Thus, this molecule may have different ways to regulate cell death under different contexts.

Role of the Bcl-2 Family Proteins in Cancer Biology and the Development of BH3-Based Therapeutic Strategies

Apoptosis, cancer development, and cancer therapy are closely associated (see Chapter 25). The ability of antideath members to maintain cell survival over a long period can be dangerous if it is not under tight control. Indeed, the abnormal expression of these molecules could lead to oncogenesis. A chromosomal translocation (14;18) that results in a deregulated expression of Bcl-2 is responsible for the etiology of 85% follicular lymphomas and 20% diffuse B-cell lymphomas in humans **(179)**. The deregulated expression of both antideath and prodeath molecules has been demonstrated in many types of cancers **(50, 180)**. This finding leads to the definition of a new type of proto-oncogenes represented by *bcl-2* that mainly affect cell death **(50, 181)**. While the overexpression of Bcl-2 could lead to increased tumorigenesis, the loss of any single prodeath molecule has not been found to cause tumor development **(7, 50)**. The combined deletion of Bim and PUMA **(157)**, or PUMA and Noxa **(155)**, or Bax

and Bak **(125)** in mice has not conclusively demonstrated an increased spontaneous tumor development, although lymphoid hyperplasia is evident in these cases **(125, 157)**. These findings may suggest that there is a significant redundancy in cell death molecules and pathways, including those not mediated by the Bcl-2 family proteins, which can work together to affect the survival of tumor cells. Alternatively, tumorigenesis may occur only when both cell death and cell cycle are deregulated, such as following p53 mutation. Notably, when the overexpression of Bcl-2 or the loss of a single prodeath gene, such as Bim, PUMA, or Bax, is combined with the overexpression of a classical oncogene, such as myc or SV40 large T antigen, tumor development could be significantly accelerated **(50)**, indicating that cell death is important for tumor development in the context of oncogenic transformation, where other cellular functions, such as cell cycle progression and energy metabolism, are also deregulated.

The concept of cell death molecules serving a role in neoplasia is helpful in treating cancers that may have developed a resistance to chemotherapy. Bcl-2, Bcl-xL, and/or Mcl-1 are overexpressed in many cancers, which correlates with poor survival, progression of the disease, and resistance to therapy **(180)**. Thus, they can be ideal targets for cancer therapy. Several strategies have thus been developed **(50, 132, 180)**.

An early approach is to use antisense oligonucleotides to reduce the expression level of Bcl-2 proteins. Oblimersen (Genasense), an antisense oligonucleotide against *BCL2*, has been clinically tested for several types of cancers, but the results were not ideal **(132, 180)**. This may be because Oblimersen does not inhibit Bcl-2 function, but merely reduces its level. There are no definite data available to address what level of reduction of Bcl-2 could be obtained *in vivo*. In addition, most cancer cells may also have an increased level of Bcl-xL and/or Mcl-1. Thus, simply targeting Bcl-2 may not be enough.

A more recent approach is based on the use of the BH3 domain to antagonize the prosurvival Bcl-2 molecules (Fig. 2.2). Synthetic BH3 peptides have been designed, which can be further chemically modifed to stablize their α-helical structure. These peptides have been shown to be cell-permeable, protease-resistant, and able to induce apoptosis **(182)**. More extensively tested are small organic molecules that mimic the BH3 peptides. A well-characterized mimetics is ABT-737, developed based on a structure-activity relationship by NMR **(183)**. ABT-737 can mimic the BH3 domain of Bad and bind to Bcl-2, Bcl-xL, and Bcl-w with subnanomolar affinity. It demonstrates synergistic cytotoxicity with chemotherapeutic drugs or radiation in various cell lines and patient-derived primary cells and in tumors xenotransplanted in mice **(180)**. Several other BH3 mimetics are also under development **(132)**.

Additional methods not based on known protein interactions of the Bcl-2 family molecules have also been employed, which include the standard high-throughput screening and structure-based virtual screening. These approaches have led to the discovery of several other molecules, including Tetrocarcin A, HA14-1, and antimycin **(180)**. Both HA14-1 and antimycin seem to act like a

BH3-domain mimetic, inhibit the function of Bcl-2 and Bcl-xL, and activate the mitochondrial apoptosis pathway.

Several key issues may need to be emphasized in the use of these chemicals in treating cancers **(132)**. First, it has to be determined in the first place whether Bcl-2 or Bcl-xL is actually important in suppressing cell death in the particular type of cancer to be targeted. As mentioned above, it seems that the BH3 mimetics will work only if the targeted antideath molecules have been primed, or in binding with a sequestered prodeath molecule. In such cases, the sequestered prodeath molecule can be released to activate the death process **(132, 134–136)**. Second, targeting Bcl-2 or Bcl-xL may not be sufficient in certain cancers, as Mcl-1 could play a role in the resistance as well **(184)**. Thus, it would be necessary to target Mcl-1 in cases that are resistant to ABT-737 or the like, which only targets Bcl-2, Bcl-xL, and Bcl-w. Although BH3 mimetics that selectively bind to Mcl-1 have not been described yet, Mcl-1 may be indirectly modulated by other agents, such as the proteasome inhibitors, which increase the level of Noxa that can inhibit Mcl-1, or a kinase inhibitor, Sorafenib, which can downregulate Mcl-1 expression **(185)**. Alternatively, a pan-suppressor that can bind to all antideath molecules should work as well. BH3 mimetics that fulfill this requirement have been developed and are found to be effective in inactivating both Mcl-2 and Bcl-xL (GX15-070/Obatoclax and TW-37) **(132, 136, 185–189)**.

Concluding Remarks

The Bcl-2 family proteins are important to a number of physiological functions that are beyond the regulation of apoptosis. Although they have been classically considered to function at the mitochondria, recent findings indicate that these molecules could work in other subcellular compartments, such as the ER and the nucleus, to fulfill their diverse roles. This family of proteins includes both antideath and prodeath members. Despite the significant sequence diversities, they share homology in certain BH domains. The prodeath and antideath members can directly or indirectly antagonize each other's activity in various functional scenarios. The significant understanding of their interactions and their fine structures in recent years has led to the development of novel therapeutic strategies and agents, which may have promising utilities in treating cancers.

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Chapter 3
The Mammalian IAPs: Multifaceted Inhibitors of Apoptosis

Eric C. LaCasse, Herman H. Cheung, Allison M. Hunter, Stephanie Plenchette, Douglas J. Mahoney, and Robert G. Korneluk

Abstract The inhibitor of apoptosis (IAP) gene family is characterized by the presence of a BIR zinc-finger domain. In this chapter, we discuss how the IAPs, through multiple mechanisms, are able to suppress apoptosis as just one of their many functions. The IAPs also affect signal transduction pathways, differentiation, immunity, and proliferation. The IAPs are central to the regulation of apoptosis, and IAP mutations or gene deletions have uncovered critical roles for the IAPs in various disorders and cellular functions. Apoptosis plays an essential role in immunity and homeostasis. It is therefore understandable that deregulation of IAP activity is associated with immune disorders and cancer. In fact, therapeutics targeting the IAPs have entered the clinic for cancer with much anticipated outcomes. Discoveries surrounding the IAPs emerge everyday, constantly changing our understanding of their function and role in normal and pathological physiology. The IAPs are sure to keep our attention for years to come.

Keywords Apoptosis · Caspase · IAPs · Inhibitors of apoptosis · XIAP · cIAP1 · cIAP2 · NAIP · Survivin · BRUCE · Livin · BIR · Birc · BIR-containing · BIR domain · disease · NFkappaB · Ubiquitin · E3 ligase · RING domain · Knockout mice · Smac · XAF1 · Signal transduction · TNFalpha · Genetics

Introduction

The inhibitor of apoptosis (IAP) gene family regulates the cell's decision to live or die in response to daily stresses and insults. In this chapter, we provide an overview of the mammalian IAPs, with particular emphasis on their many cellular roles in apoptosis suppression, signal transduction, and proliferation.

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In addition, we address the association of the IAPs with disease and discuss the results from gene ablation studies in mice.

The first two IAP genes discovered in 1993–1994 were from an insect virus. They are the baculoviral genes *CpIAP* (from *Cydia pomonella* granulosis virus CpGV) and *OpIAP* (from *Orgyia pseudosugata* nuclear polyhedrosis virus OpNPV). The IAPs are reversible caspase inhibitors, believed to be used by all baculoviruses to allow for viral propagation by preventing a defensive apoptotic response in host insect cells **(1, 2)**. Since the discovery of the baculoviral IAPs, numerous cellular orthologues and paralogues have been identified in a range of species from yeast to vertebrates [e.g., **(3–5)**]. The first discovered cellular IAP is the mammalian gene *NAIP* (encoding neuronal apoptosis inhibitory protein). NAIP was identified during a positional cloning effort in 1995 to determine the causative gene for spinal muscular atrophy (SMA) **(6)**. With the discovery of NAIP (NLRB1/BIR containing gene 1/BIRC1), the human IAP family has rapidly expanded to include seven other members: X-linked inhibitor of apoptosis (XIAP/MIHA/hILP/BIRC4/ILP-1); cellular IAP1/Human IAP2 (cIAP1/HIAP2/MIHB/BIRC2); cellular IAP2/Human IAP1 (cIAP2/HIAP1/MIHC/API2/BIRC3) **(7-10)**; IAP-like protein 2/Testis-specific IAP (Ts-IAP/hILP2/BIRC8/ILP-2) **(11, 12)**; BIR-containing ubiquitin conjugating enzyme (BRUCE/Apollon/BIRC6) **(13, 14)**; survivin (TIAP/BIRC5) **(15)**; and livin (KIAP/ML-IAP/BIRC7) **(16, 17)** (Fig. 3.1 and Table 3.1). The IAPs, deserving of their name, effectively suppress apoptosis induced by a variety of stimuli, including death receptor activation, growth factor withdrawal, ionizing radiation, viral infection, endoplasmic reticulum stress, and genotoxic damage [e.g., **(18)**].

The defining characteristic of the IAPs is a BIR domain. IAPs contain one to three quintessential baculovirus IAP repeat (BIR) domains of 70 to 80 amino acids. Each C2HC-type zinc-finger BIR domain tetrahedrally chelates one zinc atom and forms a globular structure that consists of four or five alpha helices and a variable number of antiparallel β-pleated sheets **(19, 20)**. Based on BIR sequence homology, mammalian IAPs can be divided into two classes (Fig. 3.1): those with type 1 BIR domains of approximately 70 amino acids, and those with type 2 BIR domains of approximately 80 amino acids in length. The latter class encompasses the two human IAPs, survivin and BRUCE, both with a role in cell mitosis **(21, 22)**, and both with an earlier evolutionary origin compared to the type 1 human members with roles in apoptosis and immunity **(4)**. In addition to BIR domains, IAPs contain various other domains, which are summarized in Fig. 3.1. Several mammalian IAP family members contain a carboxy-terminal RING (really interesting new gene) zinc-finger domain. This RING domain has been shown to possess E3 ubiquitin ligase activity, directly regulating self-ubiquitination and protein degradation **(23)**. Within the IAP family, the presence of a CARD domain is unique to cIAP1 and cIAP2 (Fig. 3.1). The function of CARDs in the IAPs is unknown, but they undoubtedly form protein-protein interactions **(24)**. Notably, a functional nuclear export signal exists within the CARD domain of cIAP1, which appears to be important for cell

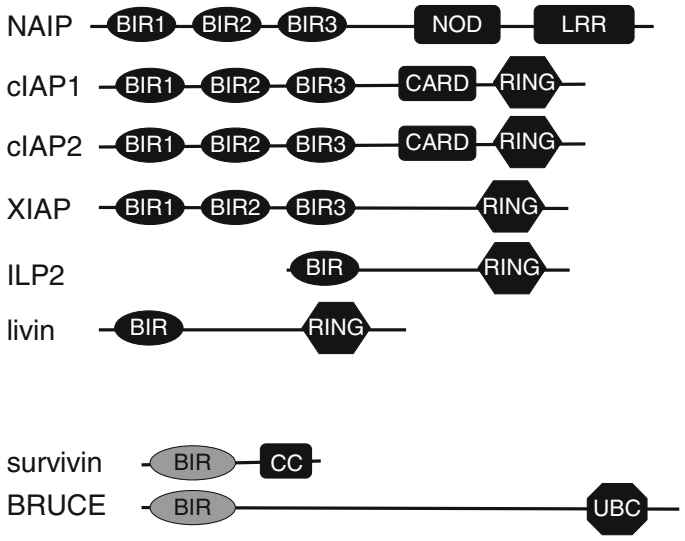

Fig. 3.1 Domain structure of the mammalian IAP family. The domain organization is shown for the eight mammalian IAPs. The presence of at least one BIR domain is the defining characteristic of the IAP family. The human IAPs possess either one (survivin, BRUCE, livin, ILP2) or three tandem amino-terminal BIR domains (XIAP, cIAP1, cIAP2, NAIP). Several IAPs contain an E3 ubiquitin ligase zinc-finger (RING) domain at the carboxy-terminus. cIAP1 and cIAP2 both possess a caspase-recruitment (CARD) domain in the linker region between the BIR domains and the RING domain. Uniquely, NAIP possesses a nucleotide-binding and oligomerization (NOD) domain, as well as a leucine-rich repeat (LRR) domain seen in other NLR proteins involved in innate immunity. BRUCE contains an ubiquitin-conjugating (UBC) domain instead of a RING domain. Survivin contains a coiled-coil (CC) domain that binds the chromosomal passenger proteins INCENP and borealin. The BIR domains shown in gray are longer type 2 domains found in the IAPs with roles in mitosis, compared to the type 1 BIR domains, in black, with roles in signal transduction and apoptosis suppression. Note that the drawings are not to scale and that the length of the IAP proteins, and their splice isoforms, is shown in Table 3.1

differentiation **(25)**, providing one such function for this domain. However, other functions likely still exist. NAIP is unique among the IAPs in that it possesses a nucleotide-binding and oligomerization domain (NOD) as well as leucine-rich repeats (LRRs) that classify it along with other proteins involved in innate immunity, called the NOD-like receptors (NLRs) **(26–28)**. BRUCE, the largest of the IAPs, lacks a RING domain, but instead possesses an ubiquitin-conjugating (UBC) domain capable of performing a similar function. Survivin, the smallest IAP, possesses a coiled-coil domain required for its interaction with the chromosomal passenger proteins INCENP and borealin and for maintenance in the nucleus **(29–31)** (Fig. 3.1).

The physiological analysis of IAP form (domain structure) and function is complicated by the presence of several alternatively spliced products for the survivin, livin, and cIAP2 genes that lead to various protein isoforms of differing size and function **(32–41)** (Table 3.1). In addition, gene duplications and

Table 3.1 Properties of the human (and mouse) IAP genes and proteins

IAP	Gene symbol	DNA locus	Full-length (aa)	Splice isoforms* (aa)	Principal function
NAIP	*birc1*	5q13.2 (13 D1)	1403 (1403, 1447)		**Specialized** *(innate immunity, neuroprotection)*
cIAP1	*birc2*	11q22 (9A1)	618 (612)	(>450)	**Signal transduction** *(TNFR, NF-kappaB activation)*
cIAP2	*birc3*	11q22 (9A1)	604 (600)	(496, 516)	**Signal transduction** *(TNFR, NF-kappaB activation)*
XIAP	*birc4*	Xq25 (X A3.2)	497 (496)		**Apoptosis inhibition** *(housekeeping gene)*
survivin	*birc5*	17q25 (11 E2)	142 (140)	74, 137, 165 (48, 121)	**Mitosis** *(cytokinesis)*
BRUCE	*birc6*	2p22 (17 E2)	4857 (4854)		**Mitosis** *(cytokinesis)*
livin	*birc7*	20q13.3 (2 H4)	298 (271)	280	**Specialized** *(developmental, other?)*
ILP2	*birc8*	19q13.42 (*NA*)	236 (*NA*)		**Specialized** *(spermatogenesis?)*

Notes: Entries with brackets in DNA locus, protein, and splice isoforms are for mouse. (*NA*) refers to where the gene does not exist.
* refers to values derived from searches with the ASD bioinformatics resource on alternative splicing **(158)** or publications cited in this text.

expansions have occurred in some vertebrates that have led to specialized functions that may not exist in humans. For example, survivin is duplicated in xenopus and zebrafish, while the *naip* locus has undergone substantial expansion and specialization in the mouse, encoding multiple functional isoforms. The presence of the two closely related genes, cIAP1 and cIAP2, in mammals allows for some redundancy and additional specialization. This gene duplication event is fairly recent, as zebrafish only possess one cIAP gene **(42)**. Ts-IAP/ hILP2 gene is present on an autosomal chromosome **(11)** (Table 3.1 and Fig. 3.1) and found only in great apes **(12)**. Ts-IAP likely represents a retrotransposed, intronless, copy of XIAP. Ts-IAP produces a mutated, truncated, and unstable version of XIAP that is no longer subject to X-chromosome inactivation **(12**, **43)**.

IAP Proteins: Multifaceted Inhibitors of Apoptosis

Inhibition of Caspase Function

All apoptotic signaling pathways converge on the caspases, making these proteases the ultimate effectors of apoptotic cell death. Not surprinsingly, it is absolutely critical that the caspases be tightly regulated, as their inappropriate

activation can have severe consequences. The first level of caspase regulation is seen in their structure and activation. Caspases are synthesized as inactive zymogens and are only activated via signaling through strictly controlled pathways. A second level of regulation involves the specific inhibition of active caspases by naturally occurring cellular inhibitors.

The IAPs are the only known endogenous proteins that regulate the activity of both initiator and effector caspases. Controlled expression of the IAPs has been shown to influence cell death in a variety of contexts and is believed to have important consequences with respect to human cancer **(18)**. The mechanism by which the IAPs inhibit apoptosis was first interrogated in John Reed's laboratory **(44, 45)**. In these early studies, XIAP prevented caspase-3 processing in response to caspase-8 activation. Therefore, XIAP was suggested to inhibit the extrinsic apoptotic signaling by blocking the activity of the downstream effector caspases, as opposed to interfering directly with caspase-8 activation **(44, 45)**. Supporting this concept, XIAP was shown to specifically bind to caspases-3 and -7, but not to caspases-1, -6, -8, or -10 **(44, 45)**. *In vitro* assays confirm that XIAP, as well as cIAP1 and cIAP2, prevents downstream proteolytic processing of pro-caspases-3, -6, and -7 by blocking cytochrome c–induced activation of pro-caspase-9 in the intrinsic pathway **(45)**.

To further dissect the mechanism of IAP-mediated caspase inhibition, the ability of various XIAP fragments to suppress caspase activation and apoptosis *in vitro* was examined. Utilizing recombinant proteins composed of the BIR1 + BIR2 domains and the BIR3 + RING domains, more precise interactions are observed in which BIR2 specifically inhibits caspases-3 and -7 and BIR3 inhibits caspase-9 activity **(46, 47)**.

Crystallography and mutagenesis studies have since established that individual BIR domains of the IAPs do indeed have different mechanisms of caspase inhibition. Complexes of XIAP BIR2 with caspases-3 and -7 indicate that the linker region between BIR1 and BIR2 is the only IAP element that has contact with caspases-3 and -7, whereas the BIR2 domain is hidden in the crystal structure **(48)**. Using GST-fusions with the XIAP linker region alone, caspase-3 is inhibited and the BIR2 domain appears irrelevant to this interaction **(48)**; however, the interaction with caspase-7 is slightly different in that the BIR2 domain is required to stabilize the linker interaction with this caspase **(49)**. In essence, the effector caspases appear to be inhibited simply by the steric hindrance due to BIR2 and/or its linker blocking the substrate entry site.

The BIR3 domain of XIAP, cIAP1, and cIAP2 **(45, 50)** and the single BIR domain in livin **(17)** and Ts-IAP **(11)** have been shown to directly bind to and inhibit caspase-9. Furthermore, the mechanism of XIAP interaction with caspase-9 differs from the interactions with caspases-3 and -7. Catalytic cleavage of caspase-9 occurs at Asp-315 to yield large (p35) and small (p12) caspase-9 subunits. The newly generated amino-terminus of the p12 subunit starts with the amino acid sequence ATPF. This peptide sequence binds to a pocket on the surface of XIAP BIR3. Fifteen amino acids downstream is a caspase-3 cleavage site at Asp-330. The co-crystal structure of caspase-9 complexed with XIAP

BIR3 reveals that the BIR3 domain forms a heterodimer with caspase-9 **(51)**. In effect, XIAP BIR3 stabilizes caspase-9 in an inactive state, by preventing caspase-9 homodimerization, which is essential for its autocatalytic activity. Interestingly, further cleavage by caspase-3 at Asp-330 in the p12 subunit of caspase-9 generates a p10 subunit that lacks the XIAP-binding motif. Therefore, a feedback proteolytic mechanism was proposed as follows: Caspase-9 activation in the apoptosome results in pro-caspase-3 cleavage, thereby activating caspase-3. Caspase-3 feedback cleavage at Asp-330 in the p12 caspase-9 subunit removes the XIAP BIR3 recognition motif of caspase-9, thereby preventing IAP inhibition and increasing the enzymatic activity of caspase-9 **(52, 53)**. Although this is an attractive model, there is evidence to suggest that removal of the IAP-binding motif may not prevent XIAP association and that surface contacts between XIAP and the caspase-3-generated p10 fragment of caspase-9 can still maintain this interaction **(53)**.

One report describes a "two-site interaction mechanism" to illustrate how BIR domains are able to achieve specificity and potent inhibition of their target caspases. This model describes a conserved IAP-binding motif (IBM) interacting groove that participates in inhibition by binding neoepitopes that are revealed once caspase activation occurs. These IBM interacting grooves are the most conserved surface feature of BIRs and are found on many BIR domains including BIR2 and BIR3 of XIAP and BIR1 and BIR2 of DIAP1, a functional orthologue in *Drosophila melanogaster* **(54, 55)**. In spite of their overall sequence conservation and similar structural fold, there is convincing evidence for the differential roles of the BIR domains in the regulation of caspase activity.

The Salvesen laboratory **(56, 57)** suggests that only XIAP is a true direct inhibitor of caspases and that other IAPs simply bind caspases but do not directly inhibit them. This suggestion is based on the fact that XIAP is stable and exhibits the greatest potency for caspase inhibition compared to the other IAPs. Importantly, while cIAP1 and cIAP2 can bind caspases, their ability to inhibit caspases *in vitro* has been attributed to an artifact of the GST-fusion moiety, which allows aggregation and steric inhibition of the caspases by the cIAP fusions **(56)**. However, cIAP1 and cIAP2 are capable of antagonizing caspases. For example, cIAP1 and cIAP2, without GST moieties, antagonize caspase activity when co-expressed in yeast **(58)**, rescuing them from cell death.

To summarize, caspase-3 is inhibited exclusively by the linker region between BIR1 and BIR2, whereas caspase-7 inhibition requires both the linker region and the BIR2 domain of XIAP. It is evident that caspase-9 is inhibited in direct response to BIR3 binding, thereby preventing further caspase-9 activation as well as suppressing downstream effector caspase activity. Thus far, none of the IAP BIR1 domains has been shown to have any caspase-inhibiting activity; however, Akt/PKB (protein kinase B)-mediated phosphorylation at serine 87 within BIR1 reduces autoubiquitination and stabilizes XIAP **(59)**. BIR1 therefore appears to play a regulatory role rather than directly participating in apoptosis suppression. For example, the BIR1 domains of cIAP1 and cIAP2

are proposed to play a role in TRAF interactions and ubiquitination reactions **(60, 61)** . Furthermore, the BIR1 domain of XIAP is proposed to interact with TAB1 and induce NFkappaB activation through the TAK1 pathway **(62)**.

Inhibition or Promotion of Caspase Activity Through Ubiquitination and Degradation of Caspases or IAPs

The posttranslational modifications of proteins with ubiquitin chains is now seen as a widely used and important regulatory mechanism that either results in their proteasomal degradation or allows signal transduction depending on the type of ubiquitin linkage used. The ubiquitination and subsequent proteasomal degradation of the IAPs may be a key regulatory event in the apoptotic program. The carboxy-terminal RING domain of the IAPs mediates both the ubiquitination and degradation of the IAPs, as well as that of their substrates **(23, 63)**. RING domain-containing proteins possess E3 ubiquitin ligase activity and function as specific adapters by recruiting target proteins to a multicomponent complex. Earlier studies on the RING domains of XIAP and cIAP1 show that this domain is involved in the ubiquitination and degradation of the IAPs in response to apoptotic triggers **(23)**. Distinctly, other studies show that XIAP and cIAP1 are able to promote the ubiquitination and proteasomal degradation of caspases-3 and -7, thereby enhancing the antiapoptotic effect of the IAPs **(63)**. Although the RING domain has been clearly shown to be involved in the ubiquitination and degradation of the IAPs, it remains unclear whether this activity enhances the antiapoptotic activity of the IAPs or actually antagonizes the activity. Studies using RING-deletion mutants provide evidence for both scenarios. In one study, overexpression of RING-deleted XIAP and cIAP1 mutants in cell culture results in the loss of autoubiquitination and proteasomal degradation and confers better protection against apoptotic stressors than wild-type XIAP and cIAP1 **(23)**. However, in a separate study, RING-deletion mutants of XIAP are found to be less effective than wild-type XIAP at preventing apoptosis induced by the overexpression of caspase-3 or Fas **(63)**.

XIAP ubiquitination sites have been identified and the role of IAP ubiquitination examined by site-directed mutagenesis. Overexpression of wild-type or XIAP RING-mutant protein in cultured cells does not reveal any differences in their ability to protect against Bax- or Fas-induced apoptosis **(64)**. These findings suggest that ubiquitin-mediated destruction of the IAPs may not be as significant as believed, at least in the context of overexpressed proteins **(64)**. Alternatively, there is a possibility that RING domains may function to suppress apoptosis in conditions of low apoptotic stimulus by the ubiquitination of caspases, whereas high levels of apoptotic stress trigger self-degradation of the IAPs, leading to cell death.

cIAP2 is typically present in low abundance in the cell; it has been proposed that cIAP2 levels are maintained at low levels via constitutive ubiquitination and subsequent degradation by cIAP1 **(65)**. Insight into cIAP1 E3 ligase function came from the generation of the cIAP1 "knockout" mouse. Although these mice appeared to be both viable and fertile, they had significantly elevated cIAP2 protein levels with normal mRNA levels, suggesting that in a normal setting, cIAP1 ubiquitination of cIAP2 leads to low protein levels of cIAP2 **(65)**. Furthermore, coexpression and *in vitro* binding studies of cIAP1 and cIAP2 along with TRAF1 or TRAF2 demonstrate that the cIAPs and TRAF proteins form a multimeric complex. In this complex, TRAF1 and TRAF2 appear to function as adaptors, bringing cIAP1 or cIAP2 together, allowing cIAP1 to ubiquitinate cIAP2 **(65)**. The cIAP1 CARD-RING fragment produced under apoptotic conditions can target all the other RING-containing IAPs for degradation. Interestingly, XIAP ubiquitination is not required for RING-mediated degradation in this case **(66)**.

The ubiquitination and degradation of XIAP depend on its phosphorylation status. More specifically, XIAP is a substrate for Akt/PKB **(59)**. Phosphorylation of XIAP by Akt/PKB inhibits cisplatin-induced autoubiquitination, thereby reducing XIAP degradation. Collectively, these observations support the concept that IAP-IAP or IAP-partner interactions play roles in regulating their relative abundance, through posttranslational modifications, to impact upon the progression of apoptosis.

Modulation of Survival Signal Transduction Pathways

In recent years, it has become clear that the IAPs are more than just caspase inhibitors. In fact, it is now widely suggested that the primary role of many IAPs is to modulate various cellular signal transduction pathways. The earliest evidence for such a role was generated in David Goeddel's laboratory, where cIAP1 and cIAP2 were shown to interact with tumor necrosis factor (TNF) receptor-associated factor (TRAF) 2 **(9, 67)**. TRAF2 is an adapter protein for the nuclear factor-κB (NF-κB) and mitogen activated protein kinase (MAPK) signaling pathways, which are involved in many biological processes, including the decision to live or die in response to stress. These pathways are typically activated by ligation of receptors in the TNF family, but can also be engaged by various intracellular stressors. Early studies showed that cIAP1 and cIAP2 get recruited to TNF receptors in response to TNFα, a process that is dependent upon their interaction with TRAF2. Accordingly, it was hypothesized that cIAP1 and cIAP2 participate in TNFα-mediated NF-κB activation. Subsequent overexpression studies supported this notion, demonstrating that cIAP2 has the capacity to activate NF-κB **(68)** and that a RING-deficient version of cIAP1 can cooperate in the activation of NF-κB by TRAF2 **(60)**.

On the other hand, the physiological involvement of cIAP1 and cIAP2 in NF-κB signaling is not supported by cIAP1 and cIAP2 "knockout" studies **(65, 69)**. Mice with whole body deletion of cIAP1 are asymptomatic, and primary cells tested from these animals display normal TNFα-induced NF-κB activation and are not sensitized to TNFα-mediated cell death **(65)**. Similarly, cIAP2-null mice do not display an overt phenotype, although their macrophages are sensitized to LPS-mediated apoptosis **(69)**. This is not due to a defect in LPS-induced NF-κB activation, which is normal in cIAP2-null macrophages. However, a confounding variable in both the cIAP1- and cIAP2-null mouse studies is the presence of the other highly similar cIAP, which may be able to compensate. Unfortunately, it has not yet been possible to create a cIAP1/cIAP2 double-"knockout" mouse, due to the close chromosomal proximity of these two genes. To circumvent this, our group recently used combined genetic knockout and siRNA-mediated knockdown methodologies to generate numerous cIAP1/cIAP2 "null" cells. Using this approach, we showed definitively that cIAP1 and cIAP2 are both involved in TNFα-mediated NF-κB activation **(70)**. Moreover, we demonstrated that the expression of either cIAP1 or cIAP2 at endogenous levels is sufficient for proper signal transduction, which explains why NF-κB signaling was normal in the cIAP1 and cIAP2 "knockout" mice. These results are consistent with recent data by Santoro and colleagues **(42)** demonstrating that cIAP1 and cIAP2 redundantly regulate TNFα-mediated NF-κB activation in human endothelial cells **(42)**. Although the mechanism whereby cIAP1 and cIAP2 regulate TNFα-mediated NF-κB activation is not entirely known, several clues suggest that they are involved in TNF receptor signalosome assembly (Fig. 3.2). In most cells, TNFα signals through its receptor TNF-R1. Upon TNFα occupancy, TNF-R1 rapidly recruits the TNFR-associated death domain (TRADD) protein and the receptor-interacting protein 1 (Rip1/RIPK1). TRADD binding in turn recruits TRAF2 and cIAP1/cIAP2 to form a large membrane complex. When this occurs, Rip1 is modified with large polyubiquitin chains conjugated at lysine 63 (K63) residues of ubiquitin, which serves as a docking site for the downstream kinases directly involved in NF-κB signaling **(71, 72)**. The E3 ligase responsible for targeting Rip1 for K63-linked polyubiquitination had originally been proposed to be TRAF2. TRAF2 is a *bona fide* E3 ubiquitin ligase, and in its absence TNFα-mediated ubiquitination of Rip1 does not occur. However, evidence that TRAF2 can directly ubiquitinate Rip1 *in vitro* has been lacking, which suggests that the loss of TRAF2 indirectly affects Rip1 ubiquitination. As cIAP1 and cIAP2 (1) are required for TNFα-mediated NF-κB activation, (2) possess a RING zinc-finger domain with strong E3 ligase activity, and (3) require TRAF2 to get recruited to TNF-R1 upon TNFα ligation, they emerged as obvious candidates. Supporting a role for cIAP1 and cIAP2 in Rip1 ubiquitination, our group has recently shown that Rip1 ubiquitination occurs normally when either cIAP1 or cIAP2 is present at endogenous levels. In contrast, the dual loss of cIAP1 and cIAP2 completely abolishes TNFα-induced Rip1 ubiquitination, consistent with their redundant roles in TNFα-mediated NF-κB activation.

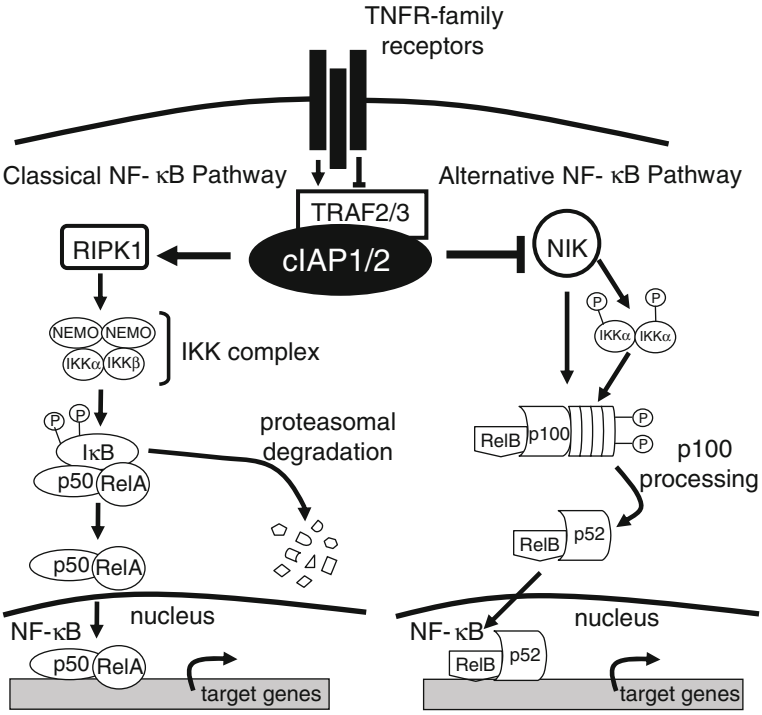

Fig. 3.2 cIAP1 and cIAP2 regulation of classical and alternative NFkB activation pathways. The two major activation pathways for NFkB are shown. Those TNF superfamily receptors that utilize TRAF2 as an adaptor can recruit cIAP1 or cIAP2, which can then positively modulate the classical pathway or negatively regulate the alternative pathway. In the classical NF-κB pathway, NF-κB dimers (*e.g.*, p50/RelA) are retained in the cytoplasm by interaction with IκB. In response to the binding of a ligand to a tumor necrosis factor receptor (TNFR)-related protein, TRAF proteins are recruited to the receptor, and through the ubiquitination of RIP kinase (RIPK1/RIP1) suggested to be mediated by the RING domain E3 ligase of cIAP1 and cIAP2, the IKK complex containing IKKα, IKKβ, and NEMO (IKKγ) is recruited and activated. The activated IKK complex in turn phosphorylates the inhibitor of κB (IκB), leading to its degradation, thereby releasing NFkB (p50/RelA) to translocate into the nucleus for transcriptional activation of targeted genes. By contrast, cIAP1, cIAP2, TRAF2 and TRAF3 repress the alternative NF-κB pathway by promoting the ubiquitination and degradation of NFkB-inducing kinase (NIK), which is required for phosphorylation of p100 in the p100/RelB complex. Phosphorylated p100 is processed into p52, and NFkB (*e.g.*, the p52/RelB dimer) translocates into the nucleus for transcriptional activation of targeted genes. Hence, the E3 ubiquitin ligase function of cIAP1 or cIAP2, as well as their binding via BIR1 to TRAF2, plays a central role in the regulation of these pathways

Importantly, cIAP2 can directly K63-ubiquitinate Rip1 *in vitro*, a requirement for signaling **(72)**. However, another study demonstrates that cIAP1 and cIAP2 can ubiquitinate Rip1 *in vitro* and lead to its degradation, suggesting K48-mediated ubiquitination events and proteasomal degradation **(73)**. This may result from the IAP overexpression. Alternatively, the IAPs may switch

between K63- and K48-mediated ubiquitination for the same substrate depending on cellular context. Taken together, these data support a model in which TRAF2 serves as an adapter protein that bridges the gap between cIAP1/cIAP2 and Rip1, which enables cIAP1/cIAP2 to target Rip1 for K63-linked polyubiquitination.

In addition to participating in TNFα-mediated NF-κB activation, cIAP1 and cIAP2 also modulate the activity of a closely related, parallel NF-κB pathway termed the alternative NF-κB pathway **(74, 75)** (Fig. 3.2). Alternative NF-κB signaling is highly regulated and normally inactive but is turned on by the ligation of various TNF receptors such as CD40-R, lymphotoxin (LT) β-R, and B-cell activation factor (BAFF)-R. Upon receptor ligation, the levels of NF-κB-inducing kinase (NIK), which is normally co-translationally degraded in a complex containing TRAF2 and the related protein TRAF3, accumulate. Following sufficient accumulation, NIK in turn phosphorylates downstream kinases that propogate the alternative NF-κB signal. Given that TRAF2 and TRAF3 proteins both have RING zinc fingers with E3 ligase activity, and that the loss of either TRAF2 or TRAF3 results in NIK accumulation and constitutive activation of alternative NF-κB signaling, it was thought that TRAF2 and/or TRAF3 target NIK for ubiquitination and proteasomal degradation. Recent studies, however, suggest or demonstrate that TRAF2 and TRAF3 serve as adapter proteins for cIAP1/cIAP2 and NIK, bringing them into a large protein complex **(75, 76)** (Fig. 3.2). Co-localization by TRAF2 and TRAF3 allows cIAP1 and/or cIAP2 to constitutively target NIK for K48-linked ubiquitination and proteasomal degradation, thereby repressing alternative NF-κB activity. Thus, a conserved mechanism involving TRAF and cIAP proteins appears to regulate both TNFα-mediated and alternative NF-κB signaling, whereby TRAF proteins serve as adapters between the E3 ligases cIAP1/cIAP2 and their substrates. Intriguingly, cIAP1 and cIAP2 have the capacity to positively or negatively regulate NF-κB signaling (Fig. 3.2), and this seems to depend upon whether they target proteins for K48- or K63-linked ubiquitination events. How these decisions are made is currently the subject of intense investigation.

It is noteworthy that NF-κB signaling strongly favors survival in both the TNFα and alternative pathways. As cIAP1 and cIAP2 positively regulate TNFα-mediated signaling yet negatively regulate alternative NF-κB signaling, an intriguing possibility exists that cIAP1 and cIAP2 may have both anti- and prosurvival roles depending on the cell type and context. Indeed, TNFα strongly induces apoptosis in the absence of both cIAP1 and cIAP2 in most cell lines tested to date. In contrast, primary B-cells undergo less spontaneous apoptosis in the absence of cIAP1 and cIAP2, as they rely on alternative NF-κB signaling for survival.

To complicate matters further, cIAP1 has been shown to facilitate TNFα-mediated apoptosis when TNF-R1 and TNF-R2 are simultaneously engaged. Unlike TNF-R1, which is ubiquitously and constitutively expressed, TNF-R2 expression is largely restricted to lymphoid cells. Although the biological role of TNF-R2 is not known, co-stimulation of TNF-R2 sensitizes cells to TNFα-mediated apoptosis through TNF-R1. Elegant work in Jonathan Ashwell's

laboratory has demonstrated two mechanisms responsible for such "crosstalk," both involving cIAP1-mediated regulation of signal transduction. The first mechanism is that cIAP1 negatively regulates TNFα-mediated NF-κB signaling when TNF-R2 is engaged **(77)**. How this occurs is somewhat controversial, but it is known that TRAF2 recruits cIAP1 to TNF-R2 upon exposure to TNFα. Following such recruitment, cIAP1 can target TRAF2 for K48-linked ubiquitination and proteasomal degradation, which likely prevents its own recruitment to TNF-R1. As such, the prosurvival NF-κB signaling pathway, which requires cIAP1 or cIAP2 for activation, is not engaged and apoptosis ensues. The second mechanism involves the ability of cIAP1 to target apoptosis signal-regulating kinase 1 (ASK1) for K48-linked ubiquitination and proteasomal degradation **(78)**. ASK1 is a key negative regulator of the stress responsive kinases Jun N-terminal kinase (JNK) and p38, which are activated by TNFα but must be deactivated quickly to prevent apoptosis. Primary B-cells from cIAP1-null mice revealed that cIAP1 targets ASK1 for K48-linked ubiquitination and degradation in response to TNF-R2 ligation. As such, cIAP1 is responsible for regulating the duration of TNFα-induced JNK and p38 signaling in TNF-R2 expressing cells **(78)**. In the absence of cIAP1, JNK and p38 signaling is prolonged and cells are sensitized toward apoptosis.

Not to be outdone, XIAP is also known to influence signal transduction pathways, particularly the TAB1/TAK1 complex via interaction with its BIR1 domain. This TAB1/TAK1 complex impacts on NFkappaB signaling pathways as well as JNK pathways **(79**, **80)**. XIAP has been shown to inhibit c-Jun N-terminal kinase 1 (JNK1) activation by transforming growth factor beta1 (TGF-beta1) through ubiquitin-mediated proteasomal degradation of the TGF-beta1-activated kinase 1 (TAK1) **(81)**. Studies from Ulevitch's laboratory suggest that XIAP's antiapoptotic activity is achieved by two separate mechanisms: one requiring TAK1-dependent JNK1 activation and the second involving caspase inhibition **(82–84)**.

Negative Regulation of the IAPs

Multiple proteins play critical roles in maintaining an adequate and fine-tuned balance between too much or too little apoptosis. To help maintain and regulate this balance are proteins that act as antagonists of the IAPs, by specifically interacting with and relieving their caspase-inhibitory effects.

Smac/DIABLO: An IAP Antagonist and a Caspase Activator

Smac/DIABLO (second mitochondria-derived activator of caspases, or direct IAP-binding protein with low PI) is a 25-kDa mitochondrial protein that promotes apoptosis via its ability to antagonize IAP-mediated caspase inhibition once released into the cytoplasm. The ability of Smac to prevent IAP

inhibition of caspases makes this protein a functional mammalian equivalent to the *Drosophila* death proteins Reaper, Grim, and Hid **(85, 86)**.

Initial mitochondrial targeting of Smac or DIABLO depends upon a 53- to 55-amino-acid leader signal sequence found at its amino-terminus. This sequence is proteolytically cleaved within the mitochondria to generate an AVPI-containing amino-terminus polypeptide **(87)**, sequestered until an apoptogenic stimulus is sensed. Upon receiving an apoptotic signal via the intrinsic mitochondrial stress pathway, both Smac and cytochrome c are released with similar, but not necessarily identical, kinetics. Although the exact mechanism of Smac release from the mitochondria is not known, one study suggests that, despite their significant size difference, the Smac dimer (100 kDa) and cytochrome c (12 kDa) are released through the same Bax- or Bak-formed membrane pores. This mechanism is supported by the fact that Bcl-2 overexpression can inhibit both cytochrome c and Smac release **(88)**, whereas Bid-induced permeabilization of the outer mitochondrial membrane induces the rapid and complete release of cytochrome c and Smac from the intermembrane space **(89)**. Bax is also suggested to be involved in this process **(90)**.

Other studies, however, suggest that Smac and cytochrome c release is mediated through different mechanisms. Although TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) induces cytochrome c release and apoptosis in wild-type, Bid-null, Bax-null, and Bak-null mouse embryonic fibroblasts (MEFs), Bax/Bak double-knockout MEFs are resistant **(91)**. In contrast, TRAIL-induced mitochondrial Smac release is blocked in all of the single-knockout and double-knockout MEFs. Therefore, it is concluded from these experiments that the release of Smac and cytochrome c from mitochondria is, in fact, differentially regulated in receptor-mediated pathways of apoptosis **(91)**. Differences in the mechanism of Smac and cytochrome c release occur in the presence of caspase inhibitors. Under these conditions, Smac release is prevented whereas cytochrome c is permitted, suggesting that Smac efflux from the mitochondria is a caspase-catalyzed event **(88)**. It may be that Smac release simply requires further membrane permeability due to increased or persistent mitochondrial damage.

Several Smac isoforms exist and studies using these proteins suggest that the proapoptotic function of Smac may be mediated by additional, non-IAP mechanisms. There are some reports that the Smac3 isoform is unique in that it is able to induce the acceleration of XIAP autoubiquitination and destruction, whereas Smac only seems to have this effect on cIAP1 and cIAP2 **(92, 93)**. However, another study describes the ability of Smac to antagonize both XIAP autoubiquitination as well as XIAP-dependent ubiquitination of caspase-7 **(94)**. By disrupting IAP-caspase interactions and repressing the ubiquitin ligase activities of the IAPs, Smac may effectively prevent caspase demise via ubiquitination **(94)**. Although Smac and its isoforms may differ in how they potentiate apoptosis, the involvement of all Smac proteins with the IAPs, at multiple levels, is an effective measure to ensure that the apoptotic program can proceed. Smac/DIABLO appears to function as a general IAP inhibitor in that it binds to

XIAP, cIAP1, cIAP2, survivin, livin, and BRUCE, but not NAIP **(85**, **86**, **95–98)**. The first four amino-terminal residues of mature Smac/DIABLO, Ala-Val-Pro-Ile, are required for Smac/DIABLO function, and deletion of this sequence abolishes Smac/DIABLO-IAP interaction **(99–101)**. The crystal structure of Smac/DIABLO indicates that it consists of three extended alpha helices bundled together to form an arc-shaped structure, exposing an unstructured amino-terminus. Smac/DIABLO homodimerizes through an extensive hydrophobic interface, which is essential for its activity **(100)** and makes it an extremely stable protein dimer **(102)**. The newly generated amino-terminus in mature Smac/DIABLO makes contacts with XIAP BIR3 and mediates XIAP inhibition. The co-crystal structure of XIAP BIR3 and Smac/DIABLO indicate that the amino-terminal four residues, AVPI, in Smac/DIABLO recognize a surface groove on BIR3, with the alanyl residue bound within a hydrophobic pocket **(99)**. The amino-terminal four residues of the caspase-9 linker peptide (Ala_{316}-Thr-Pro-Phe) share significant homology to the amino-terminal tetrapeptide in mature Smac/DIABLO and *Drosophila* death proteins. Initially, it was believed that binding of the caspase-9 linker peptide and Smac/DIABLO to the BIR3 domain of XIAP is mutually exclusive, suggesting a competition model in which Smac/DIABLO displaces XIAP from caspase-9 **(103)**. Smac/DIABLO is also predicted to bind XIAP BIR2 and disrupt caspase-3 and -7 inhibition, possibly by steric hindrance **(100)**. More recent experiments suggest that Smac/DIABLO is unable to remove caspase-3 and -7 inhibition by the linker-BIR2 domains of XIAP and inefficiently relieves caspase-9 inhibition by BIR3. However, when constructs are used that express the XIAP-BIR2 and -BIR3 domains in tandem, Smac/DIABLO effectively prevents IAP inhibition of both initiator and effector caspases. Furthermore, the affinities of the BIR2 and BIR3 domains of XIAP for Smac/DIABLO were shown to be almost identical with each Smac dimer interacting with the BIR2 and BIR3 domains of one XIAP molecule to form a 2:1 stoichiometric complex **(104)**. Although individual BIR domains of XIAP are adequate for inhibiting *in vitro* caspase activity, these findings suggest that both BIR2 and BIR3 are required not only for XIAP-Smac/DIABLO interaction but also for subsequent liberation of caspase inhibition **(104)**.

The structural analysis of Smac binding to XIAP indicates that the amino-terminal tetrapeptide recognizes a surface groove on the BIR3 domain, implying that peptides or small molecules modeled on this binding motif might serve as prototypical drugs whose activity might complement that of Smac **(105)**. When Smac peptides composed of the first four to eight amino-terminal residues are delivered into MCF-7 breast cancer cells, they are capable of interacting with XIAP, cIAP1 **(106**, **107)**, and the single BIR domain of livin **(95)**. These peptides enhance apoptosis and the long-term antiproliferative effects of a range of chemotherapeutics including paclitaxel, etoposide, and doxorubicin **(106**, **107)**.

Smac-like peptides delivered in combination with cancer therapeutics such as Apo2L/TRAIL appear to be a promising method to reduce tumor burden **(106**, **107)**. The *in vivo* delivery of Smac peptide, in conjunction with Apo2L/TRAIL,

completely eradicates established intracranial malignant glioma xenograft tumors and increases the survival time of treated mice **(107)**.

Some studies show that amino-terminal Smac tetrapeptides bind only to the BIR3 domain of XIAP with low affinity, are sensitive to proteolytic degradation, and have a poor capacity to penetrate cells **(108)**. Efforts to circumvent the limitations of Smac peptides led to the development of small molecule Smac mimetics, compounds that inhibit the IAPs with higher affinities than Smac peptides **(108–110)**. The mechanism of action of Smac mimetic compounds may be due to the induction of auto- or trans-ubiquitination of the IAPs, primarily cIAP1 and cIAP2, which results in their rapid loss from the cell within minutes of compound addition **(74, 75)**.

Other IBM-Containing Proteins as Potential IAP Antagonists

Following the identification of Smac, another mitochondrial IAP-binding protein called Omi, or HtrA2, appeared **(111–113)**. Omi/HtrA2 is a mammalian homologue of the *Escherichia coli* bacterium heat-inducible serine protease known as HtrA. Another identified IBM-containing protein is an isoform of the polypeptide chain-releasing factor GSPT1/eRF3 protein that is localized to the endoplasmic reticulum **(114)**. Checkpoint kinase 1 (Chk1), a dual-function kinase that is active during the S-M phase transition of the cell cycle, regulating Cdc25A function, and that prevents mitotic progression in the presence of DNA damage, contains an IBM sequence **(115)**. Still more IBM-containing proteins, all mitochondria-derived, exist **(116)**. If we extrapolate from the situation in *Drosophila*, with four known IAPs (DIAP1/*thread*, DIAP2, deterin, and dBruce) versus eight human IAPs, and five known *Drosophila* IBM-containing antagonists (Reaper, Hid, Grim, Sickle, and Jafrac), then we would predict that more mammalian IBM-containing proteins remain to be discovered. It is suggested that of all the mitochondrial apoptogenic proteins, Smac and cytochrome c remain the only likely mammalian "killer" proteins **(117)**.

XAF1 Is an Interferon-Inducible IAP Antagonist and a Candidate Tumor Suppressor

XAF1 (XIAP-associated factor 1) is an IAP-binding protein that does not possess an IBM motif. XAF1 is a multizinc-finger protein that was identified by a yeast two-hybrid screen with XIAP as bait **(118)**. XAF1 directly associates with XIAP to antagonize XIAP-mediated caspase-3 inhibition **(118)**. However, XAF1 also binds cIAP1 and cIAP2 (and other IAPs as well) with at least equal, if not greater, affinity than XIAP **(119)**. XAF1 is therefore likely a pan-IAP inhibitor, and not just a XIAP antagonist. Interestingly, XAF1 does not bind survivin directly. However, XAF1 can destabilize survivin via XAF1's and

survivin's mutual interaction with XIAP **(119)**. Survivin increases XIAP's stability, reinforcing the antiapoptotic activity of this IAP-IAP complex **(120)**. Therefore, XAF1 expression negates these possible cancer-promoting effects of the XIAP-survivin complex.

It is noteworthy that *xaf1* mRNA is upregulated in response to interferon-β (IFN-β) treatment **(121**, **122)**, possibly explaining the anticancer effects seen with interferon therapy. The XAF1 promoter is primarily silenced in cancer by DNA methylation [reviewed in **(123)**]) and also by HSF1-mediated transcriptional repression **(124)**. However, XAF1 expression can be restored experimentally upon inhibition of DNA methylation, and with the addition of interferon [e.g., **(125–127)**]. In summary, therefore, the downregulation or loss of *XAF1* expression in cancer cells may contribute to apoptosis suppression and tumorigenesis through unrestricted IAP activity.

Summary: Regulation of Apoptosis by the IAPs

IAP regulation of cell death, required to keep apoptotic pathways in check, either by ubiquitination and degradation pathways, or via protein-protein interactions with negative regulators such as XAF1, Smac, and/or Omi, is complex. How these various regulatory mechanisms function together in a simplified general model of apoptotic cell death is summarized in Fig. 3.3.

Apoptotic cell death can be triggered via either the intrinsic or extrinsic pathway, where eventually all signaling converges on the effector caspase-3 or -7. Apoptotic stresses acting through the intrinsic pathway result in the release of cytochrome c (most likely via Bax- or Bak-channel formation in the outer mitochondrial membrane), leading to caspase-9 activation. The consequences of cytochrome c release are determined by the activity of the IAPs. Preexposure to apoptotic stresses or the presence of high levels of IAPs may inhibit activated caspase-9 and any subsequent effector caspase activation. The ubiquitin ligase activity of the IAPs may target the bound caspases for proteasome degradation, thereby aborting the apoptotic process. This type of regulation could be considered a safety mechanism to prevent transient or accidental cytochrome c leakage from eliciting an unnecessary, full-blown apoptotic response. In the case where sufficient caspase-9 activation occurs, the effector caspases-3 and -7 will become activated, triggering more significant or persistent changes in mitochondrial membrane permeability. This in turn releases more cytochrome c as well as apoptosis-inducing factor (AIF), Smac, and Omi. AIF translocates to the nucleus, where it is involved in chromatin condensation and degradation. Meanwhile, Smac and Omi are able to bind to and neutralize IAPs so that unrestricted caspase activation can proceed (Fig. 3.3).

With Smac and Omi positioned in the mitochondria, their inhibition of IAP-caspase interactions should predictably occur only when there is apoptotic signaling via the intrinsic pathway. However, Smac exerts its proapoptotic effects

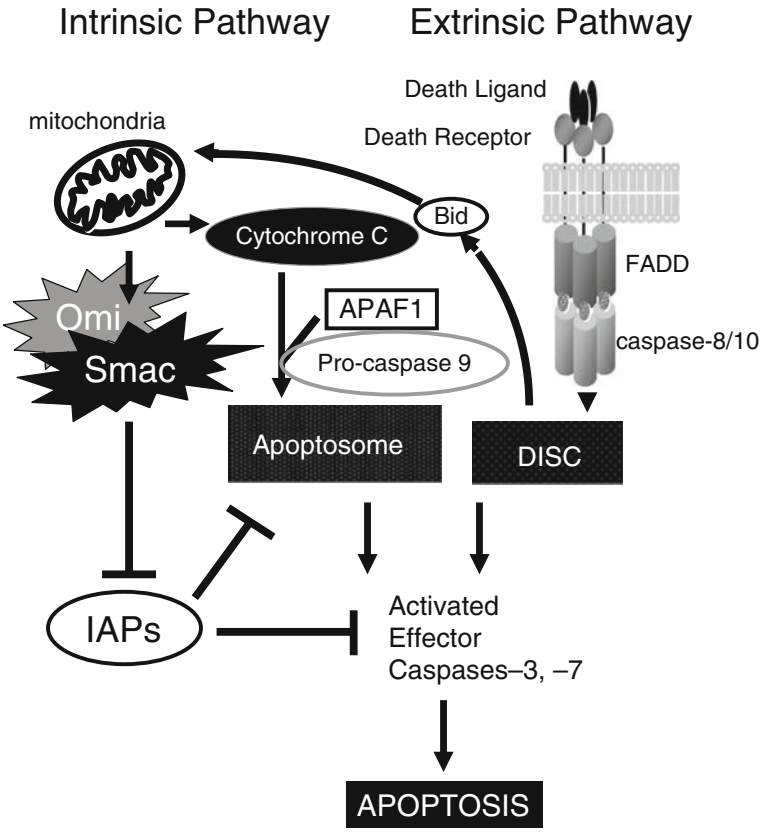

Fig. 3.3 IAPs effectively inhibit the apoptotic process initiated through either the intrinsic or extrinsic death pathways. The IAPs effectively block processing of caspase-3 into its mature, active form following the initial downstream caspase-3 cleavage, induced by caspase-8 DISC signaling (extrinsic death receptor pathway). The IAPs are also able to inhibit cytochrome c-induced activation of caspase-9 as occurs in the intrinsic (mitochondrial) death pathway. The presence of high levels of the IAPs results in caspase inhibition. Caspase-IAP interaction triggers ubiquitination of both the IAP and the caspase. The entire complex is degraded via the proteasome (not shown) and the apoptotic process is aborted. Alternatively, if a cell receives a lethal apoptotic stimulus, endogenous IAPs are saturated by interactions with mitochondrially released IAP antagonists, such as Smac/DIABLO and/or Omi/HtrA2. Proteosome-mediated degradation of IAP-Smac and or IAP-Omi complexes results in IAP depletion (not shown), allowing for unrestricted caspase activation, ultimately leading to apoptosis

through the extrinsic or death receptor pathway by functioning at the level of the effector caspases. Death receptors and their ligands, such as FasL-Fas and DR4-TRAIL, are able to initiate caspase activation independently of the mitochondria. Since both the intrinsic and extrinsic pathways converge on the effector caspases-3 and -7, high levels of IAPs are able to prevent caspase activation at this

distal point, thereby terminating the receptor pathway via the inhibition of caspase-3. Given that the mitochondria can be bypassed in the death receptor pathway, the mechanism of Smac release may not be intuitively obvious; however, it seems that in some cell types, stimulation of death receptors results in caspase-8 cleavage and the subsequent activation of the proapoptotic Bcl-2 family member Bid **(128**, **129)**. When Bid becomes cleaved, its truncated form is able to stimulate the formation of Bax- or Bak-pores in the outer mitochondrial membrane. This in turn induces the release of cytochrome c and Smac and the formation of the Apaf-1/caspase-9 apoptosome. Since caspase-8 is not sensitive to direct IAP inhibition, persistent activation of the death receptor pathway should result in relief of the inhibition on downstream effector caspases by the release of Smac via the secondary activation of the mitochondrial pathway **(130)**. Thus, there is crosstalk between several signaling pathways to ensure that once an appropriate cell death signal is given, the apoptotic pathway is fully engaged.

The IAPs in Health and Disease

Convincing data for the involvement in the IAPs in cancer and other human diseases now exists. Direct genetic evidence, as demonstrated by IAP gene amplications, gene mutations or deletions, and chromosomal translocations, indicating causality in cancer and proliferative autoimmune disorders has come to light in the last decade. These findings are supported by animal studies, and cell culture experiments, demonstrating the immunomodulatory and oncogenic potential of the IAPs as well as identifying their normal physiological roles.

Biological Roles of the IAPs: Evidence from Animal Gene Ablation Studies

The fundamental role the IAPs and their antagonists play in apoptosis control, development, homeostasis, and disease progression is addressed in part through the use of gene ablation strategies in mice and through the genetic analysis of various human and mouse disorders.

Of the six of the seven IAP genes that have been individually deleted in mice through homologous recombination **(**reviewed in **131)**, only two are embryonic lethal, both with roles in mitosis. The majority of these IAP "knockout" mice are, in fact, viable, develop normally, and at first glance do not demonstrate any overt phenotype. The argument is made that the lack of phenotype is due to the high degree of redundancy seen in the IAP family, and that possible compensatory increases occur in other IAP genes. The two lethal phenotypes are also particular, in that the apollon/BRUCE "knockout" late lethality (days E14.5 to the perinatal stage) is due to a placental development problem denoting a specific role for apollon in spongiotrophoblast proliferation **(132**, **133)**, while the early lethality

(prior to E4.5) of survivin "knockout" mice is most likely related to survivin's critical role in cytokinesis. Significantly, this phenotype is also seen in other "knockout" and RNAi depletion studies of survivin orthologues in man **(134**, **135)**, *Drosophila* **(136)**, *C. elegans* **(137)**, and yeast **(138**, **139)**. Recently, BRUCE has also been identified as crucial to the final stages of cytokinesis and midbody ring formation **(22)**, underlying its similarity to the survivin BIR domain (Fig. 3.1). Another surprising "knockout" phenotype is revealed for the cIAP1 orthologue in zebrafish (*Danio rerio*). Fish embryos were chemically mutagenized to identify genes essential for maintaining endothelial cell survival and blood vessel homeostasis during vascular development **(42)**. In the absence of functional cIAP1 gene, a caspase-8-dependent apoptotic program takes place that leads to vessel regression. This phenotype is observed in the fish due to the lack of a redundant copy of the cIAP2 gene that is normally present in other vertebrates such as mouse and man.

Although an overt phenotype is not initially apparent in the four other IAP "knockout" models (likely due to redundancy), these models all revealed specific attributes due to the loss of a specific *in vivo* IAP. The models demonstrate increased sensitivity of certain cell types to specific death, with dramatic results in some cases, such as the rescue of cIAP2-null mice to a lethal dose challenge of lipopolysaccharide (LPS) due to the death of cIAP2-null macrophages in response to LPS, and abatement of the inflammatory response **(69)**. Furthermore, primary or immortalized cells derived from these animals, such as MEFs, provide valuable tools to experimentally dissect the roles of various molecules in apoptotic pathways. Without such MEFs, we could not have clearly demonstrated the functional redundancy of the cIAP1 and cIAP2 genes in TNFalpha-mediated TNFR1 signaling **(70)**.

Pathological Roles of the IAPs in Cancer

The IAPs are clearly implicated in oncogenesis. This subject has been covered in many recent and excellent reviews [*e.g.*, **(131**, **140**, **141)**]. Therefore, we will limit our discussion here and focus on other disorders not often discussed. However, for some examples of direct IAP involvement in cancer, see Table 3.2.

IAP Dysfunction in Human Diseases Other Than Cancer

The importance of the IAPs in normal tissue homeostasis and disease progression has come to light recently with evidence of loss-of-function mutations in various disorders (Table 3.2). NAIP (*birc1*), the first mammalian IAP identified, discovered through its genetic association with the most severe cases of spinal muscular atrophy, likely represents a phenotypic modifier **(6)**. The *NAIP* locus has undergone expansion and specialization in the mouse with several polymorphic alleles

Table 3.2 Summary of IAP and antagonist natural mutations and deletions in mammals leading to disease

Gene	Disease	Comments
Birc1 (NAIP)	Type 1 spinal muscular atrophy (SMA, type 1)	Approximately 60% of type 1 SMA individuals display a deletion in the NAIP gene in addition to the deletion of the causative adjacent gene, SMN. NAIP is thought to be a phenotypic modifier, with loss of function producing a worse prognosis **(6)**.
Birc1e (NAIP5)	*Legionella* infection and susceptibility (legionnaire's disease)	Mice lacking the *naip5*/ *birc1e* gene, at the *Lgn1* locus, are susceptible to *Legionella pneumophila* infection **(142**, **143)**. NAIP5, a BIR-containing gene and a NOD-like receptor, is a major regulator of intracellular replication of *Legionella* in macrophages **(159)**.
Birc2 (cIAP1)	Multiple myeloma (MM)	cIAP1 and cIAP2 are deleted in some MM cancers, as part of a larger trend of activating mutations for the alternative NF-kappaB pathway **(76**, **160)**. Hence,
	Various carcinomas	the negative regulatory role of cIAP1 or cIAP2 as E3 ubiquitin ligases for NIK is removed, allowing for NIK stabilization and activation of the alternative NF-kappaB pathway.
		cIAP1 and cIAP2 genes, at 11q22, are commonly amplified in many cancers, with visibly increased expression of cIAP1. This phenomenon is also conserved in some murine tumors that also lead to co-amplification of YAP. Both cIAP1 and YAP have been shown to cooperate in transformation **(161)**. cIAP1 can also cooperate with Myc to transform. This results from cIAP1's ability to induce the proteosomal degradation of the Myc antagonist Mad1 **(162)**.
Birc3 (cIAP2)	MALT lymphoma	*cIAP2* is translocated in t(11;18)(q21;q21)-bearing lymphomas **(163)**. The chromosomal translocation fuses the three BIR domains of cIAP2 with the paracaspase domain of MALT1, while preserving the cIAP2, NFkappaB-responsive, promoter.
	Multiple myeloma (MM)	cIAP1 and cIAP2 genes are deleted in some MM cancers, as part of a larger trend of activating mutations for the alternative NF-kappaB pathway **(76**, **160)**. Hence, the negative regulatory role of cIAP1 or cIAP2 as E3 ubiquitin ligases for NIK is removed, allowing for NIK stabilization

Table 3.2 (continued)

Gene	Disease	Comments
		and activation of the alternative NF-kappaB pathway.
	Various Carcinomas	cIAP1 and cIAP2 genes, at 11q22, are commonly amplified in many cancers, with visibly increased expression of cIAP1, while cIAP2 expression is often overlooked. This DNA amplification is also conserved in some murine tumors, which also leads to co-amplification of YAP **(161)**. cIAP2 may also cooperate in transformation in these cases, but this remains to be conclusively proven.
Birc4 (XIAP)	X-linked lymphoproliferative disorder (XLP)	Apoptosis of lymphocytes from XIAP-deficient patients is enhanced in response to various stimuli including CD3, Fas, and TRAIL. In addition, XIAP-deficient XLP patients (XLP-2), like SAP-deficient XLP patients (XLP-1), have low numbers of NK T cells. XLP patients can exhibit a fatal response to EBV, the viral cause of infectious mononucleosis [*presumably due to a failure(s) in NK and lymphocyte homeostasis and signaling*] **(149)**.
Omi	Motor neuron degeneration 2 (*mnd2*)	The mouse mutant *mnd2* exhibits muscle wasting, neurodegeneration, and juvenile lethality. Loss of Omi protease activity increases the susceptibility of mitochondria to permeability transition and increases the sensitivity of MEFs to stress-induced cell death **(164)**.

expressed under different promoters. The *NAIP5* (*birc1e*) gene is responsible for resistance to *Legionella pneumophila* infection of macrophages **(142, 143)**. NAIP5 protein senses flagellin of *Legionella* or *Salmonella* and stimulates caspase-1 activation. This restricts bacterial infection and replication by promoting the fusion of bacteria-containing phagosomes with lysosomes and by releasing proinflammatory cytokines **(144–147)**. Recent work in cell culture models suggests that NAIP may perform a similar immunomodulatory function in humans **(148)**.

Recently, loss-of-function mutations in XIAP (*birc4*) have been found to underlie 20% of cases of X-linked lymphoproliferative (XLP) disorder, pointing to a direct role for this IAP in NK cell and lymphocyte homeostasis **(149, 150)**. XIAP-deficient XLP patients (XLP-2) have low NK T-cell numbers similar to SAP-deficient XLP patients (XLP-1, responsible for the other 80% of XLP cases) **(149)**. XLP patients can exhibit a fatal response to EBV infection, the viral cause of infectious mononucleosis, presumably due to a failure in NK and lymphocyte homeostasis and signaling.

Alterations in XIAP protein translation, or protein stability, are found to underlie other genetic disorders for which XIAP is not the primary lesion. Ruggero's laboratory demonstrates impaired cap-independent translation of XIAP, bcl-xL, and p27(Kip1) in mice and patients with X-linked dyskeratosis congenita (X-DC) **(151)**. X-DC is characterized by bone marrow failure, skin abnormalities, and increased susceptibility to cancer. A mutated DKC1 gene is responsible for X-DC, and it normally encodes a pseudouridine synthase that modifies rRNA. A specific defect in IRES (internal ribosome entry site)-dependent translation in Dkc1(m) mice and in cells from X-DC patients results in impaired translation of messenger RNAs containing IRES elements, including those encoding the tumor suppressor p27(Kip1) and the antiapoptotic factors Bcl-xL and XIAP. Moreover, Dkc1(m) ribosomes are unable to direct translation from IRES elements present in viral mRNAs. These findings reveal a potential mechanism by which defective ribosome activity leads to disease and cancer. Another disease-associated gene, PABPN1, affects XIAP translation in oculopharyngeal muscular dystrophy (OPMD) **(152)**. OPMD is a late-onset, progressive disease caused by the abnormal expansion of a polyalanine tract-encoding (GCG)(n) trinucleotide repeat in the poly-(A) binding protein nuclear 1 (PABPN1) gene. A novel beneficial function of wild-type PABPN1 is identified to reduce mutant PABPN1 toxicity in both cell and mouse models of OPMD. In addition, wild-type PABPN1 provides some protection to cells against proapoptotic insults distinct from the OPMD mutation such as staurosporine treatment and Bax expression. Conversely, PABPN1 knockdown (which itself is not toxic) makes cells more susceptible to apoptotic stimuli. The protective effect of wild-type PABPN1 is mediated by its regulation of XIAP protein translation. This normal activity of PABPN1 is partially lost for mutant PABPN1; elevated levels of XIAP are seen in mice expressing a wild-type but not a mutant PABPN1 transgene. This raises the possibility that a compromise of the antiapoptotic function of PABPN1 might contribute to the disease mechanism of OPMD. In an unrelated study, using a cell culture model of viral-induced progressive transformation of keratinocytes, a progressive shift toward IRES-dependent, rapamycin-insensitive, translation results in increased expression of XIAP **(153)**. During keratinocyte transformation, the protein synthesis network contracts, as shown by the reduction in key cap-dependent translation factors. The switch from cap- to IRES-dependent translation correlates with the progressive activation of c-Src, an activator of AMP-activated protein kinase (AMPK), which controls energy-consuming processes, including protein translation. As cellular protein synthesis is a major energy-consuming process, the observed reductions in cell size and protein amount are postulated to provide the energy required for cell survival and proliferation. The cap- to IRES-dependent switch may be part of a gradual optimization of energy-consuming mechanisms that redirects cellular processes to enhance cell growth (and survival), in the course of transformation. These three studies all support a specialized, stress-regulated, translational control mechanism for XIAP that is

exploited or altered in cancer and disease, confirming earlier studies demonstrating XIAP's unique translational properties **(154)**.

In another disease example, XIAP protein stability is compromised in patients suffering from Wilson disease (WD) and in similar animal disorders, such as dogs afflicted with a hereditary copper toxicosis **(155)** or the Jackson toxic milk mouse **(156)**, leading to the eventual complete disappearance of the XIAP protein in some cases. Previously, a novel role for XIAP in copper homeostasis was identified through its regulation of MURR1/COMMD1 levels **(157)**. WD is due to mutations in ATP7B, which encodes an intracellular metal-transporting P-type ATPase. In WD, holoceruloplasmin production and biliary excretion of copper are decreased, leading to copper overload, oxidative stress, and apoptotic cell death. Another copper-binding protein, COMMD1, is inactive in Bedlington terrier hereditary copper toxicosis. XIAP promotes the ubiquitination and degradation of COMMD1, a protein that promotes the efflux of copper from the cell **(157)**. Through its effects on COMMD1, XIAP can regulate copper export from the cell. XIAP also binds copper directly and undergoes a substantial conformational change when bound. This in turn destabilizes XIAP, resulting in lowered steady-state levels of the protein. Furthermore, copper-bound XIAP is unable to inhibit caspases, and cells that express this form of the protein exhibit increased rates of cell death in response to apoptotic stimuli. These events take place in the setting of excess intracellular copper accumulation as seen in copper toxicosis disorders such as WD, or the Jackson toxic milk mouse, and establish a new relationship between copper levels and the regulation of cell death via XIAP **(155, 156)**.

All these mutations identify physiological roles that the various IAPs and their antagonists play in normal cells (Table 3.2). This knowledge will help guide the development of future IAP-targeted drugs and delineate the possible side effects these compounds may have.

Conclusions

Apoptosis is controlled at multiple intracellular nodes, each of which is influenced by pro- and antiapoptotic proteins. The equilibrium between the cell death that induces the caspase cascade and the inhibition of that process by the IAPs constitutes a fundamental decision point. The recurrent upregulation of IAP activity in cancer cell lines and tumors indicates that this decision point is important in determining cell fate. The IAPs not only control cell death, but also influence signal transduction pathways, differentiation, protein turnover, and progression through the cell cycle. Still, many aspects of IAP function in all these processes remain to be elucidated.

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Chapter 4
Structural Biology of Programmed Cell Death

Yigong Shi

Abstract Structural and biochemical characterization on death receptors, Bcl-2 family proteins, caspases, IAPs, Smac/DIABLO, and other regulatory proteins has revealed significant insights into the molecular mechanisms of apoptosis. This chapter summarizes these advances and presents our current understanding of apoptosis from a structural and mechanistic perspective.

Keywords Macromolecule structure · Bcl-2 family proteins · Inhibitor-of-apoptosis proteins · Smac · DIABLO · Death receptor

Introduction

Structural biology is an important and integral component of the modern experimental biology. Insights revealed by X-ray crystallography, nuclear magnetic resonance, electron microscopy, and other biophysical methods have fundamentally changed our way of thinking and have tremendously improved our understanding of the biological system. Similar to other research disciplines, the concept of apoptosis and many aspects of its mechanisms have been made crystal clear through the last decade of structural and biochemical investigation **(1**, **2)**.

In mammalian cells, apoptosis can be triggered by a wide spectrum of stimuli, from both intra- and extracellular environments. The intracellular stimuli, such as DNA damage, generally cause the activation of the BH3-only Bcl-2 family proteins, which invariably leads to the release of proapoptotic factors from the intermembrane space of mitochondria into the cytoplasm. One of these factors, cytochrome c, directly activates Apaf-1 and, in the presence of dATP or ATP, induces the formation of

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a large multimeric complex "apoptosome." The apoptosome recruits and mediates the autoactivation of the initiator caspase, caspase-9, which goes on to activate caspase-3 and caspase-7, triggering a cascade of caspase cleavage and activation. The active caspases are subject to inhibition by the inhibitor-of-apoptosis (IAP) family of proteins. Another mitochondria-derived protein, Smac/DIABLO, physically interacts with multiple IAPs and removes IAP-mediated caspase inhibition during apoptosis. Thus, mitochondria play an indispensable role in the intrinsic form of apoptosis.

The extracellular stimuli, such as the withdrawal of growth factors, directly activate the death receptors through ligand-induced trimerization and assembly of a large death-inducing signaling complex (DISC) at the plasma membrane. Although the constituents of DISC have not been fully identified, one adapter protein, the Fas-associated death domain or FADD, appears to be the obligate component, which recruits and mediates the autoactivation of the initiator caspase, procaspase-8. The active caspase-8 cleaves and activates caspases-3 and -7. Thus, both extrinsic and intrinsic cell death results in the activation of caspases-3 and -7.

One physiological target of the active caspase-8 is Bid, a BH3-only protein that lacks a transmembrane region. After cleavage, the C-terminal fragment of Bid (truncated Bid, or tBid) translocates to the outer mitochondrial membrane and induces the release of proapoptotic factors. Thus, Bid mediates the crosstalk between the extrinsic and intrinsic forms of cell death.

Structural information is now available on the death ligands and receptors, all three subfamilies of the Bcl-2 proteins, both initiator and effector caspases, IAPs, Smac/DIABLO, several classes of signaling motifs, and the apoptosome. These structures and associated biochemical studies have revealed significant insights into the molecular mechanisms of the initiation, execution, and regulation of programmed cell death.

Death Receptors

Death receptors, located on the cell surface and characterized by multiple cysteine-rich extracellular domains, belong to the tumor necrosis factor (TNF) family of proteins **(3)**. They transmit apoptotic signals initiated by specific death ligands. The best-characterized death receptors include TNFR1 (also p55 or CD120a), Fas (also Apo1 or CD95), and DR3 (also Apo3 or Wsl1). Each of the death receptors contains a single death domain in the intracellular compartment, which is responsible for recruiting adapter proteins, such as FADD and TRADD, through homophilic interactions.

The activated death ligands are homotrimeric and induce trimerization of the death receptors upon binding. The associated adapter proteins further recruit other effector proteins. For example, the death effector domain of

FADD interacts with the prodomain of procaspase-8, thus bringing three procaspase-8 molecules into close proximity of one another and presumably facilitating their autoactivation.

Structures of the death ligands TNFα and TNFβ have been determined in their trimeric forms, which reveal a highly similar fold **(4–6)**. Each monomer contains a β-sandwich with a canonical jellyroll topology [Fig. 4.1(A)]. Three monomers intimately associate to form a bell-shaped homotrimer [Fig. 4.1(A)]. The extensive trimeric interface between adjacent monomers involves a mixture of hydrophobic contacts and hydrogen bond interactions. The co-crystal structure of the soluble extracellular domain of TNFR1 in complex with TNFβ reveals the specific recognition of a death ligand and the activated state of the death receptor **(7)**. In this complex, the four cysteine-rich domains (CRDs) within one TNFR1 molecule stack up vertically over a distance of 80 Å to form an elongated rod-like structure [Fig. 4.1(B)]. Each TNFR1 rod binds to two adjacent TNFβ monomers at their interface, and the three TNFR1 molecules do not directly interact with one another. Protruding loops from CRD2 (named the "50s loop") and CRD3 (named the "90s loop") bind to two distinct regions of TNFβ [Fig. 4.1(B)]. This structure allows the construction of a model in which the cytoplasmic regions of the receptors are proposed to cluster together.

Although TNFR1 contains four CRDs, only the second and third directly bind to the ligand. Another death receptor, DR5, contains only two CRDs. The structure of DR5 in complex with another death ligand, TRAIL, reveals a similar overall binding topology but significant differences that determine the specificity of this interaction **(8, 9)**. The two CRDs of DR5 correspond to CRD2 and CRD3 of TNFR1. Although CRD1 of DR5 interacts with TRAIL in a similar fashion as CRD2 of TNFR1, CRD2 of DR5 binds to TRAIL in a different conformation compared to CRD3 of TNFR1. These structural differences underlie distinct signaling specificity by different death ligands and may have important ramifications for the design of specific therapeutic agents.

In addition to the ligand-bound activated death receptors, the structure of the isolated TNFR1 was also reported **(10, 11)**. These structures reveal that the free receptors associate into dimers of two distinct types **(10)**. In one case, the two receptors are arranged in an antiparallel fashion, which would result in the separation of their cytoplasmic domains by a distance of over 100 Å [Fig. 4.1(C)]. Under this circumstance, these death receptors would prevent the intracellular adapter proteins from forming a productive signaling complex. Thus, the structure of this dimeric TNFR1 may represent the inactive form. In the other case, the two receptors are placed parallel to each other, with their TNF-binding surfaces fully exposed [Fig. 4.1(D)]. Each of the two receptors is capable of forming a trimeric assembly upon ligand binding; thus, this arrangement would result in clustering of TNF/TNFR1 trimers, a scenario that may enhance signaling efficiency.

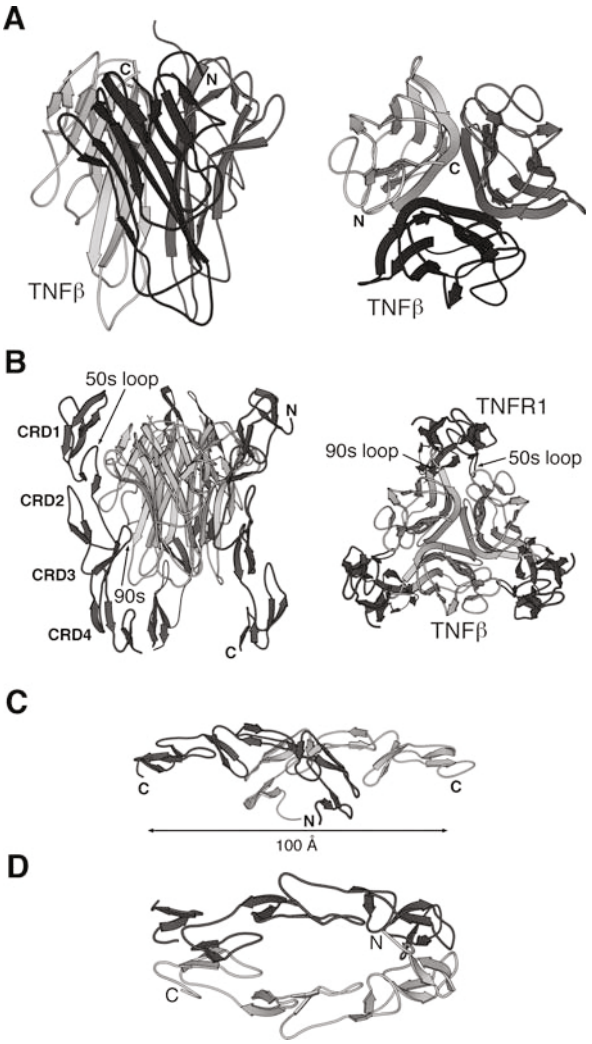

Fig. 4.1 Structure of death ligand, receptor, and their complex. (**A**) Structure of the trimeric TNFβ. Two perpendicular views are shown. (**B**) Structure of a complex between the soluble extracellular domain of TNFR1 and TNFβ. The four CRDs as well as the two important loops are labeled. (**C**) The suggested inactive form of a dimeric TNFR1 (extracellular domain). This configuration presumably prevents the formation of a productive intracellular signaling complex. (**D**) The suggested active form of a dimeric TNFR1 (extracellular domain). All figures were prepared using MOLSCRIPT **(71)**

Bcl-2 Family of Proteins

The Bcl-2 family of proteins controls the mitochondria-initiated intrinsic apoptosis and regulates the death receptor-initiated extrinsic cell death. More than two dozen Bcl-2 family proteins have been identified in multicellular organisms

examined to date **(12)**. On the basis of function and sequence similarity, the diverse Bcl-2 members can be grouped into three subfamilies. The antiapoptosis subfamily, represented by Bcl-2/Bcl-xL in mammals and CED9 in worms, inhibits programmed cell death by distinct mechanisms. For example, Bcl-2/Bcl-xL functions by preventing the release of mitochondrial proteins, whereas CED9 binds CED4 and prevents CED4-mediated activation of CED3. The proapoptosis proteins constitute two subfamilies, represented by Bax/Bak and Bid/Bim. Members in the Bcl-2/Bcl-xL subfamily contain all four conserved Bcl-2 homology domains (BH4, BH3, BH1, and BH2), while the Bax/Bak subfamily lack the BH4 domain and the Bid/Bim subfamily only contains the BH3 domain. Most members of the Bcl-2 family contain a single membrane-spanning region at their C-termini. Members of the opposing subfamilies as well as between the two proapoptotic subfamilies can dimerize, mediated by the amphipathic BH3 helix.

The first structure of the Bcl-2 family of proteins was determined on Bcl-xL by both X-ray crystallography and NMR spectroscopy **(13)**. This structure reveals two centrally located hydrophobic α helices (α5 and α6), packed by five amphipathic helices on both sides [Fig. 4.2(A)]. Interestingly, the Bcl-xL structure closely resembles the pore-forming domains of bacterial toxins such as diphtheria toxin. This similarity raised an interesting hypothesis that the Bcl-2 family of proteins may form pores at the outer mitochondrial membrane to regulate ion exchange. Indeed, this conjecture has been proven for nearly all members of the Bcl-2 family examined *in vitro*. However, it is unclear whether the pH-dependent ion-conducting property of Bcl-2 proteins occurs *in vivo* and, if so, how it contributes to the regulation of apoptosis.

Because most members of the Bcl-2 family associate with lipid membranes using their C-terminal transmembrane region, it is important to examine the structure of membrane-associated Bcl-2 family proteins. Unfortunately, due to technical difficulties, no such structure is yet available. Toward this ultimate goal, Bcl-xL was characterized in detergent micelles and, compared to the aqueous solution, exhibited significant structural differences **(14)**.

Bcl-xL or Bcl-2 interacts with the BH3-only subfamily of Bcl-2 proteins such as Bad, Bim, and Bid. The recognition mode was revealed by the solution structure of Bcl-xL bound to a BH3 peptide from Bak **(15)** ([Fig. 4.2(B)]. The BH3 peptide forms an amphipathic α helix and interacts with a deep hydrophobic groove on the surface of Bcl-xL. The binding of the BH3 domain causes a significant conformational change in Bcl-xL, including the melting of a short α helix (α3).

The structures of the other two groups of Bcl-2 family members have also been determined. Bid, containing only the BH3 domain and displaying very weak sequence homology with Bcl-xL, exhibits a conserved structure with that of Bcl-xL **(16, 17)**. The minor differences include the length and relative orientation of several helices as well as an extra α helix between helices α1 and α2 [Fig. 4.2(C)]. On the basis of the structure, a model was proposed to explain how the caspase-8-mediated cleavage of Bid (after residue 59) improves its proapoptotic function. In this mechanism, removal of the N-terminal 59 amino acids

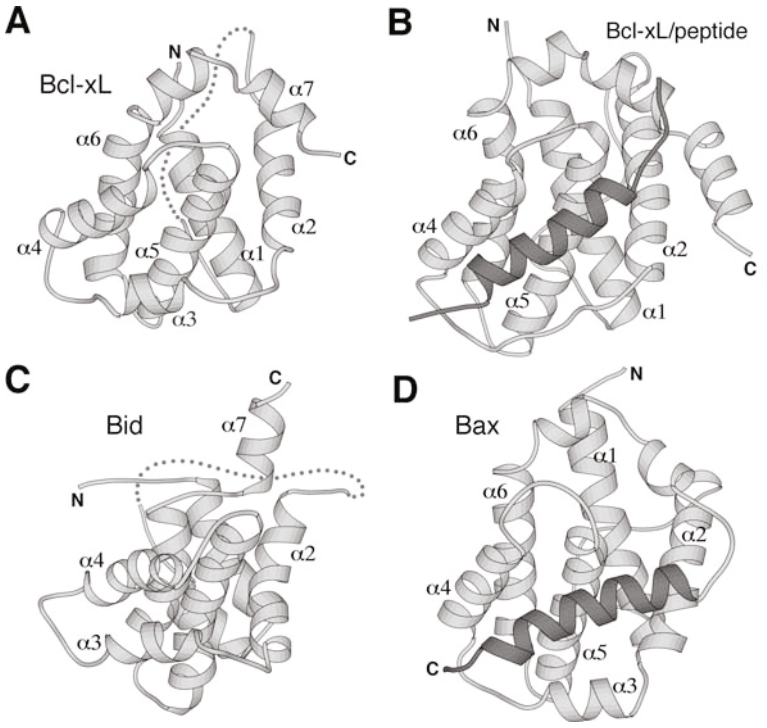

Fig. 4.2 Structure of the Bcl-2 family proteins. (**A**) Structure of Bax-xL. The flexible loop linking helices α1 and α2 are represented by a dotted line. (**B**) Structure of Bcl-xL bound to a BH3 peptide from Bad. This Bad peptide exists as an amphipathic helix, with the hydrophobic side binding to Bcl-xL. (**C**) Structure of the uncleaved form of Bid. Cleavage after Asp59 results in the activation of Bid, presumably due to exposure of the BH3 helix. (**D**) Structure of the full-length Bax. Note that the C-terminal amphipathic helix folds back to bind a hydrophobic surface groove, resembling the Bcl-xL-bound Bad peptide

leads to the exposure of the BH3 domain, which mediates binding to the other two subfamilies of Bcl-2 proteins.

Similar to Bid, the structure of the full-length Bax in aqueous solution closely resembles that of Bcl-xL **(18)**. Intriguingly, the C-terminal membrane-spanning region folds back to bind a hydrophobic groove that normally accommodates the BH3 domain of another Bcl-2 protein [Fig. 4.2(D)]. Thus, this structure may represent the inactive or closed form of the Bax/Bak group and suggests a regulatory role for their C-termini in the absence of apoptotic stimuli.

Bax and Bak exist as monomers in aqueous solution but can form homo-oligomers in the presence of detergents. These large homo-oligomers are thought to form channels with a pore size large enough to allow the passage of proteins such as cytochrome c, though direct biochemical and structural evidence is lacking. Despite structural information on all three subfamilies of Bcl-2 members, how these proteins regulate apoptosis remains largely

unknown. Biophysical and structural characterization of the Bcl-2 protein complexes under membrane-like conditions will likely reveal some surprising insights, although it is also possible that other important regulators of Bcl-2 proteins have yet to be identified.

Caspases—The Executioners of Apoptosis

Caspases are a family of highly conserved cysteine proteases that cleave after an aspartate in their substrates **(19)**. The critical involvement of a caspase in apoptosis was first documented in 1993 **(20)**, in which CED3 was found to be indispensable for programmed cell death in the nematode *Caenorhabditis elegans*. Since then, compelling evidence has demonstrated that the mechanism of apoptosis is evolutionarily conserved, executed by caspases from worms to mammals. At least 14 distinct mammalian caspases have been identified **(21)**.

Caspases involved in apoptosis are divided into two groups: the initiator caspases, which include caspases-1, -2, -8, -9, and -10, and the effector caspases, which include caspases-3, -6, and -7. An initiator caspase invariably contains an extended N-terminal prodomain (>90 amino acids) important for its function, whereas an effector caspase contains only 20 to 30 residues in its prodomain sequence. All caspases are synthesized in cells as catalytically inactive zymogens and must undergo proteolytic activation. The activation of an effector caspase, such as caspase-3 or -7, is performed by an initiator caspase, such as caspase-9, through internal cleavages to separate the large and small subunits. The initiator caspases, however, are autoactivated under apoptotic conditions.

The first caspase structure was determined on caspase-1 (or ICE, interleukin 1β-converting enzyme) bound with a covalent peptide inhibitor **(22, 23)**. Structural information is now available on caspase-3 **(24, 25)**, caspase-7 **(26)**, caspase-8 **(27, 28)**, and, more recently, caspase-9 **(29)**. In each case, caspase is bound to a synthetic peptide inhibitor [Fig. 4.3(A)]. These structures reveal that the functional caspase unit is a homodimer, with each monomer comprising a large (∼20-kDa) and a small (∼10-kDa) subunit. Homodimerization is mediated by hydrophobic interactions, with six antiparallel β-strands from each monomer forming a single contiguous 12-stranded β-sheet -[Fig. 4.3(A)]. Five α helices and five short β–strands are located on either side of the central β-sheet, giving rise to a globular fold. The active sites, highly conserved among all caspases and located at two opposite ends of the β-sheet, are formed by four protruding loops (L1, L2, L3, and L4) from the scaffold.

Caspases recognize at least four contiguous amino acids, named P4-P3-P2-P1, in their substrates, and cleave after the C-terminal residue (P1), usually an Asp. The binding sites for P4-P3-P2-P1 are named S4-S3-S2-S1, respectively, in caspases. These sites are located in the catalytic groove. The L1 and L4 loops constitute two sides of the groove (Fig. 4.3). Loop L3 and the following β-hairpin, collectively referred to as L3, are located at the base of the groove.

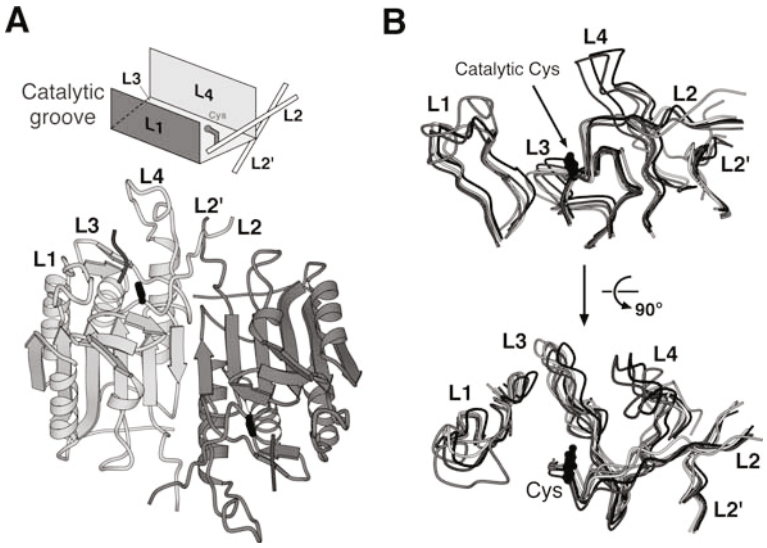

Fig. 4.3 Structural features of caspases. (**A**) A representative structure of the inhibitor-bound caspase-3 (PDB code 1DD1). The bound peptide inhibitor is shown in black. The four surface loops that constitute the catalytic groove of one heterodimer are labeled. The apostrophe denotes the other heterodimer. Note that L2' stabilizes the active site of the adjacent heterodimer. The substrate-binding groove is schematically shown above. (**B**) The active-site conformation of all known caspases is conserved. Of the four loops, L1 and L3 are relatively constant while L2 and L4 exhibit greater variability. The catalytic residue Cys is shown in black. Two perpendicular views are shown

Loop L2, which harbors the catalytic residue Cys, is positioned at one end of the groove with Cys poised for binding and catalysis. These four loops, of which L1 and L3 exhibit conserved length as well as composition, determine the sequence specificity of the substrates.

The S1 and S3 sites are nearly identical among all caspases. The P1 residue (Asp) is coordinated by three invariant residues at the S1 site, an Arg from the L1 loop, a Gln at the beginning of the L2 loop, and an Arg at the end of the L3 loop. The Arg residue on the L3 loop also coordinates the P3 residue (Glu). The S2 and S4 sites are coordinated mainly by the L3 and L4 loops. Since the sequence of L4 is most divergent among caspases, the P2 and P4 residues exhibit greater sequence variation. For example, the L4 loop in caspase-1, -8, or -9 is considerably shorter than that in caspase-3 or -7, resulting in a shallower substrate-binding groove. This observation is consistent with a bulky hydrophobic residue as the preferred P4 residue for caspase-1, -8, or -9.

The conformational similarity at the active site is extended to surrounding structural elements. In particular, loops L4 and L2 from one catalytic subunit are stabilized by the N-terminus (loop L2') of the small subunit of the other catalytic subunit, forming the so-called loop bundle **(30)**.

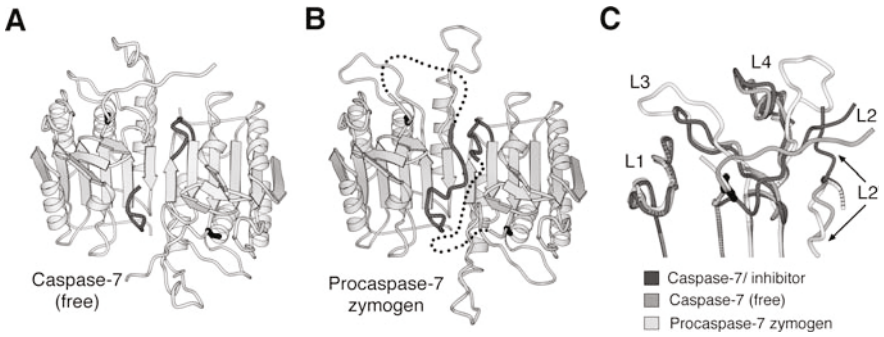

Fig. 4.4 Mechanisms of procaspase-7 activation and substrate binding. (**A**) Structure of an active and free caspase-7 (PDB code 1K88). The active-site loops are flexible. Despite an interdomain cleavage, the L2' loop still exists in the closed conformation, indicating an induced-fit mechanism for binding to inhibitors/substrates. (**B**) Structure of a procaspase-7 zymogen (PDB code 1K86). Compared to that of the inhibitor-bound caspase-7, the conformation of the active site loops does not support substrate binding or catalysis. The L2' loop, locked in a closed conformation by covalent linkage, is occluded from adopting its productive and open conformation. (**C**) Comparison of the conformation of the active site loops. Compared to the procaspase-7 zymogen or the free caspase-7, the L2' loop is flipped 180° in the inhibitor-bound caspase-7 to stabilize loops L2 and L4

Most structural information is derived from the inhibitor-bound caspases, which share the same topology at the active site. These observations give the impression that the substrate-binding grooves of caspases are preformed. However, in the structure of the free caspase-7 **(30)**, these loops are flexible and quite different from those in the inhibitor-bound caspase-7 (Fig. 4), suggesting that the inhibitor-bound state is transient and trapped by the covalent peptide inhibitors. Thus, substrate binding and catalysis may be a process of induced fit, accompanied by some large conformational changes, such as the back-and-forth flipping of the critical L2' loop.

Why are procaspase zymogens (except procaspase-9) catalytically inactive? The answer was partially provided by the crystal structure of procaspase-7 **(30**, **31)**, which reveals significant conformational changes in the four active site loops (Fig. 4.4). Except L1, all three other loops move away from their productive positions, unraveling the substrate-binding groove. Most notably, the loop bundle seen in the inhibitor-bound caspases is missing in the procaspase-7 zymogen as the L2' loop is flipped by 180°, existing in a "closed" conformation. This closed conformation is locked by the unprocessed nature of the procaspase-7 zymogen.

The ability of L2' to move freely in response to inhibitor/substrate binding is a decisive feature for the active caspase-7. This ability is acquired through activation cleavage after Asp198 in procaspase-7. Because L2' is at the N-terminus of the small subunit, inverting the order of primary sequences of

the large and small subunits could free L2' and hence constitutively activate caspases. Indeed, this prediction was confirmed for caspases-3 and -6 **(32)** as well as for the *Drosophila* caspase drICE **(33)**.

In contrast to most other caspases, procaspase-9 exhibits a basal level of activity prior to proteolytic activation **(34)**. This surprising feature may be explained by the fact that procaspase-9 contains an expanded L2 loop, which could allow enough conformational flexibility such that procaspase-9 does not need an interdomain cleavage to have the L2' loop move to its productive conformation.

Inhibitors of Apoptosis (IAP)

The inhibitor of apoptosis (IAP) family of proteins, originally identified in the genome of baculovirus, suppresses apoptosis by interacting with and inhibiting the enzymatic activity of mature caspases **(35)**. At least eight distinct mammalian IAPs, including XIAP, c-IAP1, c-IAP2, and ML-IAP/Livin, have been identified, and they all have antiapoptotic activity in cell culture. A structural feature common to all IAPs is the presence of at least one BIR (baculoviral IAP repeat) domain, characterized by a conserved zinc-coordinating Cys/His motif ($CX_2CX_{16}HX_6C$). Some IAPs, such as XIAP and ML-IAP/Livin, also contain a C-terminal RING finger, a C_3HC_4-type zinc-binding module. Most mammalian IAPs have more than one BIR domain, with the different BIR domains exhibiting distinct functions. For example, in XIAP, c-IAP1, and c-IAP2, the third BIR domain (BIR3) potently inhibits the activity of processed caspase-9, whereas the linker region between BIR1 and BIR2 selectively targets active caspase-3. The RING fingers were found to exhibit ubiquitin ligase (E3) activity, which may regulate the self-destruction or degradation of active caspases through the 26S proteasome pathway.

The structures of various BIR domains, determined by both NMR and X-ray crystallography **(36–38)**, reveal a highly conserved topology, with a three-stranded antiparallel β–sheet and four α helices [Fig. 4.5(A)]. The three cysteine and one histidine residues, invariant in all BIRs, coordinate a zinc atom. Although the BIR2 and BIR3 domains of XIAP share an identical fold, structure-based mutational analysis revealed that different regions are involved in the interaction with and inhibition of caspases-3 and -9 **(36, 37)**. Several amino acids in the linker sequence preceding BIR2 were found to be essential in targeting caspase-3, while residues on the surface of XIAP-BIR3 inhibited caspase-9.

The smallest IAP is survivin, with only one BIR domain and a C-terminal acidic stretch. In contrast to the relatively stable expression levels of other IAPs, the expression of survivin oscillates with cell cycle and peaks at the G2/M phase **(39)**. Recombinant survivin does not inhibit caspase activity *in vitro* and appears to play an important role in mitosis. Although the structures of survivin

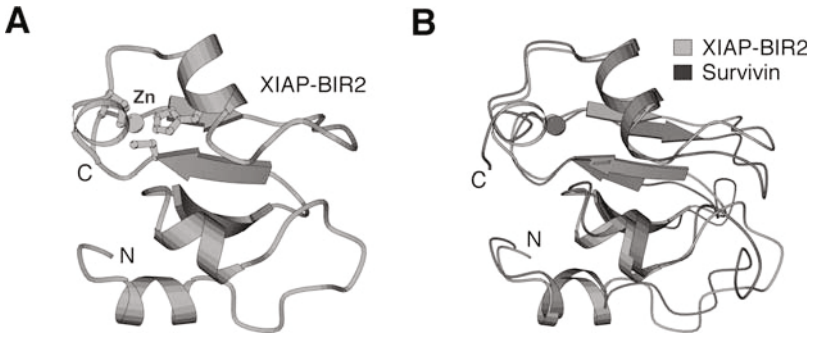

Fig. 4.5 Structure of the BIR domains. (**A**) Structure of the BIR2 domain of XIAP. The bound zinc atom as well as the four conserved Cys/His residues are labeled. (**B**) Superposition of the structure of XIAP-BIR2 with that of human survivin

reveal that the BIR domain adopts the canonical fold [Fig. 4.5(B)], two contrasting modes of dimerization were proposed **(40–42)**, each with supporting evidence.

Caspase-IAP Complex

The IAP-bound structures were determined for two highly conserved effector caspases, caspases-3 and -7 **(43–45)** [Figs. 4.6(A) and (B)]. In the structures, the linker peptide N-terminal to XIAP-BIR2 forms highly similar interactions with both caspases-3 and -7 [Fig. 4.6(C)]. Compared to the covalent peptide inhibitors, the linker segment of XIAP occupies the active site of caspases, resulting in a blockade of substrate entry. Four residues from the XIAP linker peptide, Gly144-Val146-Val147-Asp148, occupy the corresponding positions for the P1-P2-P3-P4 residues of the substrates, respectively [Fig. 4.6(D)]. The P1 position is occupied by the N-terminal Gly144 of these four residues. Thus, this orientation is the reverse of that observed for the tetrapeptide caspase inhibitors, in which the P1 position is occupied by the C-terminal Asp. Interestingly, despite a reversal of relative orientation, a subset of interactions between caspase-7 or -3 and XIAP closely resemble those between caspase-7 or -3 and its tetrapeptide inhibitor DEVD-CHO. Asp148 of XIAP binds the S4 pocket in the same manner as the P4 residue of the covalent peptide inhibitors. In addition, Val146 makes a similar set of van der Waals contacts to surrounding caspase residues as does the P2 residue.

Interestingly, although the linker sequence between BIR1 and BIR2 of XIAP plays a dominant role in inhibiting caspases-3 and -7, this fragment in isolation is insufficient. However, an engineered protein with the linker peptide fusing either the N- or C-terminal to BIR1 was able to bind and inhibit caspase-3 while neither BIR1 nor BIR2 in isolation exhibited any effect **(36)**. These observations suggest that the linker peptide needs to be presented in a "productive"

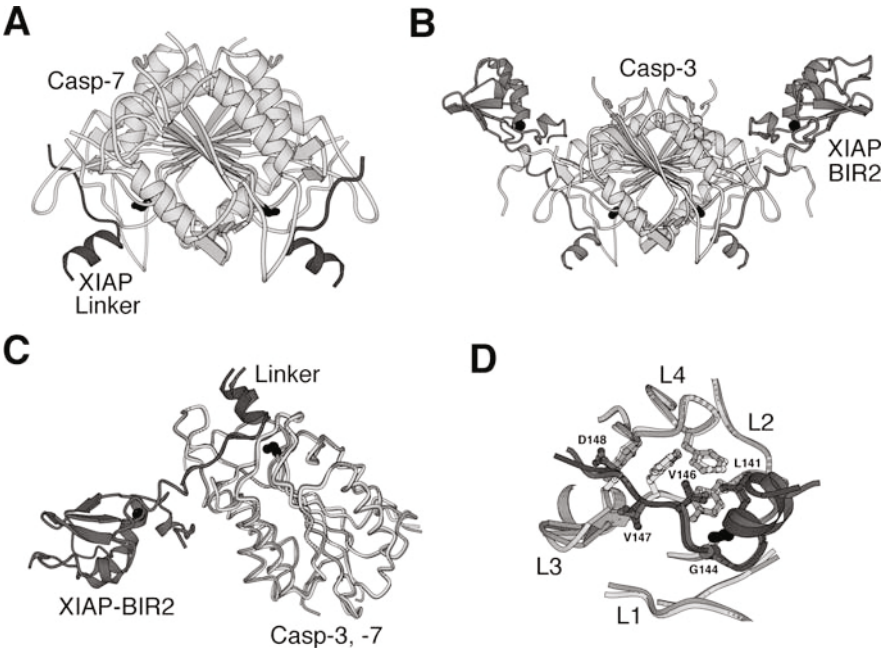

Fig. 4.6 Mechanisms of IAP-mediated inhibition of effector caspases. (**A**) Structure of caspase-7 bound with an XIAP linker peptide preceding the BIR2 domain. (**B**) Structure of caspase-3 bound with an XIAP fragment including BIR2 and its preceding linker peptide. (**C**) Superposition of the structures of caspases-3 and -7 together with their bound XIAP fragments. The interactions primarily occur between a linker segment N-terminal to the BIR2 domain of XIAP and the active site of caspase-3 or -7. (**D**) Close-up view of the active sites of caspases-3 and -7 bound to their respective XIAP fragments. Two hydrophobic residues of XIAP, Leu141 and Val146, make multiple van der Waals interactions with a conserved hydrophobic pocket on caspase-3 or -7. Asp148 of XIAP, occupying the S4 pocket, hydrogen bonds to neighboring residues in caspase-3 or -7

conformation by surrounding BIR domains while their identities do not matter. In support of this hypothesis, the linker peptide fused to glutathione S-transferase (GST) was able to inhibit caspases-3 and -7 **(43**, **44)**. Nevertheless, the BIR domains also contribute to the inhibition of caspases, as XIAP exhibits about 20-fold higher potency than the GST-linker peptide fusion. Consistent with this observation, XIAP-BIR2 also makes direct contact to caspase-3 in the crystal structure **(45)** [Fig. 4.6(B)].

Although the mode of IAP-mediated inhibition of the effector caspases is well characterized, how IAPs inhibit the initiator caspase, caspase-9, remain unclear. Nevertheless, biochemical investigation has revealed an encouraging hint. Although both BIR2 and BIR3 of XIAP can inhibit caspase-9, BIR3 displays tighter binding affinity and higher potency. Mutation of Trp310 or Glu314 in BIR3 completely abrogated XIAP-mediated inhibition of caspase-9 while amino acids outside BIR3 are unnecessary for this inhibition **(37)**. In the

structure, these two residues are located close to each other, suggesting that this region is involved in binding and inhibiting caspase-9. Indeed, BIR3 of XIAP binds to the N-terminus of the small subunit of caspase-9 that becomes exposed after proteolytic processing **(46)**. This binding presumably brings BIR3 close to the active site of caspase-9, which may block substrate entry and subsequent catalysis. Supporting this hypothesis, when the N-terminal four residues of the caspase-3 small subunit were replaced with those from caspase-9, the resulting protein could be inhibited by BIR3. Since BIR3 binds the N-terminus of a flexible 20-residue loop in caspase-9, how exactly BIR3 prevents substrate entry remains to be investigated by structural approaches.

Caspase-p35 Complex

IAPs are not the only natural inhibitors to caspases. In contrast to XIAP, which only affects caspases-3, -7, and -9, the baculoviral p35 protein is a pan-caspase inhibitor, and it potently targets most caspases both *in vivo* and *in vitro* **(47)**. Caspase inhibition by p35 correlates with the cleavage of its reactive site loop after Asp87, which leads to the translocation of the N-terminus of p35 into the active site of caspases **(48**, **49)**. The crystal structure of caspase-8 in complex with p35 reveals that the catalytic residue Cys360 of caspase-8 is covalently linked to the Asp87 of p35 through a thioester bond **(50)** (Fig. 4.7). Although a thioester bond is generally susceptible to hydrolysis, this bond is protected by the neighboring N-terminus of p35, which occludes access by water molecules.

Compared with the structure of the uncleaved p35 **(51)**, the cleavage after Asp87 of the reactive site loop produces a dramatic conformational switch **(50**, **52)**. The C-terminal end of the reactive site loop flips 180° and folds back to form a β–strand as part of an antiparallel β-sheet. The vacated space is in turn occupied by the N-terminus of p35, which springs from a closed conformation into an open form, moving over a distance of 20 Å (Fig. 4.7(C)]. Much of this conformational change occurs in the absence of binding to caspase-8.

Another protein, the serpin CrmA derived from the cowpox virus, can also inhibit several caspases, likely through a similar covalent modification. This unique covalent mechanism adds to the complexity of caspase inhibition by natural proteins. It is important to note, however, that the equivalent of p35 in the mammal genome has not been identified.

Smac/DIABLO

In normal surviving cells that have not received an apoptotic stimulus, the aberrant activation of caspases can be inhibited by IAPs. In cells signaled to undergo apoptosis, however, this inhibitory effect must be suppressed. This process is mediated by a mitochondrial protein named Smac (second

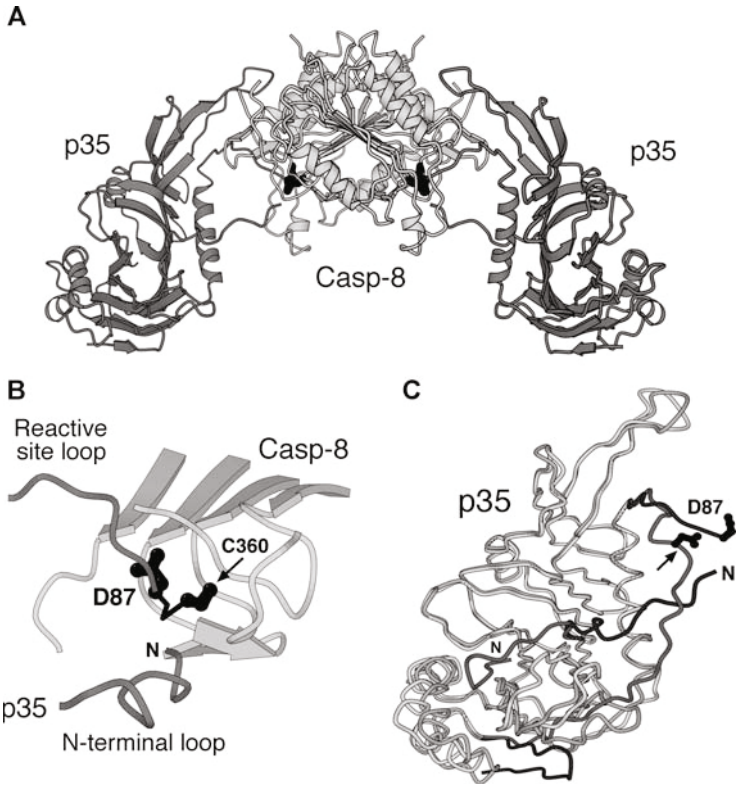

Fig. 4.7 Mechanisms of p35-mediated pan-caspase inhibition. (**A**) An overall view of caspase-8 covalently bound to its inhibitor p35. (**B**) A close-up view of the covalent inhibition of caspase-8 by p35. The thioester intermediate is shown between Asp87 of p35 and Cys360 (active-site residue). The N-terminus of p35 restricts solvent access to this intermediate. (**C**) Superposition of the structure of uncleaved p35 with that bound to caspase-8. The arrow indicates the position of proteolytic cleavage

mitochondria-derived activator of caspases) **(53)** or DIABLO (direct IAP binding protein with low pI) **(54)**. Smac, synthesized in the cytoplasm, is targeted to the intermembrane space of mitochondria. Upon apoptotic stimuli, Smac is released from mitochondria into the cytosol, together with cytochrome c. Whereas cytochrome c directly activates Apaf-1 and caspase-9, Smac interacts with multiple IAPs and relieves their inhibitory effect on both initiator and effector caspases.

Structural analysis reveals that Smac exists as an elongated dimer in solution, spanning over 130 Å in length **(55)** [Fig. 4.8(A)]. The wild-type Smac protein binds to both the BIR2 and the BIR3 domains of XIAP but not BIR1. In contrast, the monomeric Smac mutants retain strong interaction with BIR3 but can no longer form a stable complex with BIR2. Because the linker sequence immediately preceding BIR2 is involved in binding and inhibiting caspase-3,

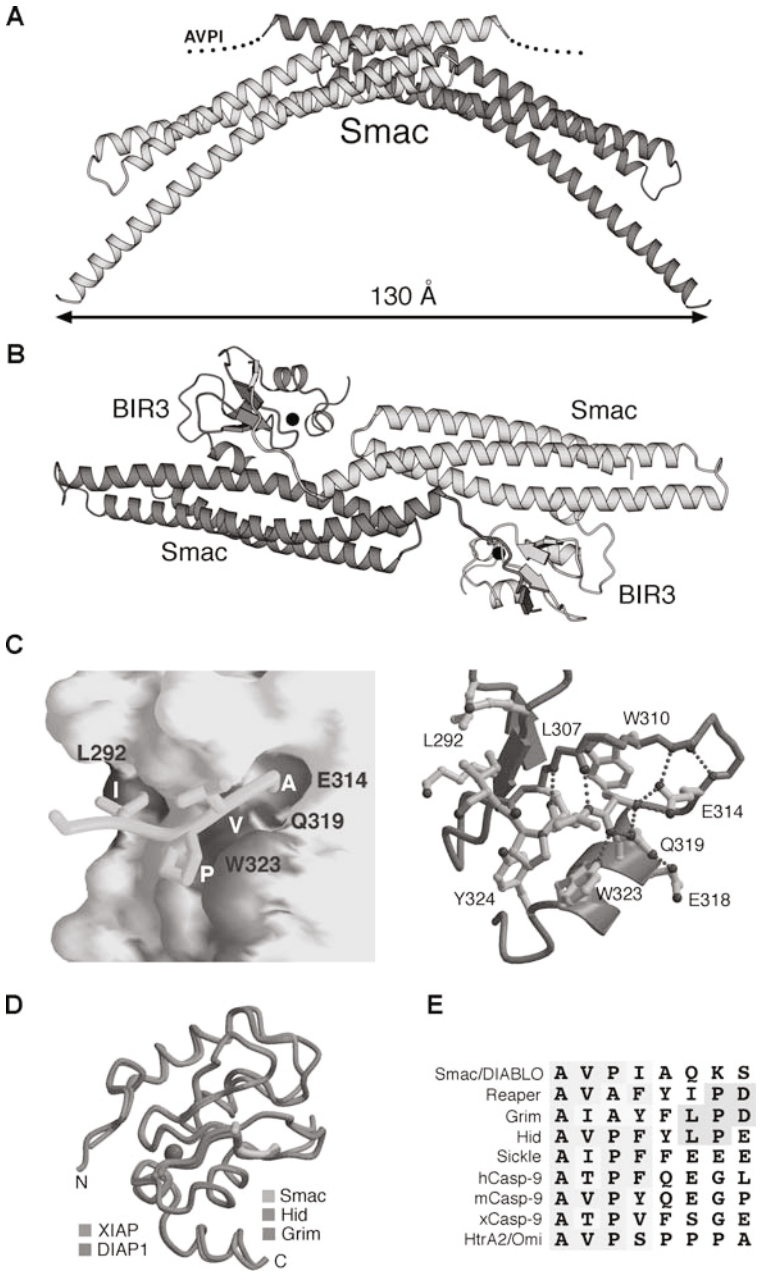

Fig. 4.8 Smac structure and function. (**A**) Structure of the mature Smac. The disordered N-terminal residues are shown as dotted lines. (**B**) Structure of a monomeric Smac bound to the BIR3 domain of XIAP. (**C**) A close-up view of the Smac N-terminal tetrapeptide bound to the BIR3 surface groove. The BIR3 domain is shown either by degree of hydrophobicity (left panel) or in ribbon diagram (right panel) to highlight the interactions. The amino and carbonyl

Smac monomers cannot relieve the IAP-mediated inhibition of caspase-3. Despite maintenance of interactions with BIR3, the monomeric Smac mutants also exhibit compromised activity in relieving IAP-mediated inhibition of caspase-9.

The N-terminal mitochondria-targeting sequence of Smac is proteolytically removed upon import. The freshly exposed N-terminal residues play an indispensable role for Smac function; a seven-residue peptide including these residues can remove the IAP-mediated inhibition of caspase-9. Strikingly, a missense mutation of the N-terminal residue Ala to Met in Smac leads to a complete loss of interactions with XIAP and the concomitant loss of Smac function **(55)**.

In fruit flies, the antideath function of the *Drosophila* IAP, DIAP1, is removed by four proapoptotic proteins, Reaper, Grim, Hid, and Sickle, which physically interact with the BIR2 domain of DIAP1 and remove its inhibitory effect on *Drosophila* caspases **(56)**. Thus, Reaper, Grim, Hid, and Sickle represent the functional homologues of the mammalian protein Smac/DIABLO. Although generally regarded as having similar functions in *Drosophila* development, Hid, Grim, Reaper, and Sickle only share homology in the N-terminal's four residues in their primary sequences. Interestingly, these four residues are very similar to the N-terminal residues of mature Smac.

Smac-IAP Complex

The molecular explanation for the indispensable role of the Smac N-terminal sequences is provided by structures of XIAP-BIR3 bound to either a monomeric Smac protein **(57)** [Fig. 4.8(B)] or a nine-residue Smac peptide **(58)**. The Smac N-terminal tetrapeptide (Ala-Val-Pro-Ile) recognizes an acidic surface groove on BIR3, with the first residue Ala binding a hydrophobic pocket and making hydrogen bonds to neighboring XIAP residues [Fig. 4.8(C)]. The next three residues also interact with surrounding hydrophobic residues in BIR3. To accommodate these interactions, the N-terminus of Smac must be free, thus explaining why only the mature Smac can bind to IAPs. Modeling studies indicate that replacement of Ala by a bulkier residue will cause steric hindrance while Gly substitution may result in an entropic penalty as well as loss of

Fig. 4.8 (continued) groups of the N-terminal Ala make several hydrogen bonds to conserved residues in XIAP. (**D**) A conserved IAP-binding mode from mammals to fruit flies. The structure of DIAP1-BIR2 is superimposed with that of the XIAP-BIR3 domain, with their corresponding bound peptides Hid, Grim, and Smac/DIABLO. (**E**) A conserved motif of IAP-binding tetrapeptides. The tetrapeptide motif has the consensus sequence A-(V/T/I)-(P/A)-(F/Y/I/V/S). The *Drosophila* proteins have an additional binding component (conserved sixth to eighth residues)

binding in the hydrophobic pocket. This analysis explains why mutation of Ala to Met or Gly abrogated interactions with the BIR domains.

The Smac-binding surface groove on XIAP-BIR3 comprises highly conserved residues among the BIR3 domains of c-IAP1 and c-IAP2 and the BIR2 domain of DIAP1, suggesting a conserved binding mode. Indeed, the crystal structures of DIAP1-BIR2 by itself and in complex with the N-terminal peptides from Grim, Hid, and Sickle reveal that the binding of these N-terminal tetrapeptides precisely match that of the Smac-XIAP interactions **(59)** [Fig. 4.8(D)]. For Grim and Hid, the next three conserved residues also contribute to DIAP1-binding through hydrophobic interactions.

Thus, the tetrapeptides in the N-termini of the mammalian Smac/DIABLO and the *Drosophila* Reaper, Grim, Hid, and Sickle define an evolutionarily conserved family of IAP-binding motifs [Fig. 4.8(E)]. Interestingly, caspase-9 also contains such a tetrapeptide motif (Ala-Thr-Pro-Phe) in the N-terminus of the small subunit. Subsequent experiments confirmed that this sequence is indeed primarily responsible for the interactions between the processed caspase-9 and XIAP **(46)**. In the absence of proteolytic processing, procaspase-9 is unable to interact with IAPs. Proteolytic processing of procaspase-9 at Asp315 leads to the exposure of an internal tetrapeptide motif, which recruits IAPs to inhibit caspase-9. The mature Smac binds IAPs, again using a similar N-terminal tetrapeptide. A conserved IAP-binding motif in caspase-9 and Smac mediates opposing effects on caspase activity.

During apoptosis, caspase-9 can be further cleaved after Asp330 by downstream caspases such as caspase-3. This positive feedback not only permanently removes XIAP-mediated caspase-9 inhibition but also releases a 15-residue peptide that is able to relieve IAP-mediated inhibition of other caspases. This mechanism ensures that less than stoichiometric amounts of Smac can remove the IAP-mediated caspase-9 inhibition, as transient activation of caspase-9 may lead to the activation of caspase-3 and ensuing positive feedback.

Although the Smac tetrapeptide in isolation can remove IAP-mediated caspase-9 inhibition, it plays a less direct role in the removal of IAP-mediated inhibition of effector caspases. The binding site for this tetrapeptide motif maps to the surface of BIR2 or BIR3, whereas the fragment responsible for inhibiting caspase-3 or -7 is located between BIR1 and BIR2 of XIAP. Although a conclusive mechanism remains elusive, modeling studies of a Smac/BIR2/caspase-3 complex suggest that steric clashes preclude XIAP-BIR2 from simultaneously binding to caspase-3 and Smac **(43)**. In this model, binding to the BIR2 domain requires not only the N-terminal tetrapeptide of Smac but also an extensive surface available only in the wild-type dimeric Smac protein. This model is consistent with the observation that monomeric Smac mutants only weakly interacted with BIR2 and were unable to remove the IAP-mediated caspase-3 inhibition.

Signaling Modules in Apoptosis

During apoptosis, caspase recruitment and activation require three highly conserved families of signaling modules, the caspase recruitment domain (CARD), the death domain (DD), and the death effector domain (DED) **(1)**. These motifs share the same structural topology with different surface features, which give rise to specific homophilic recognition.

Structures of representative members of these signaling motifs have been determined by both NMR and X-ray crystallography **(60–62)**. In each case, the topology consists of six antiparallel helices, with minor variation in interhelical packing (Fig. 4.9). Mutational analyses on these domains revealed critical residues important for interactions with other signaling motifs. In two cases, these mutagenesis results were further confirmed with crystal structures of complexes, each involving two similar domains.

The recognition of procaspase-9 by Apaf-1, primarily through a CARD-CARD interaction, is essential to the formation of the apoptosome holoenzyme and the subsequent activation of caspases. The positively charged surface of procaspase-9 CARD formed by the helices H1a/H1b and H4 is recognized by Apaf-1 CARD through a negatively charged surface formed by the helices H2 and H3 **(63)** [Fig. 4.9(D)]. In *Drosophila*, recruitment of the Ser/Thr kinase

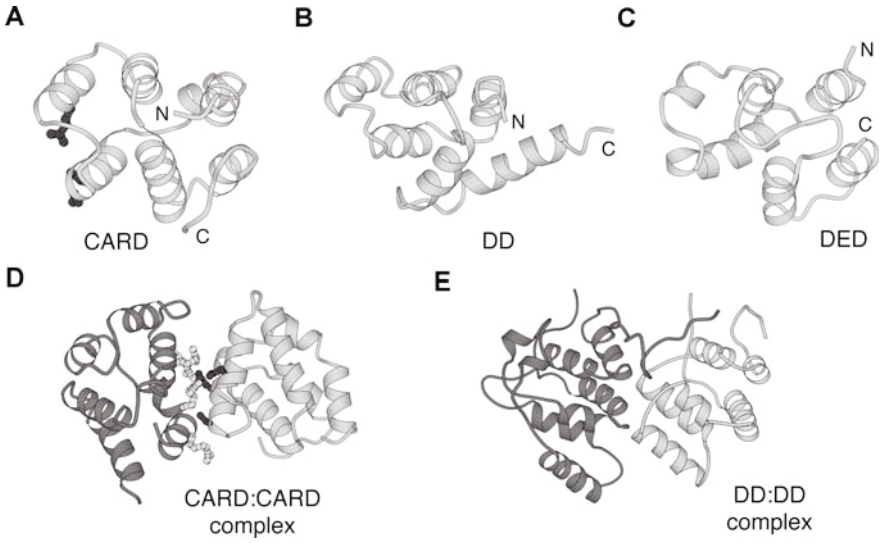

Fig. 4.9 Structure of signaling modules. (**A**) Structure of Apaf-1 CARD. The acidic residues important for caspase-9 binding are shown in black. (**B**) Structure of the death domain (DD) of Fas. (**C**) Structure of the death effector domain (DED) of FADD. (**D**) Structure of a heterodimer between the CARD domains of Apaf-1 and caspase-9. Critical interface residues are shown. (**E**) Structure of a heterodimer between the death domains of Pelle and Tube proteins in *Drosophila*

Pelle to the plasma membrane by the adapter protein Tube is important for embryogenesis. The structure of a death domain complex between Pelle and Tube reveals an interesting addition to the diverse recognition mechanisms by these simple motifs **(64)** [Fig. 4.9(E)]. Despite these advances, it is structurally unclear how these signaling motifs function in the context of a large signaling complex such as the DISC or the apoptosome.

Apoptosome and the Activation of Initiator Caspases

The initiator caspases invariably contain one of two protein-protein interaction motifs, the CARD or the DED. These motifs directly interact with similar motifs present on oligomerized adapter proteins, thus bringing multiple initiator caspase molecules into close proximity with one another and presumably facilitating their autoactivation. This hypothesis is summarized as the induced-proximity model **(65)**. For example, procaspase-8 contains two copies of the DED, which interact with the DED of FADD. Through association with FADD, procaspase-8 is brought into the DISC, resulting in its autoactivation. Another well-characterized paradigm involves procaspase-9 activation. During apoptosis, cytochrome c is released from mitochondria into the cytoplasm, where it assembles Apaf-1 into an apoptosome in the presence of dATP or ATP **(2)**. The primary function of the apoptosome is to recruit procaspase-9 and facilitate its autoactivation.

The induced-proximity model is consistent with a number of observations. For example, bacterially expressed caspases can be processed to their mature forms, presumably due to high local concentrations. In mammalian cells, the forced oligomerization of procaspase-8 led to its activation and subsequent apoptosis **(66, 67)**. Similar results were obtained for the mammalian caspase-9 and the *C. elegans* CED3 **(68)**.

Although induced-proximity undoubtedly leads to caspase activation, it remains unclear whether this is indeed how the initiator caspases are activated under physiological conditions. The strongest supporting evidence for the induced-proximity hypothesis also serves as a cautionary reminder. For example, effector caspases can also be autoactivated through induced proximity, yet they are activated *in vivo* by the initiator caspases. In addition, unprocessed procaspase-9 is nearly as active as the mature caspase-9 and the primary function of the apoptosome is to allosterically enhance caspase-9 activity rather than to facilitate its autoactivation. Furthermore, forced oligomerization of the initiator caspases may not recapitulate the physiological context, in terms of protein expression levels, and more importantly, in terms of the specific protein-protein interactions that are required for the precise positioning and activation of the initiator caspases.

Surprisingly, the isolated caspase-9 is only marginally active in the absence of the apoptosome, prompting the concept of a holoenzyme **(69)**. Through

association with the apoptosome, the catalytic activity of caspase-9 is enhanced about three orders of magnitude. Intriguingly, the unprocessed caspase-9 can be similarly maintained in this "hyperactive" state, demonstrating that the proteolytic processing is unnecessary for the activation of procaspase-9.

Although most other caspases exist exclusively as a homodimer in solution, caspase-9 was found to exist mostly as a monomer at micromolar concentrations **(29)**. Fractions corresponding to dimers and monomers from gel filtration were separately analyzed for their catalytic activity, and only the dimer fractions were found to be active. Biochemical as well as structural analyses revealed that dimerization resulted in the formation of only one functional active site. Based on these observations, it was proposed that dimer formation may drive the activation of caspase-9 **(29)**.

The three-dimensional structure of the ~1.4-MDa apoptosome at 27 Å resolution reveals a wheel-shaped heptameric complex, with the CARD domains located at the central hub and the WD40 repeats at the extended spokes **(70)** [Fig. 4.10(A)]. Docking of caspase-9 to this apoptosome resulted in a dome-shaped structure in the center; however, the bulk of the caspase-9 was not visible in these EM studies [Fig. 4.10(B)]. The central domes were thought to be complexes involving the CARD domains from Apaf-1 and caspase-9.

Based on structural information on caspase-9 and the apoptosome, a model was proposed to explain the activation of procaspase-9 **(70)**. In this model, a heptameric apoptosome binds seven monomers of inactive caspase-9. The high local concentrations of caspase-9 within this apoptosome drive the efficient recruitment of additional inactive caspase-9 monomers, which become activated upon binding. This interesting model has a strong assumption that

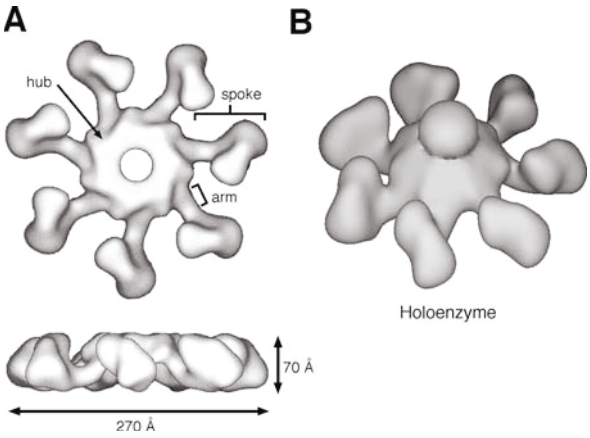

Fig. 4.10 Structure of the apoptosome. (**A**) Structure of the apoptosome at 27 Å resolution by electron microscopy. The CARD domain of Apaf-1 was interpreted to reside in the central hub of this wheel-shaped structure. (**B**) Structure of the caspase-9-bound apoptosome. Caspase-9 binding induces a dome-shaped structure in the center of the apoptosome

caspase-9 activity in the active dimers is identical to that in the apoptosome holoenzyme, which remains to be experimentally tested.

Conclusion

The rapid progress in the characterization of apoptosis by structural biology has significantly enhanced our understanding of the underlining mechanisms. However, many daunting tasks remain. For example, we still know very little about the activation mechanisms of the initiator caspases. In this respect, future effort should be directed at solving structures of higher-order protein complexes and performing associated biochemical and biophysical analysis. Furthermore, the apoptotic mechanisms will likely become more complex with the discovery and characterization of additional players and pathways, which will present structural biologists exciting new challenges for years to come.

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Chapter 5
The Death Receptor Pathway

Maria Eugenia Guicciardi and Gregory J. Gores

Abstract The TNF receptor superfamily is a large group of membrane-associated receptors characterized by structural similarities in their extracellular and intracellular domains. A subgroup of these receptors, the so-called death receptors, shares an intracellular motif termed the death domain. When engaged by their cognate ligands, these receptors trigger an apoptotic pathway often referred to as the extrinsic pathway. This is mediated by the recruitment of various adaptor proteins, which, in turn, recruit and facilitate the activation of initiator caspases, initiating a proteolytic cascade. Death receptor deregulation has been associated with several pathological conditions; for this reason, death receptors have been the subject of intensive studies. These studies have also revealed, in different settings, that death receptors have additional apoptosis-independent functions, including regulation of cell proliferation and differentiation, chemokine production, and tumor-promoting activity. This chapter provides an updated overview on the signaling pathways mediated by the death receptors and on their role in human physiology and pathophysiology.

Keywords Death receptors · Fas/CD95 · TNF · TRAIL · Mitochondria · Bcl-2 family · Caspases · MAP kinases

Introduction

Death receptors are cell surface cytokine receptors belonging to the tumor necrosis/nerve growth factor (TNF/NGF) receptor superfamily that can trigger apoptosis after binding a group of structurally related ligands or specific antibodies **(1–3)**. The members of this family are type I transmembrane proteins with a C-terminal intracellular tail, a membrane-spanning region, and an

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extracellular ligand-binding N-terminal domain. They are characterized by a significant homology in a region containing one to five cysteine-rich repeats in their extracellular domains, and in a 60- to 80-amino acid cytoplasmic sequence known as the death domain (DD), which typically enables death receptors to initiate the death signal.

Death receptors are activated through an interaction with their natural ligands, a group of complementary cytokines that belongs to the TNF family of proteins. With the exception of the soluble, lymphocyte-derived cytokine LTα, these proteins (known as death ligands) are type II transmembrane proteins comprised of an intracellular N-terminal domain, a transmembrane region, and a C-terminal extracellular tail. Death ligands also can be released as soluble cytokines by the cleavage of metalloproteases.

Currently, six death receptors are known: Fas (also called CD95 or APO-1), TNF-R1 (also called p55 or CD120a), TRAIL-R1 (also called Death Receptor 4 or DR4), TRAIL-R2 (also called Death Receptor 5 or DR5 or APO-2 or KILLER), Death Receptor 3 (DR3; also called APO-3 or TRAMP or WSL-1 or LARD), and Death Receptor 6 (DR6) (Fig. 5.1).

Signal transduction by death receptors is initiated by the oligomerization of the receptor triggered upon juxtaposition of the intracellular domains that follows the engagement of the ligand to the receptor's extracellular domain. This event leads to recruitment of different adapter proteins, which provide the

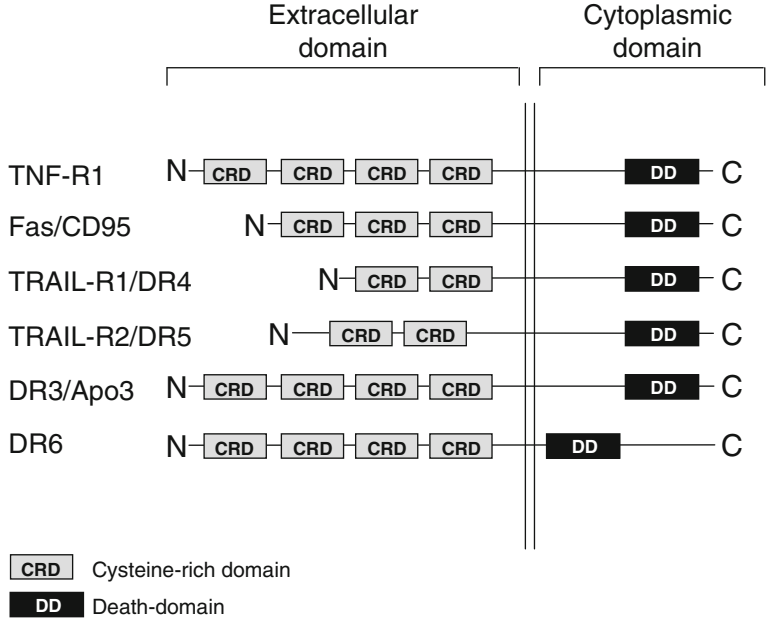

Fig. 5.1 Structural comparison of the death receptors. The extracellular domains are characterized by the presence of a variable number of cysteine-rich motifs (CRD), whereas the intracellular tails contain the death domain (DD), essential for signaling apoptosis

link between the receptor and the cell death effectors, namely, the so-called initiator caspases (caspase-8 and caspase-10) and the long form of the cellular FLICE/caspase-8-like inhibitory protein (cFLIP). Adapter proteins generally have no enzymatic activity of their own but are able to associate with receptors through homophylic interaction of the receptor's death domain and an analogous death domain on the adapter itself. Adapter proteins may also contain a death effector domain (DED) that mediates the recruitment of caspases through the association with a corresponding death effector domain or a caspase recruitment domain (CARD) in the prodomain of the inactive initiator caspases or $cFLIP_L$. The resulting complex is called the death-inducing signaling complex (DISC) and generates an apoptotic signaling cascade initiated by the activated caspases. Three hypotheses have been proposed to explain how initiator caspases are activated at the DISC. The first model, which is referred to as the "induced-proximity model" **(4)**, suggests that recruitment of the initiator caspase zymogens to the receptor complex via interaction with the adapter protein FADD leads to clustering and self-processing of the caspases that are brought into close proximity of each other. A second slightly different model is the so-called proximity-induced dimerization **(5)**. According to this model, recruitment and accumulation of initiator caspases at the DISC promotes their dimerization, which, in turn, results in their activation. Therefore, dimerization, and not cleavage, is required for initiator caspase activation, although the processing of the caspases stabilizes the active form. Recently, a third model for caspase activation called the "induced conformation model" has been proposed **(6)**. This model suggests that the activation of the initiator caspase is achieved through a conformational change in its active site as a consequence of the interaction of the initiator caspase with the adaptor protein complex.

Caspase-8 and -10 activation at the DISC can be inhibited by cFLIP **(7)**. Three different splice variants of cFLIP are expressed as polypeptides: cFLIP long ($cFLIP_L$), cFLIP short ($cFLIP_S$), and a short variant cloned from the Raji B-cell line ($cFLIP_R$) **(8)**. All cFLIP forms have two death effector domains; the long variant also contains a caspase-like domain and closely resembles caspase-8, except that it lacks the catalytic cysteine embedded in the conserved pentapeptide QACRG or QACQG motif present in all caspases, and other key residues involved in catalysis and substrate binding, and therefore has no cysteine protease activity (Fig. 5.2). All cFLIP forms are recruited to the DISC by DED-DED interactions. $cFLIP_S$ was shown to block caspase-8 processing and activation at the DISC, probably by competitive binding to FADD. Although much less is known about $cFLIP_R$, its structure closely resembles $cFLIP_S$. It is therefore likely that the two proteins inhibit death receptor-mediated apoptosis through similar mechanisms. However, the role of $cFLIP_L$ at the DISC is controversial **(9)**. Originally, $cFLIP_L$ was described as an antiapoptotic molecule that inhibits death receptor-induced apoptosis by interfering with caspase-8 activation at the DISC **(10, 11)**. Indeed, although the overexpression of $cFLIP_L$ results in the recruitment of both cFLIP and caspase-8 to the DISC, caspase-8 undergoes only partial processing, with the

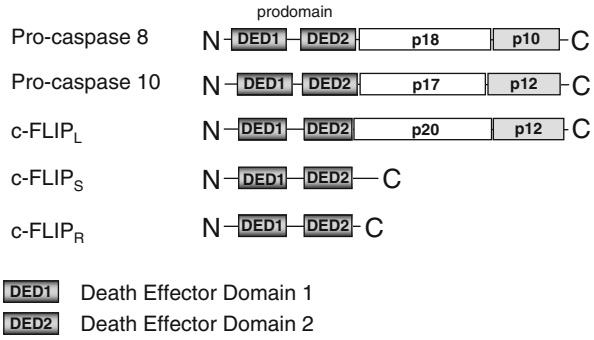

Fig. 5.2 Structural comparison of caspase-8 and FLIPs. Schematic models of caspase-8, c-FLIP$_L$, c-FLIP$_S$, and c-FLIP$_R$. Each molecule contains two death effector domains (DED) at the N-terminal. Caspase-8 and c-FLIP$_L$ also show high sequence homology in the C-terminal domain, containing both the large (p18–20) and the small (p10–12) catalytic subunits. The pentapeptide QACQG (or QACRG), highly conserved in all caspases but lacking in c-FLIP$_L$, is critical for the catalytic activity of the proteins

cleaved intermediates remaining bound to the DISC and no generation of the active heterodimeric form **(12)**. However, recent studies have demonstrated that cFLIP$_L$ can also have a proapoptotic function by actually facilitating the oligomerization and activation of caspase-8 at the DISC **(13**, **14)**. This hypothesis is supported by the evidence that cFLIP$_L$-deficient mice show the same phenotype as caspase-8- and FADD-deficient mice, which is characterized by heart failure and embryonic lethality **(10**, **15**, **16)**. Moreover, all three proteins are required for the survival of activated T and B cells **(15)**. The specific function of cFLIP$_L$ seems to be determined by its expression level, with high levels being antiapoptotic and low levels contributing to apoptosis.

The activated initiator caspases starts a cascade of caspase activation by processing and activating the so-called effector caspases (e.g., caspases-3, -6, and -7), which are directly or indirectly responsible for the cleavage and degradation of several crucial cellular proteins and for the execution of cell death. Alternatively, initiator caspases may cleave the proapoptotic BH3-only protein Bid, inducing mitochondrial dysfunction and linking the receptor-mediated extrinsic pathway with the intrinsic pathway **(17**, **18)**.

It was initially thought that all death receptor-induced cell killing was achieved only through caspase activation. However, this concept has been challenged by numerous recent reports of programmed cell death in response to activated death receptors involving proteases other than caspases, suggesting that caspase-independent pathways can be stimulated by death receptors. These important findings could potentially lead to the development of new therapeutic options in tumors, where cells often have acquired mutations to inactivate canonical, caspase-dependent death pathways. One of these proteases is the lysosomal cathepsin B, which mediates TNF-α and TRAIL-induced cell death

in some cancer cell lines through caspase-independent pathways or in collaboration with caspases **(19–24)**. Information on how alternate effectors, like cathepsin B, are activated and/or released upon death receptor stimulation is still incomplete. Recent finding suggest that Bcl-2 proteins may be involved in lysosomal membrane permeabilization and the release of cathepsins after death receptor engagement, possibly through a mechanism similar to the one leading to mitochondria permeabilization **(24)**. While the caspase-dependent pathway is almost universally working in all cells (with the notable exception of many cancer cells), death receptor pathways involving proteases other than caspases seem to be highly cell-type specific. Another molecule involved in death receptor-mediated caspase-independent cell death is the adaptor serine/threonine kinase RIP1. Indeed, it was demonstrated that death receptors stimulate a necrotic cell death dependent on the kinase activity of RIP1 in cells lacking caspase-8 or FADD **(25, 26)**.

Since the cloning of the first death receptor more than a decade ago, hundreds of reports have been published providing valuable information on these receptors, yet the understanding of the complex signaling originating from them seems still to be incomplete. Intriguingly, it has becoming increasingly evident that death receptors mediate not only apoptosis, but also diverse nonapoptotic functions, such as cell proliferation and differentiation, inflammation, and tumor migration and invasiveness, depending on the tissue and the conditions. The purpose of this chapter is to provide an updated overview of the signaling pathways initiated by death receptors and their role in both human and animal physiology and pathophysiology.

Fas (CD95/APO-1) and Fas Ligand (FasL/CD95L)

Fas (CD95/APO-1)

Fas (CD95/APO-1) is a glycosylated protein expressed on the cell surface as preassociated homotrimers. The gene encoding for Fas is located on the long arm of chromosome 10 in humans and on chromosome 19 in the mouse. It is ubiquitously expressed in various tissues but is particularly abundant in the liver, heart, kidney, pancreas, brain, thymus, and activated mature lymphocytes. Although the membrane-bound form is largely predominant, several splice variants have been described that generate soluble forms of the receptor, the function of which is still unclear **(27)**. These soluble forms of the receptor may antagonize Fas-mediated cytotoxicity by binding to and inactivating Fas ligand (FasL), thereby exerting an antiapoptotic effect. A tight regulation of Fas-mediated apoptosis is essential for the proper physiology of the cell; this is achieved through a variety of mechanisms, some of which operate directly at the level of the receptor. Fas localizes on the plasma membrane as well as in the cytosol, in particular, in the Golgi complex and the trans-Golgi network

(28, 29). Translocation of Fas-containing vesicles to the cell surface has been observed upon stimulation, providing an effective mechanism to regulate the plasma membrane density of the death receptor and avoid its spontaneous activation **(29, 30)**. Fas-mediated apoptosis can also be modulated by post-translational modifications such as glycosylation **(31)** or palmitoylation of the receptor **(32)**, as well as at the transcriptional level, by directly regulating Fas expression. A composite binding site for the transcription factor NF-κB has been described at position 295 to 286 of the Fas gene promoter, which regulates activation-dependent Fas expression in lymphocytes **(33)**. A p53-responsive element is also located within the first intron of the Fas gene and cooperates with three sequences in the promoter to upregulate Fas receptor expression during drug-induced apoptosis of leukemic and hepatocellular carcinoma cell lines **(34–36)**.

Fas Ligand (FasL/CD95L)

FasL (CD95L) is a type II transmembrane protein with a homotrimeric structure **(37)**. It is expressed on the cell surface of activated T cells and, together with its receptor, plays an important role in the maintenance of the peripheral T- and B-cell homeostasis and in the killing of harmful cells, such as virus-infected cells or cancer cells **(38–40)**. FasL can also be proteolytically cleaved by a metalloprotease between Ser126 and Leu127 in its extracellular domain, generating a soluble, trimeric form whose biological activity remains controversial **(41)**. Indeed, whereas soluble FasL with apoptosis-inducing ability has been described, serum FasL levels are often high in hepatitis, AIDS, and several types of tumors where they are not associated with increased apoptosis **(42)**. Species-related differences in the function of the soluble form are possible, as soluble human FasL is able to induce apoptosis, whereas soluble mouse FasL is not **(43)**. However, the apoptotic-inducing capacity of the soluble form is over 1,000 times lower compared to the membrane-bound FasL, providing an explanation for the absence of tissue damage in diseases associated with elevated circulating levels of FasL **(41, 44, 45)**. In lymphoma cells, the expression of soluble FasL does not trigger apoptosis, but it has been shown to suppress the inflammatory response **(46)**.

Fas Signaling

Engagement of Fas by either agonistic antibodies or FasL leads to the recruitment of the adaptor molecule FADD (Fas-associated protein with death domain)/MORT-1 (mediator of receptor-induced toxicity) **(47)**. FADD/MORT-1 is a ubiquitously expressed cytosolic protein with a C-terminal death domain and a death effector domain at the N-terminus. FADD associates

with the receptor through its death domain, while its death effector domain is required for self-association and binding pro-caspase-8 and pro-caspase-10. The recruitment and accumulation of pro-caspases at the DISC results in their spontaneous activation and initiation of the apoptosis signal (Fig. 5.3). Pro-caspase-8 and -10 are proteolytically processed with similar kinetics, and both can initiate apoptosis independently of each other **(48**, **49)**.

The early events occurring in Fas signaling are complex and highly regulated (Fig. 5.3). Upon stimulation, the preassociated receptors form SDS-stable microaggregates associated with palmitoylation of the membrane proximal cysteine 199 in the receptor **(32)** and formation of low levels of DISC. Subsequently, the receptor organizes into higher-order aggregates **(50)**, which are recruited into lipid rafts to form signaling protein oligomerization structures

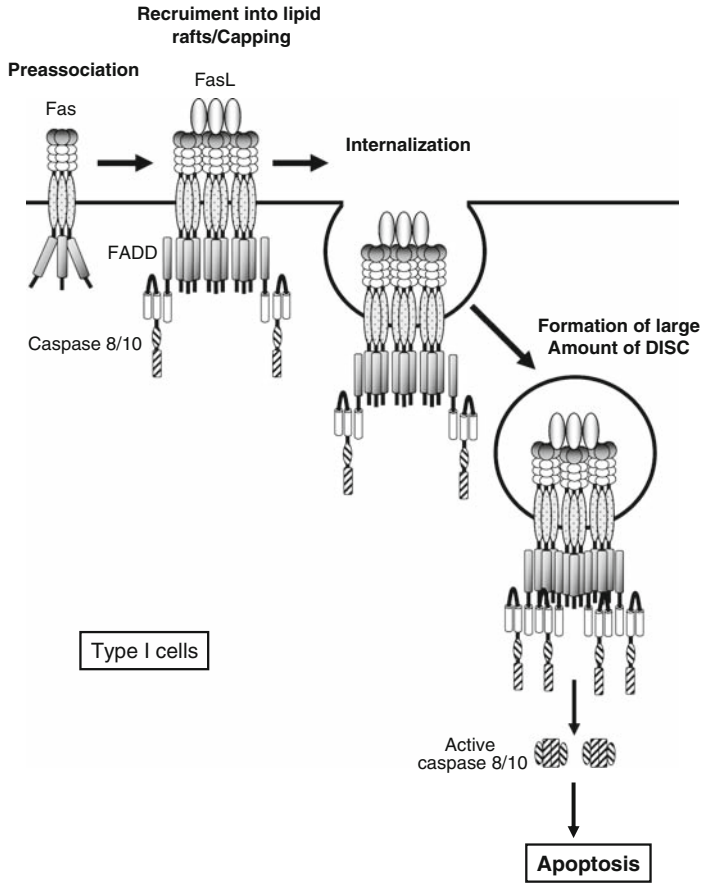

Fig. 5.3 The Fas/CD95 DISC. Schematic representation of the early steps in Fas/CD95 signaling and the formation of the death-inducing signaling complex (DISC) triggered by engagement of FasL/CD95L to Fas/CD95. See the text for details

(SPOTS) visible by immunofluorescence microscopy **(51)**. Active caspase-8 generated in these complexes then drives the receptor clustering (also referred to as "capping") to form large lipid raft platforms **(52–54)** that are internalized through clathrin-mediated endocytosis **(55)**. The receptor-containing vesicles are delivered to the early endosomal compartment, where most of the DISC formation occurs. Both internalization of the receptor and localization to the endosomal compartment are required steps for efficient DISC assembly, amplification of the signal, and execution of apoptosis. In the absence of internalization, the receptor aggregates induce activation of prosurvival signals such as NF-κB- and ERK1/2-mediated pathways, demonstrating that compartmentalization of the Fas signal is critical to determine the cellular outcome after Fas stimulation **(55)**.

The signal downstream of DISC formation differs between cell types. Two classifications of Fas-mediated apoptosis signaling pathways have been described **(56)** (Fig. 5.4). In the so-called type I cells, large amounts of DISC are rapidly assembled and internalized, caspase-8 is mainly activated at the DISC and is responsible for direct cleavage of the effector caspase-3. Fas is associated with membrane rafts in type I cells, consistent with its internalization (57). Overexpression of the antiapoptotic proteins Bcl-2 or Bcl-xL does not prevent activation of caspase-8 or caspase-3 in these cells, nor does it inhibit apoptosis, suggesting a mitochondria-independent activation of a caspase cascade. In contrast, DISC formation in the so-called type II cells is slower and strongly reduced; Fas does not co-localize with lipid rafts and is not efficiently internalized; and the activation of caspases, including caspase-8, occurs mainly downstream of mitochondria, as both caspase activation and apoptosis can be blocked by the overexpression of Bcl-2 or Bcl-xL. Notably, Fas triggers the activation of mitochondria in both type I and type II cells, and in both cell types, the apoptogenic activity of mitochondria is blocked by overexpression of Bcl-2 or Bcl-xL. However, only in type II, and not in type I, cells, overexpression of Bcl-xL or Bcl-2 blocks apoptosis, suggesting that only in type II cells are mitochondria essential for the execution the apoptotic program, whereas in type I cells, mitochondrial dysfunction likely functions as an amplifier of the apoptotic signal. Mitochondrial dysfunction during Fas signaling is mediated by caspase-8 cleavage of Bid, a cytosolic proapoptotic, BH3-only member of the Bcl-2 family of proteins **(17)**. The resulting 15-kDa fragment (tBid) translocates to the mitochondria and induces the release of apoptogenic factors, such as cytochrome c, AIF (apoptosis-inducing factor) **(58)**, and SMAC (second mitochondria-derived activator of caspases)/DIABLO (direct IAP binding protein with low pI) **(59, 60)**. Once in the cytosol, cytochrome c binds to the co-factor Apaf-1 (apoptosis-activating factor 1) and to pro-caspase-9 in a complex called apoptosome. Through an energy-requiring reaction, pro-caspase-9 is processed to the mature enzyme and, in turn, activates caspase-3, starting a caspase cascade downstream of the mitochondria.

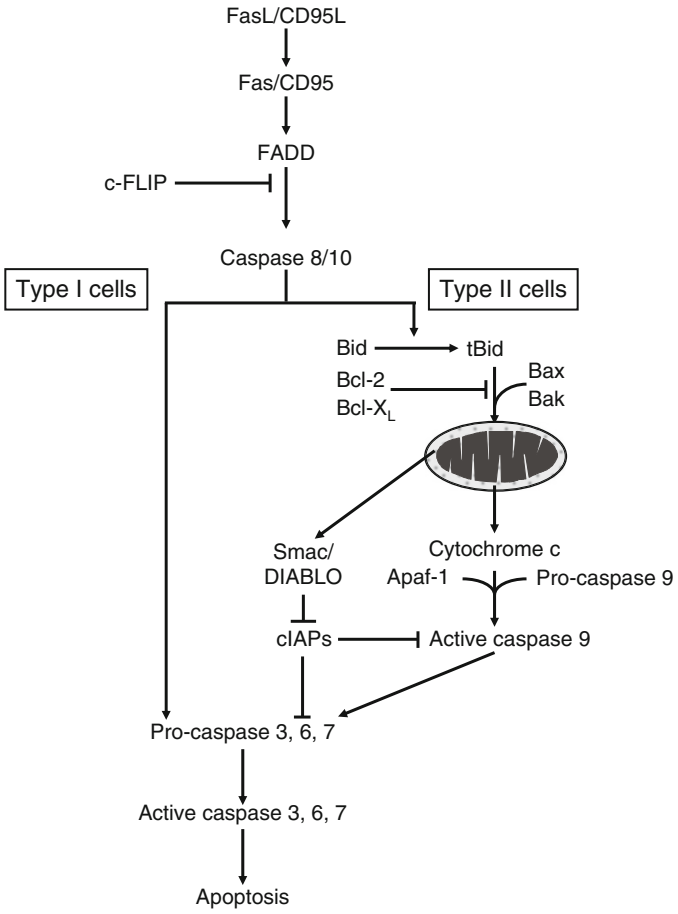

Fig. 5.4 Fas/CD95-mediated apoptotic pathways. Schematic representation of Fas-mediated apoptotic pathways in Type I and Type II cells. See the text for details

Physiology and Pathophysiology

A balance between cell death and cell proliferation is required to maintain tissue homeostasis. Not surprisingly, an excess or lack of apoptosis always leads to disease pathogenesis. As long as death receptors are appropriately expressed, they represent a powerful tool to execute apoptosis in a controlled manner. However, several diseases have been associated with either the loss of function or the overexpression of death receptors, which results in too little or too much apoptosis, respectively. The Fas/FasL system plays a major role in morphogenesis, tissue homeostasis, elimination of transformed or pathogen-infected cells, and termination of the immune response. The system's crucial role in T-cell regulation was postulated after the study of three spontaneous recessive

mutations in mice, *lpr* (lymphoproliferation), *gld* (generalized lymphoproliferative disease), and lpr^{cg}. These mutations are phenotypically associated with systemic autoimmunity, lymphadenopathy, and splenomegaly with significant accumulation of $CD4^-$ $CD8^-$ T-lymphocytes **(61, 62)**. Molecular analyses of *lpr*, lpr^{cg}, and *gld* mutations showed that they correspond to loss-of-function mutations in the Fas (*lpr* and lpr^{cg}) and FasL (*gld*) genes **(63)**. In particular, the gene mutation in the *lpr* mouse, which is caused by the insertion of a transposable element into intron 2, and the one in lpr^{cg}, which is a point mutation in the death domain of Fas that prevents the recruitment of FADD, both result in the generation of defective Fas **(64)**. The *gld* mouse instead has functional Fas but expresses a defective FasL. Impairment of the Fas/FasL system causes increased resistance of T-cells to activation-induced apoptosis. As a result, mature $CD4^-$ $CD8^-$ T-cells can no longer be eliminated and therefore accumulate in lymph nodes and the spleen **(65, 66)**. Similar phenotypes have also been described in humans affected by autoimmune lymphoproliferative syndrome (ALPS) **(64)**. These patients, most of whom are children, carry mutations in either the Fas gene (type Ia) or the FasL gene (type Ib), which generate defective proteins that either lack their normal function or work as dominant-negative when expressed with normal Fas or FasL. As a consequence, ALPS patients are incapable of effectively downregulating the immune reaction and develop lymphoadenopathy, splenomegaly, hypergammaglobulinemia, and, in some cases, autoimmune diseases, such as hemolytic anemia, thrombocytopenia, and neutropenia, due to the production of antibodies against red blood cells and platelets. Indeed, the abnormally prolonged survival of activated lymphocytes may result in a persistent humoral immune response and potentially harmful cross-reactions with self-antigens, which can eventually lead to the development of autoimmune diseases.

Downregulation of Fas expression or inactivating mutations in Fas have also been observed in several tumors, often associated with constitutive expression of FasL **(67, 68)**. This adaptation allows the cancer cell to survive the attack by cytotoxic T-lymphocytes and NK cells by binding Fas on their surface and inducing apoptosis while simultaneously increasing its own resistance to Fas-mediated apoptosis **(69–71)**. The role of Fas in carcinogenesis is consistently supported by the evidence that Fas-defective animals show an increased risk of developing tumors. Interestingly, although a large number of tumors have congenital or somatic mutations in the Fas death domain, they very rarely display loss of heterozygocity, suggesting that maintaining one wild-type receptor may confer an oncogenic advantage **(72)**. This advantage is likely due to the fact that very low levels of Fas are unable to induce apoptosis but can efficiently activate survival pathways, including NF-κB **(73)**. Therefore, mutations in one allele of the receptor may abrogate the tumor-suppressor function of Fas and, at the same time, may activate proliferative pathways that promote tumor growth. The same nonapoptotic signals can be activated by Fas in apoptosis-resistant tumor cells that bear mutations in the apoptotic machinery other than mutations affecting Fas or FasL, including, but not limited to, upregulation of

antiapoptotic Bcl-2 family members **(74)** or cFLIP **(75)**, or downregulation of proapoptotic Bcl-2 proteins, or silencing of the caspase-8 gene **(76)**. The product of the activation of these survival pathways is a set of proteins that promote invasiveness, motility, and metastasis of the apoptosis-resistant tumor cells **(77)**. These novel observations could potentially lead to a radical change in the current strategy for cancer therapy, as chemotherapy often causes upregulation of FasL in both tumor and nontumor cells, which could result in the increased tumorigenicity of apoptosis-resistant cells.

On the other hand, unregulated activation the Fas/FasL system can be equally deleterious, causing massive tissue destruction and even leading to death of the organism. Injection of an agonistic anti-Fas antibody or recombinant FasL into mice has been shown to strongly activate the Fas/FasL system in the liver, causing acute liver failure and rapid death **(78)**. The symptoms of this liver failure closely resemble those of fulminant hepatitis, which results from massive hepatocyte apoptosis caused by the reaction between abnormally activated T cells and hepatitis B or C virus-transformed hepatocytes overexpressing Fas. Thus, overexpression of Fas may be a cause of fulminant hepatitis. The best example of this in human pathology is a disease characterized by an accumulation of copper in the liver. This transition metal promotes oxidative stress and *de novo* expression of FasL in the liver. The FasL-expressing hepatocytes induce apoptosis in Fas-expressing neighboring cells (fratricide killing), causing liver damage. Similar mechanisms may also be important in alcoholic hepatitis. The overexpression of Fas is also known to play a role in AIDS.

Tumor Necrosis Factor-Receptor 1 (TNF-R1) and TNF-α

TNF-R1

The TNF/TNF-receptor signaling system consists of two distinct receptors, TNF-R1 (also called p55 or CD120a) and TNF-R2 (also called p75 or CD120b), and three ligands, the membrane-bound TNF-α (mTNF-α), the soluble TNF-α (sTNF-α), and the soluble lymphocyte-derived cytokine (LTα, also called TNF-β) **(47)**. TNF-R1 and TNF-R2 are both type I transmembrane proteins containing an amino-terminus, disulfide-rich, extracellular domain that recognizes TNF-α, a transmembrane helix, and a cytoplasmic tail. However, TNF-R1 only, and not TNR-R2, possesses an intracellular death domain and is therefore likely to be the sole mediator of the apoptotic signal. Both receptors are ubiquitously expressed in cells; however, TNF-R1 expression seems to be constitutively low and controlled by a noninducible promoter, whereas TNF-R2 expression is inducibly regulated by a number of extracellular stimuli. TNF-R1 and TNF-R2 interact with both forms of TNF-α as well as with LTα. Nonetheless, TNF-R1 appears to be entirely responsible for TNF signaling in most cell types, and *in vivo* studies in a TNF-R1-deficient mouse

model showed that TNF-R1 is essential for TNF-induced apoptosis of pathogen-infected cells **(79**, **80)**.

TNF-α

Tumor necrosis factor-α (TNF-α) was originally named after its ability to elicit the hemorrhagic necrosis of transplanted mouse tumors and for its selective cytotoxicity for transformed cells. In the years that followed, TNF-α was found to play a key role in inflammation and immunity. Moreover, TNF-α is able to induce the proliferation and differentiation of many different target cells **(81)**. TNF-α is mainly produced by macrophages, monocytes, and T cells in response to infection and inflammatory conditions, but also by other cell types, such as B cells, fibroblasts, and hepatocytes. TNF-α is expressed as an integral type II transmembrane protein with a homotrimeric structure. From this precursor, a soluble form is released after cleavage by the metalloprotease TNFα-converting enzyme (TACE) **(82)**. Both soluble and membrane-bound TNF-α are biologically active, and while the soluble form acts as an effector molecule at a distance from the producer cell, the membrane-bound form likely has a specific role in localized TNF-α responses.

TNF-R1 Signaling

Intracellular signals originating from TNF-R1 are extremely complex and can lead to multiple, even opposite, cell responses, from cell proliferation to inflammation to cell death. Most cells treated with TNF-α, however, do not undergo apoptosis unless protein or RNA synthesis is blocked, which suggests the predominance of survival signals over death signals under normal circumstances and the requirement that neo-synthesized proteins suppress the apoptotic stimulus. Indeed, recent studies have demonstrated that engagement of TNF-R1 leads to the formation of two subsequent signaling complexes, one of which results in apoptosis while the other leads to the expression of antiapoptotic proteins that prevent apoptosis **(83)** (Fig. 5.5). The expression of these antiapoptotic proteins is likely to be controlled by the activity of the transcription factor NF-κB, as the inhibition of NF-κB sensitizes cells to TNF-α-induced apoptosis. JNK (c-Jun NH_2-terminal kinase)/SAPK (stress-activated protein kinase) is also activated by TNF-α, and considerable data now suggest that JNK induces the transcription of proapoptotic genes in many cell types. Indeed, NF-κB complexes downregulate the JNK cascade through upregulation of *gadd45β*/*myd118*, a gene associated with cell-cycle control and DNA repair, and *xiap*, which encodes the endogenous inhibitor of apoptosis, XIAP, and promotes cell survival **(84**, **85)**.

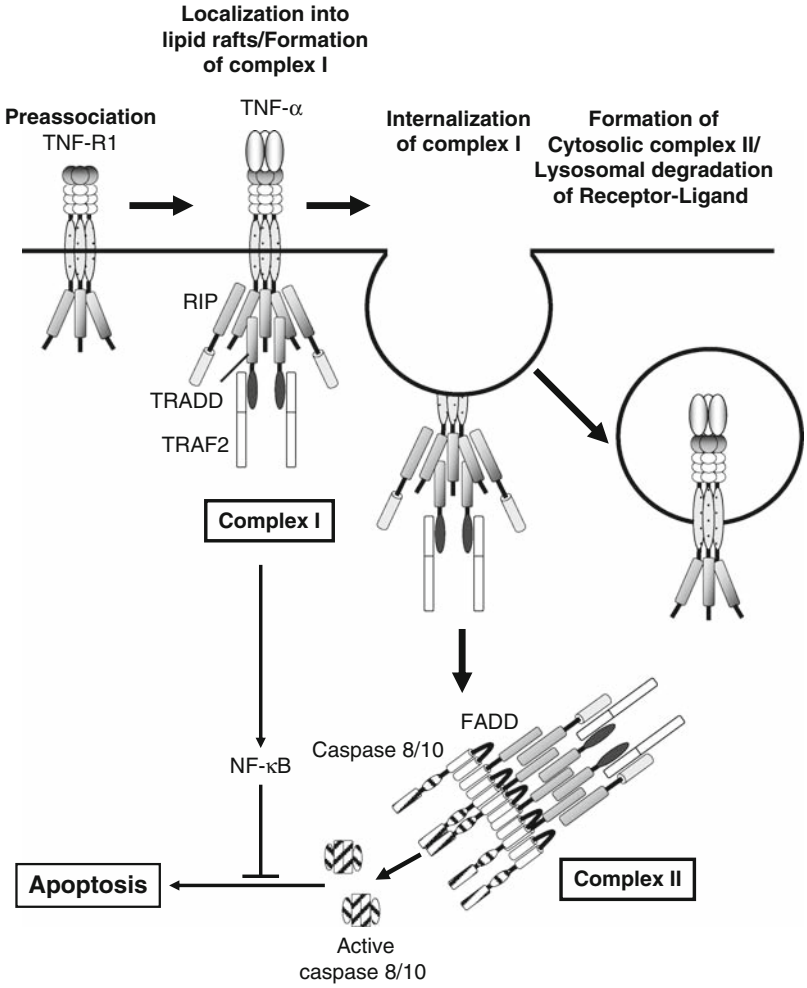

Fig. 5.5 The TNF-R1 DISC. Schematic representation of the early steps in TNF-R1 signaling and the formation of signaling complexes triggered by engagement of TNF-α to TNF-R1. See the text for details

Engagement of TNF-R1 by TNF-α results in conformational changes in the receptor's intracellular domain, resulting in rapid recruitment of several cytoplasmic death domain-containing adapter proteins via homophylic interaction with the death domain of the receptor **(47)** (Fig. 5.5). Within minutes, a plasma membrane-bound complex (called complex I) is formed following the recruitment of TRADD (TNFR-associated protein with death domain), RIP1 (receptor-interacting protein 1), TRAF2 (TNF-associated factor 2), and c-IAP1 (cellular inhibitor of apoptosis 1) to TNF-R1. This complex triggers the activation of NF-κB and mitogen-activated protein (MAP) kinase pathways but is

not capable of inducing apoptosis. Two distinct pathways originate from the association of RIP and TRAF-2 to the receptor **(86)**. The first one signals through the activation of the protein kinase NIK (NF-κB-inducing kinase), which further activates the catalytic IKK complex (IκB kinase complex), comprised of the three proteins IKKα (IKK1), IKKβ (IKK2), and IKKγ (NEMO), leading to phosphorylation of the NF-κB inhibitory protein IκBα. Phospho-IκBα is then degraded via the ubiquitin-proteasome pathway, allowing NF-κB to translocate to the nucleus and initiate transcription of target genes. The second pathway involves the MAP kinases and leads to activation of JNK, via the consequential activation of MEKK-1 (mitogen-activated protein/ERK kinase kinase-1), and JNKK (JNK kinase). JNK phosphorylates and activates of a number of transcription factors, including c-Jun, ATF-2 (activating-transcription factor 2), and AP-1. Moreover, TRAF-2 has been shown to bind to the antiapoptotic factors cIAP-1 and -2 (cellular inhibitor of apoptosis-1 and -2) and TRAF-1, to form a receptor signaling complex that inhibits the apoptotic TRADD/FADD/caspase-8 pathway possibly by facilitating the ubiquitination and degradation of caspase-8 **(87, 88)**. Ubiquitination of RIP1 at Lys377 is required for TNF-α-induced activation of NF-κB, as an inactivating mutation of this residue prevents the recruitment of its downstream signaling components into the TNF-R1 complex and fails to activate NF-κB **(89)**. Assembly of complex I occurs in lipid rafts, where several proteins in the complex undergo posttranslational modifications **(90)**. Subsequently, TRADD, RIP1, and TRAF2 dissociate from TNF-R1 and the receptor internalized by endocytosis **(91)**. After dissociation from TNF-R1, the DD of TRADD and RIP1 become available for interaction with the DD of FADD, which, in turn, act as a platform to recruit caspase-8. This second complex (complex II), which contains TRADD, TRAF2, RIP1, FADD, and caspase-8, initiates apoptosis, provided that the NF-κB signal from complex I fails to induce the expression of anti-apoptotic proteins such as $cFLIP_L$ and c-IAP1 (Fig. 5.6). A second model of TNF signaling has been proposed, in which TRADD, FADD, and caspase-8 are recruited during endocytosis of the receptor to endocytic vesicles called receptosomes to form a cytosolic TNF-R1-associated DISC **(91)**. Regardless of the presence of TNF-R1, the formation of a cytosolic DISC results in autocatalytic activation of caspase-8 and apoptosis. RIP has also been described as engaging a death pathway by binding RAIDD (RIP-associated ICH-1/CED-3-homologous protein with a death domain) and recruiting caspase-2 through homophylic interaction between homologous sequences in its amino-terminal domain and the prodomain of caspase-2 **(92)**.

Physiology and Pathophysiology

The TNF-α/TNF-R system is an important mediator in various physiological and pathophysiological conditions. The complicated signaling pathways

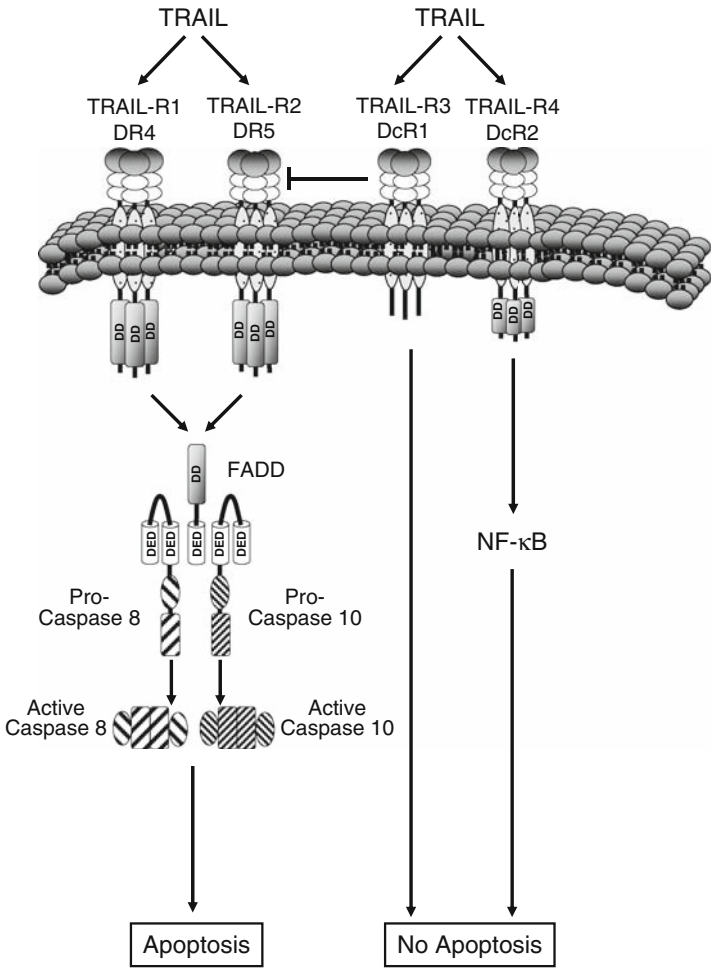

Fig. 5.6 TRAIL receptors and the TRAIL DISC. Schematic representation of TRAIL receptors and the signaling complex triggered by engagement of TRAIL to TRAIL-R1 or TRAIL-R2. Because TRAIL-R3 and TRAIL-R4 lack a functional death domain, they do not induce cell death and function as decoy receptors. Further details are found in the text

originating from the receptor complex indeed impact different biological processes, such as cell proliferation, cell death, and inflammation.

After partial hepatectomy, biliary epithelial cells and venous endothelial cells rapidly secrete large amounts of TNF-α, thus elevating the hepatic level of the cytokine. By signaling through TNF-R1, TNF-α promotes the proliferative response of liver cells by both stimulating the transcriptional activity of AP-1 and NF-κB in the hepatocytes and inducing the secretion of another cytokine critical in hepatic regeneration, interleukin-6 (IL-6), from Kupffer cells or other nonparenchymal cells **(93)**. IL-6, in turn, binds to its receptor on the

hepatocytes and induces the activation of a third transcription factor, STAT 3 **(94)**. As a result of this concerted transcriptional activity, hepatocytes are forced to leave G_0 and enter the proliferative stages of the cell cycle. Thus, it seems that TNF-α acts as an initiator and potentiator of hepatocyte proliferation and liver regeneration. Consistently, liver regeneration results are severely impaired in TNF-R1 knockout mice **(94)**.

The role of TNF-α as mediator of cell death has been described in a wide variety of liver diseases. Serum TNF-α levels are elevated in patients with alcoholic hepatitis and directly correlate with increased mortality. In these patients, TNF-α has been shown to signal via the TNF-R1 pathway, inducing both apoptosis and necrosis of the hepatic parenchyma **(95)**. Serum TNF-α levels are also higher in patients with both fulminant and chronic hepatitis and seem to correlate, at least in fulminant hepatitis, with the severity of the disease. The serum levels of soluble TNF-R1 and TNF-R2 are also significantly elevated in chronic hepatitis B. A massive production of TNF-α by blood mononuclear cells has been observed in chronic hepatitis B patients undergoing interferon-α treatment at the time of successful antigen seroconversion, suggesting that TNF-α may be involved in viral clearance **(96)**.

The study of genetic models of knockout animals has demonstrated the crucial role of the TNF/TNF-R1 system in immune and inflammatory responses. The apparently normal phenotype of TNF-R1 and TNF knockout mice suggests that TNF is not essential for embryonic development. However, mice lacking TNF-R1 show some abnormalities in the lymphoid organs, such as a lack of Peyer's patches, and impaired differentiation of follicular dendritic cells and formation of germinal centers. They also produce a reduced antibody response after immunization and display an increased susceptibility to *Listeria monocytogenes* and *Mycobacterium tuberculosis*, infections usually controlled by the mice **(79, 97, 98)**. TNF knockout mice show essentially the same characteristics, except that they do develop Peyer's patches **(99)**. Those genetic models allowed the demonstration that TNF has a dual role during the inflammatory process: a pro-inflammatory role in the initial phase of infection and inflammation, and an antiinflammatory/repair function after the infectious or toxic agent has been localized and controlled **(99)**.

Several human immune diseases have been found to be associated with a deregulation of the TNF/TNF-R system. In a genetic disease called TNF receptor-associated periodic syndrome (TRAPS), heterozygous dominant alleles of TNF-R1, with amino acid changes in the extracellular domain, cause a decrease in TNF-R1 shedding and an enhancement of the pro-inflammatory effects of TNF, which results in severe localized inflammation and the development of familial periodic fever **(100)**. TRAPS has been successfully treated with soluble TNFR2-Ig fusion protein constructs, which act as decoy receptors for the cytokine. Fusion constructs containing immunoglobulin or leucin zipper oligomerization domains allow a high-level activity of the soluble ligand or receptor to be achieved.

Overproduction of TNF has been associated with several autoimmune conditions. Elevated levels of TNF-α have been detected in diseases such as rheumatoid arthritis, inflammatory bowel disease (Crohn's disease), multiple sclerosis, and systemic lupus erythematosus. Several clinical trials for the treatment of rheumatoid arthritis and Crohn's disease have utilized anti-TNF-α mAbs to neutralize the pro-inflammatory cytokine with successful results, demonstrating that this approach may be an effective therapeutic option in the treatment of chronic inflammatory diseases **(101–103)**.

TRAIL Receptors and TRAIL

TRAIL Receptors

Another proapoptotic member of the TNF family, TNF-related apoptosis-inducing ligand (TRAIL, also called APO-2L), can bind to five different cognate receptors. Two of them, TRAIL-R1 (also called DR4) and TRAIL-R2 (also called DR5 or Killer or TRICK2), are considered actual death receptors, as engagement of TRAIL with these receptors results in apoptosis. TRAIL-R1 and TRAIL-R2 are plasma membrane receptors consisting of an extracellular region containing two cysteine-rich domains, which enable the receptors to bind TRAIL, a transmembrane domain, and a cytoplasmic tail containing a death domain essential for transducing the apoptotic signal **(104, 105)**. TRAIL-R1 is expressed in most human tissues, including the spleen, thymus, liver, peripheral blood leukocytes, activated T cells, small intestine, and some tumor cell lines **(106)**. TRAIL-R2 expression has a ubiquitous distribution in both normal tissue and tumor cell lines but is particularly high in the spleen, peripheral blood leukocytes, and activated lymphocytes. Two other receptors, TRAIL-R3 (also called DcR1 or TRID or LIT) and TRAIL-R4 (DcR2/TRUNDD), are so-called decoy receptors **(107, 108)**. Although quite similar to TRAIL-R1 and TRAIL-R2 in their extracellular and transmembrane regions, TRAIL-R3 lacks the entire intracellular domain, including the death domain, and TRAIL-R4 possesses a nonfunctional death domain. Therefore, binding of TRAIL to these receptors fails to trigger apoptosis. Indeed, transient overexpression of TRAIL-R3 or TRAIL-R4 can confer resistance to TRAIL-induced apoptosis in normal and cancer cells **(108–110)**. Recent studies suggested that TRAIL-R3 and TRAIL-R4 prevent TRAIL-induced apoptosis via a distinct mechanism. While TRAIL-R3 seems to inhibit the formation of TRAIL-R2-associated DISC merely through a competitive binding of TRAIL within the lipid rafts, TRAIL-R4 is co-recruited with TRAIL-R2 within the DISC, where it impairs the processing and activation of initiator caspases **(111)**. TRAIL-R4 has also been shown to activate NF-κB and induce the transcription of antiapoptotic genes **(108)**. TRAIL-R3 and TRAIL-R4 transcripts are almost ubiquitously expressed in healthy human tissues, but not in most cancer cell lines **(106)**. The

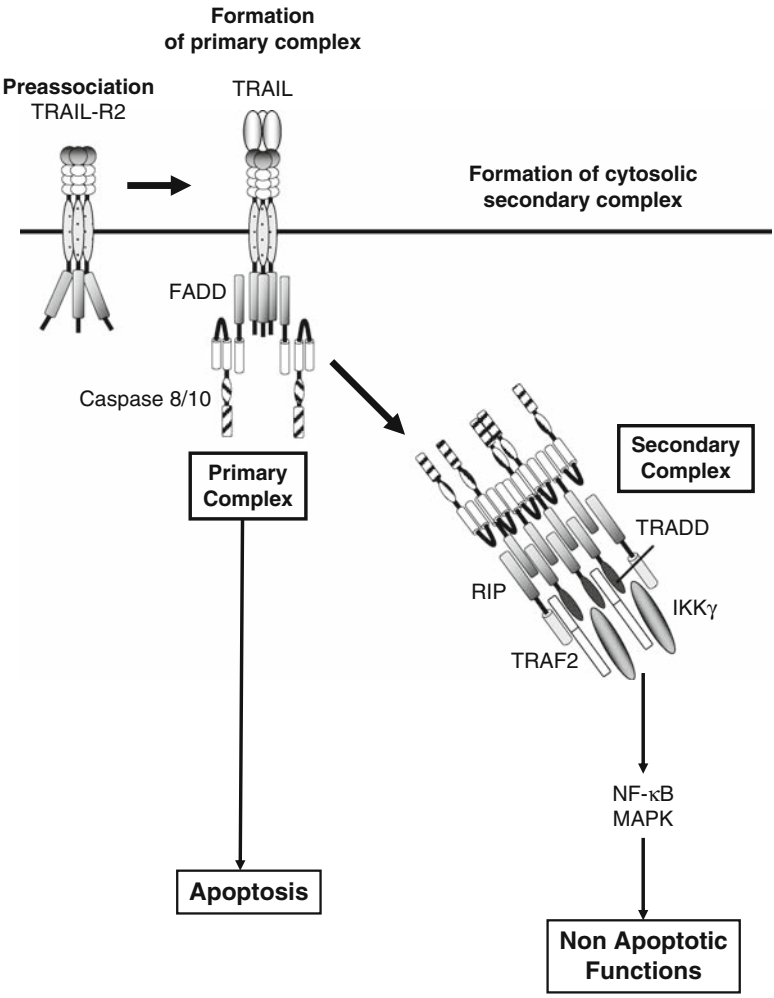

Fig. 5.7 The TRAIL-R2 DISC. Schematic representation of the early steps in TRAIL-R2 signaling and the formation of signaling complexes triggered by engagement of TRAIL to TRAIL-R2. See the text for details

preferential distribution of the decoy receptors in normal tissues, together with their ability to compete with the death receptors for binding to TRAIL, has been thought to account for the higher resistance of normal cells to TRAIL-induced apoptosis. However, a potential decoy function of these receptors has so far only been shown in overexpression experimental models, whereas their role in a physiological setting has never been proved. It is now widely recognized that intracellular regulation of TRAIL signaling more likely accounts for the resistance of healthy cells to TRAIL-mediated apoptosis rather than the surface density of decoy receptors. The fifth identified receptor for TRAIL is the

soluble osteoprotegerin receptor (OPG), which also binds the osteoclast differentiation factor (ODF), another member of the TNF family. OPG mainly acts as a regulator of the development and activation of osteoclasts in bone remodeling through its interaction with ODF. OPG can also act as a decoy receptor since it efficiently binds TRAIL but does not induce apoptosis. At 37 °C, TNR-R2 has the highest affinity for TRAIL, while OPG has the weakest **(112)**.

TRAIL

TRAIL/APO-2 was first identified in 1995 by screening DNA databases based on sequence homology with other members of the TNF family **(113, 114)**. In particular, among the TNF family, TRAIL was found to share the highest sequence homology with FasL but did not bind Fas or any of the other previously known receptors of the TNF-R-family. The TRAIL gene is located in chromosome 3, and its mRNA is expressed constitutively in many tissues. TRAIL is expressed mainly by cells of the immune system and plays a role in T-cell homeostasis as well as in NK- and T-cell-mediated killing of virally and oncogenically transformed cells **(115–117)**. Like the other ligands of the same family, TRAIL is synthesized as a type II transmembrane protein (281 amino acids) that can also be proteolytically cleaved by a cysteine protease to generate a soluble form **(118)**. Its carboxy-terminal extracellular domain shows significant homology to other TNF family members, whereas the cytosolic amino-terminus is considerably shorter and not conserved among species. The biologically active form of TRAIL is a homotrimer with the cysteine residues in position 230 coordinating a zinc ion, essential for proper folding, trimer association, and activity of the cytokine itself. TRAIL seems to trigger apoptosis more specifically in tumor cell lines and tumor xenografts rather than in normal cells, although the reason for this differential sensitivity has yet to been explained **(119–121)**.

TRAIL-R1 and TRAIL-R2 Signaling

Similar to Fas, activated TRAIL-R1 and TRAIL-R2 recruit FADD, caspase-8, and caspase-10 to their respective DISCs **(122)**. FADD and caspases-8 and -10 not only are integral components of the TRAIL receptor DISC, but are also essential for TRAIL-induced apoptosis. Therefore, TRAIL-R1 and TRAIL-R2 seem to trigger apoptosis through a pathway similar to that activated by Fas **(123)** (Fig. 5.7). However, unlike Fas and TNF-R1, which have been shown to depend on the internalization of the receptor to induce apoptosis, TRAIL-R1 and TRAIL-R2 do not require internalization for the formation of DISC and the transmission of an apoptotic signal in type I cells **(124)**. Indeed, despite ligand-engaged TRAIL-R1 and TRAIL-R2 being rapidly internalized via both

clathrin-dependent and clathrin-independent pathways, the inhibition of endocytosis does not prevent recruitment of FADD and caspase-8, or apoptosis. TRAIL-induced apoptosis is effectively inhibited by overexpression of c-FLIP (often observed in tumors), which appears to be a key factor in determining cell sensitivity to TRAIL-induced apoptosis **(125)**. Recently, it has also been suggested that a posttranslational modification, O-glycosylation of the TRAIL receptors, increases TRAIL sensitivity by promoting ligand-induced receptor clustering and consequent caspase-8 activation. Elevated expression of O-glycosyltransferases, the enzymes responsible for O-glycosylation of the receptors, correlates with sensitivity to TRAIL-mediated apoptosis in a large number of tumor cell lines and could represent a potential new biomarker to screen cancer patients to be included in TRAIL-based clinical trials **(126)**.

Several reports also show that in addition to inducing cell death, TRAIL promotes the activation of NF-κB and MAP kinases through distinct, independent pathways **(127)**. In particular, TRAIL-R1, TRAIL-R2, and TRAIL-R4 have been shown to activate NF-κB via a TRAF-2-NIK-IKKα/β-dependent signaling cascade, whereas TRAIL-R1 induces JNK (c-Jun NH2-terminal kinase) activation via a TRAF-2-MEKK1-MKK4-dependent pathway. This suggests a bifurcation in the signaling pathway at the level of TRAF-2 similar to that described for TNF-R1. Interestingly, activation of NF-κB is not sufficient to block TRAIL-induced apoptosis **(128)**. It has recently been proposed that TRAIL-induced activation of MAP kinase pathways is mediated by the formation of a secondary intracellular signaling complex subsequent to the primary apoptosis-inducing DISC (Fig. 5.7). Ligation of TRAIL-R2 by TRAIL quickly leads to recruitment of FADD and caspase-8 to the receptor, forming the primary complex (DISC) that signals apoptosis. Subsequently, the primary complex dissociates and multiple proteins, including RIP1, TRAF2, IKKγ, and TRADD, organize into a secondary complex that lacks the ligand and the receptor but contains FADD and caspase-8. This secondary complex is responsible for activation of NF-κB and MAP kinase pathways, such as JNK and p38 **(129)**. In different settings, TRAIL can also stimulate the activation of ERK (extracellular signal-regulated kinase) pathways, which is generally associated with antiapoptotic functions. Indeed, ERK activation inhibits TRAIL-mediated apoptosis by suppressing caspase-8 activation and Bid cleavage **(130, 131)**, and, consistently, the inhibition of ERK phosphorylation sensitizes cells to TRAIL-induced apoptosis **(23, 132)**. Since the primary response to TRAIL treatment is apoptosis in many cell lines, these prosurvival pathways must be kept under control by mechanisms that either are constitutively active in the cell or are driven by TRAIL stimulation. On the contrary, in TRAIL-resistant cells, the triggering of antiapoptotic signals, in particular activation of NF-κB, seems to be the prominent response to TRAIL. In these cells, TRAIL treatment has been described to promote oncogenic features, such as tumor metastasis and invasion **(133, 134)**, in an NF-κB-dependent manner, as well as NF-κB-dependent production of pro-inflammatory cytokines **(135)**. Given this oncogenic activity of TRAIL, it is crucial to investigate the players and

pathways involved in TRAIL-mediated NF-κB activation in order to optimize TRAIL-based anticancer therapies.

Physiology and Pathophysiology

Studies with TRAIL knockout mice have confirmed the role of TRAIL in antitumor immune surveillance by NK cells, in particular in host defense against tumor initiation and metastasis **(136–138)**. Indeed, TRAIL has been found to be constitutively expressed in a large number of mouse liver NK cells and likely accounts for the antimetastatic function of liver NK cells against TRAIL-sensitive tumor cells. As TRAIL has been shown to be regulated by interferon-γ endogenously produced by virus-infected cells, it appears to be, at least in part, responsible for the interferon-γ-dependent pathway of NK-cell-mediated antitumor immunity. Therefore, TRAIL is an important player in immune surveillance against oncogenically transformed and virally infected cells. Studies in TNF receptor-deficient mice (mice express only one TRAIL receptor) support the role of TRAIL-R as an inflammation and tumor suppressor in multiple tissues *in vivo*. Indeed, TRAIL-R knockout mice display increased susceptibility to develop lymphomas with high metastatic potential and diethylnitrosamine-induced (DEN-induced) liver tumors **(139)**. In particular, TRAIL-R seems to specifically suppress metastasis **(140)**.

The apparent selectivity of TRAIL to kill tumor cells renders it a promising candidate for cancer therapy. Initial studies seemed to suggest that the tumoricidal activity of TRAIL was not accompanied by any significant systemic toxicity when administered to mice and nonhuman primates **(119**, **121)**, unlike Fas, which induces fulminant hepatic failure, or TNF-α, which elicits hypotension and systemic inflammatory syndrome. Subsequent reports challenged that conclusion, showing that TRAIL has cytotoxic effects *in vitro* on isolated hepatocytes from different species, including humans, and demonstrating significant species-related differences in TRAIL sensitivity **(141**, **142)**. These studies, however, were performed using a tagged TRAIL to induce apoptosis, and careful studies later established that the toxicity of the ligand toward normal cells is associated with aberrant biochemical and structural properties of recombinant variants of the protein rather than the protein itself **(120)**. Studies using a nontagged, soluble zinc-replete form of TRAIL showed no hepatotoxicity **(143**, **144)**. Indeed, recombinant TRAIL as well as agonistic antibodies against TRAIL receptors is currently in Phase I/II clinical trials for solid tumors and hematologic malignancies. The early results of these studies report no major dose-limiting toxicity with any of the recombinant TRAIL or the TRAIL-specific monoclonal agonistic antibodies tested **(145**, **146)**. More importantly, preliminary data show therapies with TRAIL as a single agent or in combination with other chemotherapeutic agents (i.e., carboplatin and paclitaxel) have yielded promising results in the treatment of chemotherapy-refractory Hodgkin's disease and chondrosarcoma **(147)**.

Death Receptor 3 (DR3/APO-3/TRAMP/WSL-1/LARD) and APO-3L

DR3 was identified and characterized in 1996 by its high homology with TNF-R1, particularly in its death domain **(148–151)**. DR3 is a 393-amino-acid protein of approximately 47–54 kDa. Like the other death receptors, DR3 has an extracellular, N-terminal domain, which contains four cysteine-rich motifs, followed by a transmembrane domain, and a 193-amino-acid-long, C-terminal cytosolic tail. Its mRNA has been detected in spleen, thymus, and peripheral blood lymphocytes, but not in the heart, brain, placenta, lung, liver, skeletal muscle, kidney, or pancreas. DR3 is upregulated during T-cell activation, and the activation of DR3 has been described to regulate osteoblast maturation **(152)**. A natural ligand of DR3 was identified shortly after the receptor and was named Apo3L **(153)**. Apo3L is a 249-amino-acid protein with a molecular weight of approximately 27 kDa and, like the other ligands of the TNF family, is a type II transmembrane protein. Like the receptors, Apo3L and TNF also share high protein sequence identity. However, their localization is substantially different, as Apo3L is ubiquitously expressed in fetal and adult tissues, whereas TNF is expressed mainly in activated macrophages and lymphocytes. The responses and signaling, triggered by either the overexpression of DR3 or the binding of DR3 to Apo3L, resemble that mediated by TNF-R1. Indeed, DR3 activates NF-κB through TRADD, TRAF-2, RIP, and NIK and also induces apoptosis, through a pathway mediated by TRADD, FADD, and caspase-8. The different expression of the ligands and receptors, however, suggests that, despite the similarities in the signaling mechanisms, DR3 and TNF-R1 likely have distinct biological roles. Indeed, negative selection and anti-CD3-induced apoptosis have been found to be significantly impaired in DR3-null mice, suggesting a unique, nonredundant role for this receptor in the removal of self-reactive T-cells in the thymus **(154)**.

Another ligand for DR3 has recently been identified, namely the endothelial cell-derived TNF-like factor, TL1A, whose expression is inducible by TNF and IL-1α. TL1A induces NF-κB activation and apoptosis in DR3-expressing cell lines. Interestingly, in T-cells, TL1A acts as a co-stimulator that increases IL-2 responsiveness and the secretion of pro-inflammatory cytokines both *in vitro* and *in vivo*, suggesting that the interaction of TL1A with DR3 promotes T-cell expansion during an immune response **(155)**.

Death Receptor 6 (DR6)

DR6 was identified as a novel death receptor in 1998 based on the presence of the characteristic extracellular cysteine-rich domain and the intracellular death domain **(156)**. However, unlike the other death receptors, the death domain is not localized at the C-terminus, but it locates adjacent to the transmembrane

domain, followed by a 150-amino-acid tail. Following the death domain, there is a putative leucin zipper sequence overlapping a proline-rich region, whose functional relevance is still obscure. No specific cognate ligand for DR6 has yet been identified. DR6 is highly expressed in thymus, spleen, and lymphoid cells. The signaling pathway originating from this receptor has been poorly defined so far. Exogenous expression of DR6 induces apoptosis in untransformed or tumor-derived cells, and the apoptotic function of DR6 is inhibited by the co-expression of Bcl-2, Bcl-xL, or the inhibitor-of-apoptosis (IAP) family member survivin. However, the pathways engaged downstream of the receptor differ from those initiated by the other known death receptors, as DR6 does not associate with death domain-containing adaptor molecules such as TRADD, FADD, RAIDD, or RIP. Upon overexpression, DR6 is a potent inducer of JNK and NF-κB activation. The ability to activate the JNK pathway and the predominant expression in lymphoid organs suggest that DR6 may have a role in the immune system. In particular, studies employing DR6 knockout mice demonstrated that DR6 works through JNK to regulate the differentiation of naïve $CD4^+$ into Th1 and Th2 cells **(80)**.

Despite its ability to induce apoptosis, high levels of DR6 mRNA and protein have been found in tumor cell lines and advanced tumors. The absence of DR6 cytotoxicity can be explained by the concomitant activation of NF-κB and expression of NF-κB-induced antiapoptotic protein in the same cells, suggesting that NF-κB-regulated survival proteins may protect from DR6-induced apoptosis and that DR6 is a target of NF-κB regulation. Moreover, TNF-α treatment upregulates DR6 expression through activation of NF-κB **(157)**.

Experimental Tools for Inhibiting Death Receptor-Mediated Apoptosis

From the above overview, it is clear that several approaches can be used to inhibit Fas-mediated apoptosis, or to implicate death receptor signaling in an apoptotic process. One of the most common approaches is the inhibition of caspase-8 by using pharmacological inhibitors such as IETD-flouromethylketone (fmk). These compounds are peptide-based inhibitors developed as specific probes for caspase activity based on the favored substrates of the caspases. However, since the general motifs of the cleavage sites can overlap between caspases, these inhibitors often show problems of specificity, as some of them can inhibit more than one caspase with little selectivity **(158)**.

Another widely used approach consists of transiently transfecting the cells with an expression vector encoding the viral serpin CrmA (cytokine response-modifier A), a toxin produced by the cowpox virus that blocks apoptosis by binding to the active protease, thus preventing further activation of effector caspase-8. It is still controversial whether CrmA also inhibits caspase-10. Because of its high specificity, CrmA is a very effective tool to block

caspase-8-mediated cell death. The assembly of the DISC can also be disrupted by the forced overexpression of FLIP, which ultimately prevents caspase-8 activation. Likewise, the overexpression of a dominant-negative FADD, which contains the death domain but lacks the death effector domain and, therefore, cannot bind to caspase-8 or -10, also prevents DISC assembly. However, toxicity due to reagents commonly used for transfection or utilization of difficult-to-transfect cell lines can limit the use of these approaches.

Finally, the recent introduction of short interfering RNA (siRNA) techniques in common laboratory practice has largely expanded the ability to downregulate the expression of several key proteins involved in death receptor-mediated pathways. The inhibition mechanism is triggered by introducing a short interference double-stranded RNA (siRNA, approximately 19–27 bp) into the cytoplasm, where the guide strand of siRNA (usually antisense strand) binds to its target messenger RNA and the expression of the target gene is blocked. Designing siRNAs with high efficiency and high specificity to their target gene is critical to achieving effective inhibition. Although many algorithms have been developed for this purpose, it is still difficult to design siRNAs that completely suppress the target protein expression.

Conclusions

In summary, multiple death receptors are expressed differentially in mammalian cells. Their role in maintaining tissue homeostasis through regulation of cell death and survival is crucial, as demonstrated by the apparent redundancy of their signaling pathways. The death receptors do not have catalytic activity, but they undergo oligomerization by binding to selective, noncross-reacting ligands, and recruit adapter molecules by interaction of their common cytoplasmic death domains. The adapter molecules, in turn, recruit initiator procaspases, which undergo self-processing in the receptor complex, likely by induced proximity and weak, but intrinsic catalytic activity. The activated caspases then initiate complex cell signaling cascades, culminating in cell death by apoptosis. Several intrinsic cell proteins (i.e., cFLIPs, cIAPs) and survival signaling pathways (i.e., NF-κB) can inhibit death receptor-mediated apoptosis. The ultimate fate of the cell depends upon the cell context, simultaneous signaling events, and other stimuli. Death receptors are particularly important in immune regulation and tissue injury in disease states, and they have already been targeted for the treatment of several human inflammatory and autoimmune diseases. The purposeful induction of apoptosis by death receptors, especially TRAIL, may ultimately be useful also in cancer therapy, where it could bypass the need for a functional p53 associated with several conventional chemotherapies.

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Chapter 6
The Mitochondrial Pathway: Focus on Shape Changes

Silvia Campello and Luca Scorrano

Abstract Mitochondria are key participants in cell death. They amplify death signals by releasing proapoptotic proteins normally stored in their intermembrane space, such as cytochrome c. In recent years, cytochrome c release has been demonstrated to be not only highly regulated by the proteins of the Bcl-2 family, but also influenced by changes in mitochondrial shape, including remodeling of the cristae and fragmentation of the cytosolic network, both orchestrated by a large group of "mitochondria-shaping" proteins. We focus our attention in this chapter on the involvement of mitochondrial shape changes in apoptosis and on their regulatory mechanisms. In particular, we discuss the roles of the pro-fusion OPA1 protein and of the inner mitochondrial membrane rhomboid PARL on cristae remodeling and apoptosis in mammals, and on the relationship among Bcl-2 family members, mitochondrial fragmentation, and cell death. These results open the possibility to modulate mitochondrial morphological changes in order to influence apoptosis and thus to intervene in the natural history of human diseases, from neurodegeneration to cancer.

Keywords Apoptosis · Mitochondria · Cytochrome c release · Fusion · Fission · Cristae remodeling

Mitochondria

Mitochondria are dynamic and versatile organelles, the only ones among the subcellular components with their own DNA (mtDNA). Their functional versatility is paralleled by their morphological complexity. In certain cell types, they are organized in networks of interconnected organelles **(1)**. Their internal compartment, the matrix, is enclosed by an inner membrane (IM) and an outer

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(OM) membrane, which delimit the intermembrane space (IMS). Recent advances in electron tomography revealed that the inner membrane can be further subdivided into a peripheral inner boundary membrane; in the cristae compartment, baglike folds of the IM connect to it via narrow tubular junctions **(2)**.

From a functional point of view, mitochondria represent the site of oxidative phosphorylation, which ensures production of most of the cellular ATP. During oxidative phosphorylation, ATP synthesis results from the transfer of four electrons to O_2, giving rise to two molecules of H_2O. As a byproduct of their respiratory activity, mitochondria produce reactive oxygen species (ROS) that the cell can use as second messengers **(3)**. Cristae represent the site of oxidative phosphorylation, while the inner boundary membrane is enriched in structural proteins and in components of the import machinery of mitochondria **(4)**.

Not only do mitochondria provide most of the cellular ATP, but they also participate as key players in several signaling pathways crucial for the life and death of the cell. They shape cytosolic Ca^{2+} transients by uptaking Ca^{2+} following release from the endoplasmic reticulum **(5)**. They are part of essential anabolic and catabolic pathways that generate or dispose of cellular constituents or molecules involved in signaling pathways **(6)**. Last, but not least, mitochondria are core apoptotic components that amplify the death signals coming from within the cell or from the outside **(7)**.

Mitochondria and Apoptosis

Evidence indicates that mitochondria are key participants in cell death **(8)**, following a variety of stimuli such as DNA damage and growth factor deprivation. In certain cells like hepatocytes, they amplify the death signals induced by death receptors **(9–11)**. In the last 10 years, research has focused on the mitochondrial involvement in apoptosis. Today we can say that the basics of this process have been well defined.

The best-known aspect of the mitochondrial involvement in apoptosis is represented by the release of proteins normally stored in the intermembrane space, such as cytochrome c **(12, 13)**. This is one of the two crucial mitochondrial alterations during apoptosis, together with the onset of multiple parameters of mitochondrial dysfunction **(14)** including membrane depolarization. The order in which these events occur and whether one causes the other have been hotly debated in the literature. The majority of reports suggest that outer membrane permeabilization precedes membrane depolarization **(15)**, but it is also clear that certain apoptotic stimuli induce a cascade of mitochondrial dysfunction *followed* by permeabilization of the outer membrane **(16)**. Molecular mechanisms that are still not entirely clear and cross-talk probably mediate these two events **(17)**.

Cytochrome c release is tightly regulated by proteins of the Bcl-2 family, described in depth in another chapter of this book. Irrespective of the fine

details, their mechanism of action ultimately involves the regulation of the mitochondrial participation in apoptosis. Here we summarize the known, widely accepted mechanisms of action of Bcl-2 family members that impinge on mitochondria.

Proapoptotic BAX and BAK constitute a requisite gateway to the intrinsic pathway operating at the mitochondrion **(18)**. In viable cells, multidomain BAX and BAK exist as monomers. Upon a death signal, cytosolic BAX translocates and inserts into the OM by forming homo-oligomers, while inactive BAK that resides at the mitochondria undergoes a conformational activation, which includes its oligomerization. The final result is the permeabilization of the OM, with release of apoptogenic proteins of the IMS.

The proapoptotic "BH3-only" proteins serve as upstream sentinels to "sense" and selectively respond to specific, proximal death, and survival signals **(15)**. Recent evidence indicates that "BH3-only" proteins derepress BAX and BAK by direct binding and inhibition of BCL-2 and other antiapoptotic family members **(19)**. By contrast, an opposing model postulates direct activation of BAX and BAK by some "BH3-only" proteins **(20)**. For example, a reconstituted mitochondrial assay reveals that tBID serves as a membrane-targeted ligand and triggers oligomerization of BAX and BAK, with its intact BH3 domain, to release cytochrome c **(18, 21)**.

"BH3-only" protein expression can be induced by transcriptional regulation or posttranslational modification. NOXA and PUMA, for example, are under p53-mediated transcriptional control in response to DNA damage **(22)**. The activation of "BH3-only" proteins actually requires BAX/BAK for executing apoptosis. In contrast, antiapoptotic proteins, such as BCL-2 and BCL-xL, could inhibit apoptosis by binding and sequestering "BH3-only" proteins, preventing BAX/BAK activation, although their mechanism of action is widely debated **(19)**. For example, the antiapoptotic member MCL-1 is a cytosolic inhibitory factor whose degradation is required to initiate cytochrome c release before the mitochondrial translocation of BAX and BCL-xL **(23)**. In healthy cells, it is bound to BAK **(24)** together with BCL-xL **(25)**; upon apoptotic stimuli, this interaction is disrupted by "BH3-only" proteins such as BIM **(25)**.

Released cytochrome c triggers a postmitochondrial pathway that activates the initiator caspase-9 in a sophisticated way to subsequently cleave the effector caspases-3 and -7 **(26)**. Mitochondrial-released cytochrome c binds Apaf-1 via its WD40 domains and becomes competent to recruit caspase-9 in the presence of ATP/dADP. Binding of ATP/dADP to Apaf-1 is proposed to cause a conformational change facilitating the formation of a heptameric complex of cytochrome c/Apaf-1/caspase-9, known as the "apoptosome" **(13, 27)**. Cells deficient for cytochrome c **(28)**, Apaf-1 **(29)**, caspase-9 **(30)**, or caspase-3 **(31)** display defects in apoptosis following intrinsic signals, providing further substantiation to the importance of this pathway.

Curiously, certain cell types (such as cardiomyocytes) can survive the cytochrome c release step, at least for a limited amount of time **(32)**. In such cells, caspases might be stringently regulated by caspase-inhibiting IAPs. Mammalians

IAPs are controlled and antagonized by several mechanisms including binding of SMAC/DIABLO and OMI/HTRA2, two mitochondrial IMS proteins released during apoptosis **(33)** that relieve the IAP inhibition and thereby allow caspase activation.

The inability of caspase inhibitors to completely protect cells and their organelles from damage following intrinsic death signals suggests that caspase-independent death might also occur. Two mitochondrial IMS proteins, the endonuclease G and the apoptosis-inducing factor (AIF), are released upon stimuli and induce caspase-independent apoptotic DNA degradation in mammalian cells by translocating to the nucleus upon release **(34)**.

Mechanisms of Cytochrome c Release

While considerable detail has been revealed, uncertainties still exist concerning the precise mechanism(s) by which proapoptotic BCL-2 family members regulate the release of cytochrome c from mitochondria. Data from our laboratory support the existence of a bifurcated cytochrome c release pathway controlled in concert by "BH3-only" and multidomain proapoptotic BCL-2 family members. One arm activates the multidomain proapoptotics and grants a physical pathway for cytochrome c release across the outer mitochondrial membrane; the other, independent of BAX and BAK, orchestrates changes to the mitochondrial inner membrane necessary to grant complete release of cytochrome c and mitochondrial dysfunction, although this has been recently challenged and proposed to be a byproduct of caspase activation. In this part of the chapter, we will discuss the "canonical" proposed mechanisms for cytochrome c release at the OM level.

Following a variety of intrinsic and extrinsic death signals, "BH3-only" proteins undergo posttranslational modifications including dephosphorylation **(35)** and cleavage **(10**, **11**, **36)**. These result in their activation and often in their translocation to mitochondria, where they exert their biological function. Several mechanisms have been proposed by which "BH3-only" proteins can exert their biological action: (1) "BH3-only" proteins autonomously induce cytochrome c release, without the participation of BCL-2 family or intrinsic mitochondrial proteins but with a marked requirement for negatively charged lipids such as cardiolipin **(37**, **38)**; (2) they interact and inhibit antiapoptotic BCL-2 family members **(15**, **19**, **39–41)**; (3) they activate the multidomain proapoptotic BAX and BAK to mediate cytochrome c release **(18**, **26**, **42)**; (4) they interact with intrinsic mitochondrial proteins such as the adenine nucleotide exchanger (ANT) or the voltage-dependent anion channel (VDAC) to induce mitochondrial dysfunction and cytochrome c release **(43**, **44)**.

The "BH3-only" molecules BID, BIM, BAD, and NOXA require BAX and BAK to exert their mitochondrial proapoptotic activity **(26**, **42)**. Yet BAD, BIM, NOXA, and BIK display a marked binding preference for the

antiapoptotic members BCL-2 and BCL-xL versus the multidomain proapoptotics in multiple interaction assays of yeast two-hybrid, pulldown, or co-immunoprecipitation from detergent-solubilized lysates **(45**, **46)**. Moreover, functional and mutational analysis suggests that BAD promotes death only when it binds to BCL-xL **(45**, **47)**. Insight into this apparent dichotomy came from the functional analysis of BH3 peptides from the different "BH3-only" proteins. Thus, "sensitizing" BAD-like BH3 domains can preferentially act on antiapoptotic members and thus enable subliminal concentrations of BID-like activators to induce activation of the multidomain proapoptotics BAX and BAK **(48**–**50)**. We believe that these convergent genetic and functional analyses indicate that "BH3-only" molecules require the multidomain proapoptotic to induce cytochrome c release and death.

What is the activation event for the multidomain proapoptotic BAX and BAK? The measurable hallmarks of BAX/BAK activation are an exposure of new epitopes and their homo-oligomerization in the outer mitochondrial membrane **(51**–**53)**. Gel filtration and cross-linking experiments show that BAX oligomers are found only in the mitochondrial membranes after apoptotic stimulus or BAX activation by BID **(53**, **54)**, while protein cross-linking reveals a predominance of BAX homodimers, trimers, and tetramers **(54)**. Following activation by BID **(51)**, BIM **(42)**, or BH3 domains of "BH3-only" proteins **(50)**, BAK undergoes a similar homo-oligomerization, generating distinct complexes revealed by cross-linking, again consistent with dimers, trimers, and prominently tetramers. A major question is how the oligomerized multidomains BAX and BAK allow the release of proapoptotic activators from mitochondria. Current models consist of (1) a global effect on the permeability of the OM by a number of possibilities, including a concept of "lipid" channels in the bilayer **(37**, **38)** and interaction with proteins controlling the mitochondrial morphology **(55**, **56)**, (2) interactions between BAX and resident mitochondrial proteins such as VDAC **(57)** or ANT **(58)**, which are proposed to release cytochrome c directly or trigger the mitochondrial permeability transition (PT), (3) BAX and BAK oligomers generate in the OM a large pore permeable to cytochrome c and possibly to other proapoptotic proteins.

Irrespective of the exact mechanism by which active BAX and BAK release cytochrome c, it appears clear that these multidomain proapoptotic molecules act as the essential gateway to the mitochondria, serving as the critical steps of their engagement. Recent data indicate that while this step is required, there is an additional complex process downstream of the "BH3-only" proteins, ensuring complete release of cytochrome c and mitochondrial dysfunction, resulting in a massive remodeling of the mitochondrial ultrastructure. This additional pathway includes fragmentation of the mitochondrial network and remodeling of the cristae characterized by fusion of individual cristae and opening of the cristae junctions **(59**, **60)**, two aspects we will describe in the second part of the chapter.

Changes in Mitochondrial Shape During Apoptosis

The notion that mitochondrial shape remained untouched during cell death stood unchallenged for many years and was classically used as one of the major criteria to distinguish apoptosis from necrosis. In recent years, this assumption has been challenged by at least two main observations: the mitochondrial network undergoes fragmentation, and the topology of the inner membrane is altered during the functional and proteomic changes that occur to the apoptotic mitochondrion **(1)**. These observations prompted several groups to investigate the mechanisms regulating mitochondrial morphology in healthy and dying cells. In this section, we give a general overview of the highly complex and controlled machinery that regulates mitochondrial morphology.

Regulation of Mitochondrial Morphology

Mitochondrial morphology in living cells is heterogeneous and can range from small spheres to interconnected tubules **(61)**. This heterogeneity results from the balance between fusion and fission. Individual mitochondrial tubules continuously move back and forth along their long axes on radial tracks. Two mitochondrial tubules can seldom encounter each other and fuse. On the other hand, tubules can also undergo fission events, giving rise to two or more mitochondrial units **(61)**. Moreover, as mentioned above, the mitochondrial ultrastructure is also extremely complex, with several subcompartments whose shape is also tightly regulated **(2)**. Growing evidence indicates that mitochondrial morphology is critical for the physiology of the cell and that changes in mitochondrial shape have been related to many different processes such as development, neurodegeneration, calcium signaling, ROS production, cell division, and apoptotic cell death **(1)**.

The shape of mitochondria is controlled by a growing set of proteins that regulate the fusion-fission equilibrium of the organelle and that have been identified only recently. Members of this family include dynamin-related proteins, large mechanoenzymatic GTPases involved in the tubulation and severing of biological membranes (62), as well as other "nonconventional" proteins whose molecular function is less characterized that appear to regulate these processes or participate in them via an as-yet unclear mechanism.

Pro-fusion

The first identified mitochondrial shaping protein was the *D. melanogaster* Fuzzy onion 1 protein (Fzo1p), a large transmembrane GTPase of the OM, required for the formation of the giant mitochondrial derivative during spermatogenesis. Fzo1p has two homologues in mammals, MFN1 and MFN2 **(63)**, which are highly similar and control mitochondrial fusion, even though it seems

that they have different roles in cell physiology. Indeed, it is still to be clarified whether the two MFNs are completely interchangeable in terms of mitochondrial fusion as well as of other cellular functions. They possess an N-terminal GTPase domain, two transmembrane domains spanning the OM, and two regions crucial for protein-protein interactions **(63**, **64)**. Deletion of either MFN gene impairs embryonic development in the mouse at different stages **(65**, **66)**.

The only dynamin-like GTPase so far identified in the IM is OPA1. This protein is mutated in dominant optic atrophy (DOA), the most common cause of inherited optic neuropathy, and promotes fusion **(67**, **68)**. At least eight different splice variants of OPA1 have been identified **(69)**. Posttranscriptional mechanisms, including proteolytic processing, tightly regulate OPA1 activity, and single isoforms are therefore retrieved in several processed forms, whose functional significance is still not clear. The N-terminal matrix-targeting signal of OPA1 is removed by the mitochondrial processing peptidase (MPP) in the matrix during import to form the mature OPA1 isoform (l-OPA1), which is subjected to further processing to form the short isoform (s-OPA1). The presence of both long and short forms is essential for the maintenance of normal mitochondrial morphology and mitochondrial DNA **(70)**.

LETM1 is an inner membrane protein, deleted in Wolff-Hirschhorn syndrome, a complex genetic condition, homologue to the yeast regulator of mitochondrial morphology Mdm38p **(71)**. Its ablation results in mitochondrial fragmentation **(72)**. However, it appears that LETM1 does not impinge on the core mechanism of mitochondrial morphology, since its downregulation cannot be complemented by inhibition of the fission machinery **(72)**. Work performed in yeast suggests that Letm1/Mdm38p is involved in the regulation of mitochondrial K^+/H^+ exchange, indicating possible cross-talk between ion homeostasis and regulation of mitochondrial shape **(73)**.

The mechanism of mitochondrial fusion has been closely analyzed during recent years, especially in yeast. It appears that fusion of the outer mitochondrial membrane requires high levels of GTP, but not mitochondrial membrane potential, while fusion of the inner membrane does **(74)**. In mammalian cells, MFN1 docks two juxtaposed mitochondria via its second coiled domain to promote fusion **(75)**. MFN1 has higher GTPase activity and induces fusion more efficiently than MFN2 **(76)**. Furthermore, OPA1 requires MFN1 to mediate fusion, while MFN2 functions independently of OPA1 **(67)**. MFN2 seems to have an additional role in determining the efficiency of mitochondrial oxidative metabolism **(77)**.

The list of proteins involved in the control of mitochondrial fusion recently grew to include a phospholipase, PLD, associated to the OM, where it hydrolyzes cardiolipin to generate phosphatidic acid, a lipid involved in SNARE-mediated vesicular fusion **(78)**. This is the first evidence of a possible relationship between the SNARE-mediated vesicle formation and the mitochondrial fusion process.

Pro-fission

In mammalian cells, mitochondrial division is regulated by DRP1 and FIS1 **(79, 80)**. The large GTPase DRP1 is a cytosolic dynamin-related protein. Its inhibition or downregulation results in a highly interconnected mitochondrial network **(81)**. The same phenotype is caused by downregulation of FIS1 **(79)**. FIS1 is a 16-kDa integral protein of the outer mitochondrial membrane, containing a single transmembrane domain and a hydrophobic domain facing the cytosol **(82)**, suggesting the existence of hydrophobic interactions with DRP1 or with an as-yet unidentified adaptor during mitochondrial fission. Some evidence indeed exists that FIS1 is the receptor on the outer membrane for DRP1. The latter is recruited to mitochondria, and constriction of the membranes takes place by direct or indirect interaction with hFIS1 **(83)**. Levels of FIS1 and DRP1 and therefore mitochondrial fission are proteolytically controlled by the opposing action of sumoylation and ubiquitination **(84, 85)**.

In addition to these two players, endophilin B1, a member of the endophilin family of fatty acid acyl transferases that participate in endocytosis, has been shown to play a role in mitochondrial fission **(86)**. The mechanism by which endophilin B1 regulates mitochondrial shape is unclear, as it can be mediated either by a direct effect on membrane curvature or by its putative acyl-transferase activity **(86)**.

More recently, ganglioside-induced differentiation-associated protein 1 (GDAP1), a protein upregulated during cholinergic differentiation of a mouse neuroblastoma cell line induced by ganglioside, was identified as a mediator of mitochondrial fission, although its mechanism of action is completely unknown **(87)**.

Proteolytic Control of Mitochondrial Shape

A further level of complexity in the regulation of mitochondrial fusion was added by the analysis of the role of PARL, the mammalian orthologue of the rhomboid protease Rbd1p of yeast. Rhomboids are intramembrane proteases that cleave and activate a variety of membrane-inserted proteins. They participate in crucial biological processes such as Notch signaling. Considerable interest came from the discovery that one of the members of this family of proteins is targeted to the IM. The ablation of Rbd1p in yeast results in mitochondrial dysfunction, as substantiated by the petite phenotype of the deleted strains (impaired cellular growth on nonfermentable carbon sources), and abnormal mitochondrial morphology represented by fragmented mitochondria. Both phenotypes are similar to the one caused by the deletion of Mgm1p, the yeast orthologue of OPA1, which turned out to be a substrate for Rbd1 **(88)**. The short isoform of Mgm1p produced by Rbd1 is required to maintain mitochondrial morphology and fusion **(88)**. Thus, rhomboids and intramembrane proteolysis appear to control mitochondrial dynamics and function in yeast.

PARL, the mammalian orthologue of Rbd1p, is a mitochondrial protein that shares considerable homology with the yeast Rbd1p, except for its N-terminus, which is highly conserved among vertebrates but has no significant homology with orthologues found in lower eukaryotes. This part of the protein is self-cleaved to generate small peptides involved in mitochondrion-to-nucleus signaling **(89)**. A genetic model of *Parl*-/- mouse helped to verify whether PARL could also operate like Rbd1p, activating OPA1 to maintain morphologically and functionally normal mitochondria. The ablation of PARL does not result in embryonic lethality, but *Parl*-/- mice die between weeks 8 and 12 of muscular, thymic, and splenic atrophy. Lack of PARL does not affect mitochondrial function and morphology in all the tissues analyzed, but it results in an increased degree of apoptosis *in situ* and induced by intrinsic stimuli in fibroblasts derived from knockout embryos. Thus, at a major difference from yeast, the rhomboid PARL is not essential for mitochondrial fusion and function but plays a role in controlling apoptosis **(90)**. It participates in the production of a soluble IMS form of OPA1 **(90)** that together with the integral IM form constitutes a multimolecular complex at the IM **(91)**, which is responsible for the control and maintenance of the correct shape of the mitochondrial cristae and for the antiapoptotic activity of OPA1 **(91)**. A so-called β-cleavage event at the PARL N-terminal domain confers a gain of function in PARL-mediated regulation of mitochondrial morphology. Mitochondrial fragmentation induced by the overexpression of PARL was abolished when blocking the β-cleavage **(92)**. This process is sophisticatedly regulated *in vivo* by PARL phosphorylation. Since hyperphosphorylation of PARL blocks the β-cleavage **(92)**, its dephosphorylation appears to be required to allow the PARL regulation of mitochondrial dynamics.

Levels of IMS OPA1 were reduced by approximately 50% in *Parl*-/- mitochondria **(90)**. The retrieval of a substantial fraction of soluble OPA1 in *Parl*-/- mitochondria implies that PARL is probably not the only protease involved in OPA1 processing. For example, Paraplegin, an inner mitochondrial membrane mAAA metalloprotease, participates in the cleavage of OPA1 to produce a short isoform that is fusion-incompetent **(70)**. Like other AAA proteases of the inner mitochondrial membrane, Paraplegin cuts OPA1 in response to changes in the energetic status of mitochondria **(70, 93, 94)**. This in turn provides a mechanism to translate mitochondrial dysfunction into changes in the morphology of the organelle. It should be noted that Paraplegin faces the matrix and that the MPP already trims most of the matrix residues of OPA1 **(95)**. At this stage, a plausible scenario deserving further investigation is that Paraplegin and PARL somehow cooperate in the cleavage of OPA1. This would imply the existence of a network of regulation that extends from protein precursor processing to mitochondrial morphology to apoptosis. This network could be crucial in the "quality control" of mitochondria, coding damages of different intensities into an array of different responses that can vary from autophagy to cell death. Cells lacking PARL or Paraplegin have normal OPA1 processing **(96)**, suggesting that other proteases remain to be identified. For example,

Yme1L, another AAA protease with the catalytic domain located within the IMS, affects the constitutive proteolysis of a subset of OPA1 isoforms but is not required for inducible proteolysis **(93, 94)**. It remains to be elucidated whether the action of PARL is somehow in concert with that of the other proteases like Paraplegin and Yme1L that sense mitochondrial dysfunction to coordinate the modulation of OPA1's multiple functions.

Which Physiological Role for Mitochondrial Morphology?

It has been highly demonstrated in recent years that normal membrane dynamics are necessary for mitochondrial function and that changes in mitochondrial shape influence crucial cellular functions, from Ca^{2+} signaling **(97)** to the generation of reactive oxygen species **(98)**, to neuronal plasticity **(99)**, to intermediate metabolism **(77)**, even to life span **(100)**. Evidence points to a role for these proteins in several aspects of cell life and death.

Mitochondria are not randomly positioned in the cytosol: they probably accumulate at sites of high energy demand, or where they are required to regulate Ca^{2+} signaling. Mitochondrial movement is highly coordinated with changes in organelle morphology, required to produce mitochondria whose size is compatible with their transport by microtubule-associated cargoes **(101)**. The expression of pro-fusion shaping proteins such as OPA1 and MFN1 decreases the number of dendritic spines and synapses, showing a role for mitochondrial morphology in the determination of complex cellular patterns **(99)**. Ablation of Milton, a kinesin-associated protein responsible for mitochondrial movement in *Drosophila*, results in the absence of mitochondria from synapses that are otherwise normally formed **(102)**. This would suggest that mitochondrial fusion-fission plays a specific role in morphogenesis, additional to its influence on movement of the organelle. Similarly, it has recently been demonstrated that the shape of the mitochondrial reticulum regulates the ability of lymphocytes to migrate. Mitochondrial fragmentation is a required step in the determination of a migrating phenotype. Moreover, induction of mitochondrial fission per se is able to induce a polarized appearance in these lymphocytes, further strengthening the role of mitochondrial fusion-fission in cellular morphogenesis and ultimately function **(103)**.

Mitochondria, together with peroxisomes, are the major source of reactive oxygen species (ROS). This metabolic role assigns to these organelles a perhaps crucial function in the determination of life span **(104)**. Mitochondrial dynamics seem to play a role in the production of ROS as well as in longevity. The accumulation of ROS in pathological conditions such as oxygen/glucose deprivation critically depends on DRP1-dependent fragmentation of the mitochondrial reticulum **(98)**. How mitochondrial fission is required for ROS production and life span remains unclear, although a link between the two processes seems plausible. Hence, factors other than mitochondrial metabolism per se could have a role in the pathogenesis of ROS-related diseases **(98)**.

Mitochondria actively participate in the regulation of Ca^{2+} signaling by taking up and releasing Ca^{2+} in response to physiological inositol triphosphate-coupled agonists. This process relies on the relative position of mitochondria in the cytosol as well as on their juxtaposition to the endoplasmic reticulum **(5)**. It is therefore conceivable that changes in mitochondrial shape influence mitochondrial participation in the Ca^{2+} game. This hypothesis is substantiated by the finding that excessive fission by DRP1 blocks propagation of Ca^{2+} waves **(97)**, while FIS1 reduces the refilling of endoplasmic reticulum Ca^{2+} stores, probably by impairing capacitative Ca^{2+} entry from the plasma membrane **(105)**. There are indications that Ca^{2+} signaling can influence mitochondrial dynamics. Mitochondria move at resting $[Ca^{2+}]_i$, while inositol 1,4,5-trisphosphate- or ryanodine receptor-mediated Ca^{2+} signals decrease mitochondrial motility **(106)**. As movement is critically controlled by dynamics, Ca^{2+} is likely to coordinate both processes.

As we describe in detail ahead, mitochondrial shaping proteins also have a crucial role in the complete release of cytochrome c and other proapoptotic cofactors in the apoptotic process, extending the role of mitochondrial dynamics to death of the cell.

Changes in the Internal Shape of Mitochondria

Studies of the kinetics of cytochrome c release in response to apoptotic stimuli indicated that the extent of the release is remarkably complete **(107)**. However, high-voltage electron microscopic tomography of mitochondria has revealed a very narrow IMS (the average distance between the OM and IM is only ~20 nm) that possesses only 15–20% of the total cytochrome c. The majority of cytochrome c resides in the cristae, consistent with the fact that the majority of oxidative phosphorylation complexes preferentially localize there **(2)**. The cristae are pleomorphic involutions of the inner mitochondrial membrane (IM) that form a highly sequestered compartment separated from the IMS by narrow cristae junctions, with average diameters of ~20–25 nm. In most apoptotic deaths, substantial cytochrome c is released prior to any swelling of the mitochondria, which could easily explain the release of the cristae stores of cytochrome c, simply by unfolding of the IM. A possible solution came from studies that addressed changes in the mitochondrial ultrastructure during apoptosis. A set of ultrastructural changes called "cristae remodeling" has been identified that may justify how the stores of cytochrome c that appear to be so heavily subcompartmentalized can be fully released in the absence of mitochondrial swelling **(60)**.

A pathway controlled by "BH3-only" proapoptotic BCL-2 family members (especially tBID) and independent from the multidomain proapoptotics BAX and BAK triggers the remodeling of mitochondrial cristae that accounts for cytochrome c mobilization to the IMS and allows its complete release across the

OM **(60)**. The structural changes that characterize the remodeling of the cristae include the enlargement of the tubular cristae junction, which widens from its normal diameter of 20–25 nm up to 50–70 nm, and the fusion of individual cristae in a single, highly interconnected compartment. These ultrastructural changes are accompanied by the mobilization of the cytochrome c pool confined into the cristae that now becomes available in the IMS of mitochondria **(60)**.

Given the IM localization of only OPA1 among the mitochondrial shaping proteins, the protein appeared as a natural candidate to regulate the cristae-remodeling pathway. Nevertheless, its role in mitochondrial fusion **(67)** argues against a unique function in controlling the shape of the cristae and therefore their remodeling during cell death. As expected, downregulation of OPA1 leads to mitochondrial fragmentation. This is accompanied by organelle dysfunction and cytochrome c release and interestingly by changes in mitochondrial ultrastructure **(95)**. These results raised the possibility that OPA1 could, in fact, participate in the biogenesis of the cristae and regulate the cristae-remodeling pathway.

Indeed, OPA1 influences apoptosis independently from its effect on mitochondrial fusion **(90, 91)**. High levels of wild type, but not mutated OPA1, delay apoptosis induced by intrinsic stimuli by preventing mobilization of cytochrome c from the cristae stores and remodeling of the cristae. We recently also postulated a molecular mechanism by which OPA1 could control the structure of the cristae **(91)**. We already mentioned that in healthy cells, OPA1 is retrieved in high-molecular-weight (MW) complexes in the IM **(91)** and that both the integral long and the soluble short forms of OPA1 are crucial for the formation of the oligomer **(91)**. In principle, this oligomer could function as a molecular staple that participates in keeping the tubular junction of the cristae narrow. In line with this model, mechanical swelling of mitochondria leads to the loss of this oligomer; furthermore, OPA1 oligomers are disrupted early during cristae remodeling and before the release of the cristae stores of cytochrome c. OPA1 is found in lower-MW complexes upon an apoptotic stimulus **(91)**. The mitochondrial rhomboid PARL participates in the generation of the short form of OPA1 that is required to generate the high-MW complex OPA1 and ultimately to control the cristae-remodeling pathway **(90)**. PARL and OPA1 are indeed genetically positioned in the same pathway, with PARL upstream of OPA1 **(90)**. Thus, the PARL-OPA1 axis regulates the remodeling of the cristae, although the full molecular characterization of the OPA1-containing complexes is still incomplete and is likely to include a number of crucial regulators of the antiapoptotic activity of OPA1.

Changes in the Mitochondrial Reticulum

Another important structural alteration encountered by mitochondria of an apoptotic cell is the fragmentation of the network of organelles. Mitochondrial

fragmentation during apoptosis appears to be a highly conserved phenomenon, from worms to mammals. Furthermore, it is the only known and documented mitochondrial change that occurs during apoptosis in all the organisms investigated to date.

Mammals

Upon apoptotic stimuli, mitochondria undergo massive and reversible fragmentation prior to or around the same time of cytochrome c release **(32, 59)**. A transition from a mitochondrial network into vesicular punctiform mitochondria was detected in cells treated with various apoptotic stimuli, beginning at the very early stages of the cell death cascade **(108)**, but the mechanism by which the apoptotic fragmentation occurs is still unclear. DRP1 is involved in this fragmentation process; a dominant-negative mutant of DRP1 protects from fragmentation, release of cytochrome c, decrease of the membrane potential, and nuclear fragmentation of DNA, all markers of apoptosis **(59)**. Moreover, chemical inhibition of DRP1 blocks apoptosis *in situ* and release of cytochrome c in an *in vitro* system, further substantiating the role of pro-fission proteins in the cascade leading to outer membrane permeabilization. Supporting this scenario, the partner of DRP1 on mitochondrial membranes, FIS1, is also a player in apoptosis: Its overexpression leads to cytochrome c release, while its ablation protects from cell death **(79, 109)**. Not only are the pro-fission proteins activated during apoptosis, but MFN1-dependent fusion is impaired **(110)**. In principle, this could occur by blockage of the function of MFN1 itself, or of its inner membrane partner OPA1 **(67)**. This latter option is supported by the release of a fraction of OPA1 together with cytochrome c early in the course of apoptosis **(111)**. Upon induction of apoptosis, BAX translocates to the OM and, almost instantly after translocation, concentrates into submitochondrial punctate foci **(108)**. DRP1 and MFN2 co-localize with BAX in the same foci in different cell types treated with various inducers of stress-dependent apoptosis **(112)**, suggesting that BAX may be targeting the subdomains of mitochondria that are most prone to dynamic membrane rearrangements. A dominant-negative of DRP1 does not affect the dynamics of BAX translocation and foci formation but inhibits BAX-induced mitochondrial fragmentation. Thus, cytosolic DRP1 has to translocate to the OM. There are different theories concerning the mechanism and the proteins involved in the recruitment of DRP1 to the OM and its activation. For example, the "BH3-only" protein BIK induces DRP1 recruitment to mitochondria by promoting calcium release from the ER (Ca^{2+} homeostasis is at the center stage of apoptosis modulation). Another candidate that could induce recruitment of DRP1 to mitochondria could be the cytosolic phosphatase calcineurin, whose activity increases after stimuli that induce Ca^{2+} overload. DRP1 is, in fact, dephosphorylated by calcineurin at a conserved serine residue **(113)**, but it is unclear whether this dephosphorylation is a signal that leads to Drp1 recruitment on mitochondria. If this was proven correct, the subcellular localization of Drp1 could be regulated in a manner

similar to that of BAD, for example, which is also dephosphorylated by calcineurin during apoptosis **(114)**. Alternatively, recruitment of DRP1 during apoptosis could require additional components, such as proteins released from mitochondria. TIMMP8a is a protein of the IMS that is released from mitochondria and has been reported to be involved in the translocation of DRP1 from the cytosol to the organelle **(111)**, raising the question of whether DRP1-dependent fission occurs up- or downstream of OM permeabilization.

In addition to translocation, during cell death the membrane-associated pool of DRP1 becomes irreversibly locked and stabilized on the membrane upon a sumoylation event that is dependent on the presence of BAX/BAK **(115)**.

The notion that mitochondrial fission is a step toward death is reinforced by the ability of overexpressed pro-fusion MFN1 and MFN2 to block apoptosis induced by stimuli that recruit the mitochondrial pathway, perhaps by interfering with BAX activation **(116)**. Whether mitochondrial shaping proteins physically interact and regulate pro- and antiapoptotic members of the BCL-2 family is still unclear.

Yeast

Programmed cell death was generally considered to be unnecessary for single-cell organisms that do not form multicellular structures. This hypothesis was supported by the fact that yeast lack several key components (BCL-2 family proteins and caspases) of the mammalian cell death machinery. However, yeasts encode for a homologue of DRP1. The machinery responsible for mitochondrial fission was identified in yeast as a complex of three proteins, Dnm1 (the homologue of DRP1), Fis1 (the homologue of FIS1), and Mdv1/Net2, which has no obvious homologue in mammals **(117, 118)**. Deleting any one of these three causes yeast mitochondria to become fused into a large network.

In support of a primordial origin of mitochondria-dependent apoptosis, there is evidence that Dnm1 has a role in promoting mitochondrial fragmentation and cell death following treatment with several death stimuli **(119)**. Moreover, the two other Dnm1-interacting factors involved in the normal fission machinery, Mdv1/Net2 and Fis1, also regulate yeast cell death. Mdv1/Net2 promotes cells death, consistent with its role in mitochondrial fission. In contrast to its pro-fission function in healthy cells, Fis1 unexpectedly inhibits Dnm1-mediated mitochondrial fission and cysteine protease-dependent cell death in yeast. Fis1-deficient yeast has significantly reduced viability when treated with apoptotic stimuli as H_2O_2 **(119)**. Yeast knockout for Mdv1/Net2, as well as cells expressing a Dnm1 dominant-negative mutant, were significantly more resistant to death compared with wild type and Fis1-deficient cells. Fragmentation was reduced in the double-deficient Δdnm1/Δfis1 yeast compared with Δfis1, indicating that Fis1 inhibits Dnm1-mediated fission. It appears that fragmentation of mitochondria is not sufficient for cell death in yeast and that the commitment point to cell death is represented by a mitochondrial dysfunction checkpoint **(119)**.

Furthermore, the ability of yeast Fis1 to inhibit mitochondrial fission and cell death can be functionally replaced by mammalian BCL-2 and BCL-xL **(119)**. In conclusion, the common component Dnm1/Drp1 regulates a conserved programmed death pathway in yeast and mammals, inhibitable by a Bcl-2-like function in both organisms.

C. elegans

In invertebrate models of apoptosis, it is less clear which role mitochondria play. Recent data, however, have implied mitochondrial shape changes in the developmentally programmed cell death in *C. elegans*. In the well-known "direct pathway," the assembly and activity of the apoptosome appear to be blocked in healthy cells **(120)** since the Apaf-1-like protein CED-4 is sequestered to the OM by binding to the antiapoptotic Bcl-2-like protein CED-9. In response to apoptotic stimuli, the "BH3-only" protein EGL-1 induces a conformational change in CED-9 that results in the release of CED-4 **(121)**. In the "mitochondrial or indirect pathway," upon apoptotic stimuli, the tubular mitochondrial network of *C. elegans* embryonic cells undergoes fragmentation into numerous and punctiform units, called a "worm-to-beetle transition," to reflect the change in their appearance **(56, 122)**. Mitochondrial fragmentation can occur in the absence of caspase activity and has been proposed to be causally involved in apoptosis induction. DRP-1 has been shown to be important during developmental apoptosis in *C. elegans* **(123)**. Reduction of DRP-1 activity by expressing a dominant-negative mutant prevents fragmentation and cell death in about 20% of cells **(123)**. Interestingly, the mitochondrial fragmentation process mediated by DRP-1 occurs downstream of CED-9 but upstream of CED-4, in between two proteins that bind one another on the OM. The ectopic expression of DRP-1 in *C. elegans* induced mitochondrial fragmentation and cell death **(123)**. Drp1-mediated fragmentation was unexpectedly inhibited in cells lacking expression of CED-9, indicating that CED-9 is actually required for the mitochondrial fragmentation in apoptotic cells. This is consistent with the previous indication that CED-9 not only inhibits cell death but can also change conformation to promote cell death **(124)**. As we mentioned, the proapoptotic activity of CED-9 correlates with the fragmentation process and requires DRP-1. A CED-9 gain-of-function mutation also inhibited mitochondrial fragmentation induced by ectopic DRP-1 expression, suggesting that this mutant maintains cell death inhibition activity but lacks cell death induction activity. These results are compatible with the hypothesis that mammalian BCL-2 family members could convert between anti- and proapoptotic conformations **(119)**. In this new model, proposed binding of the "BH3-only" EGL-1 protein to CED-9 could throw this conformational switch to activate mitochondrial activity **(122)**.

The question of whether fission and fusion proteins other than DRP-1 play a role in apoptosis induction in *C. elegans* has yet to be explored. Indeed, fragmentation is the only known and essential involvement of mitochondria

during developmental apoptosis of *C. elegans* **(123)**. It appears more feasible that mitochondrial fragmentation in response to apoptotic stimuli induces changes in specific mitochondrial functions (i.e., respiration, biogenesis, etc.) rather than changes in the OM permeability and that these mitochondrial dysfunctions might promote cell death in *C. elegans*.

Drosophila

As in *C. elegans*, despite conservation of Bcl-2 family members, mitochondria appear to be dispensable in *Drosophila*, or to be just platforms where the reactions that regulate apoptosis happen to take place. The proapoptotic Debcl/Drop-1 and the antiapoptotic Buffy/dBorg appear to play a limited role in a cell death pathway mainly regulated by inhibitor-of-apoptosis (IAP) proteins **(13, 125)**. Nevertheless, mitochondria undergo changes in their shape following the expression of Debcl/Drop-1 in *Drosophila* cells. Debcl/Drop-1 localizes to the mitochondrial membranes **(126)**, and its C-terminal hydrophobic tail, which probably confers mitochondrial localization, appears to be important for its proapoptotic activity **(126)**. Debcl/Drop-1 expression in *Drosophila*, as well as in mammalian cells, causes a caspase-independent fragmentation of the mitochondrial network similar to the *C. elegans* one **(126)**. When overexpressed heterologously in mammalian cells, Debcl/Drop-1 can induce cytochrome c release from mitochondria, but it has not been reported to do so in fly cells **(127)**. Mitochondrial fragmentations probably represent the only mitochondrial changes that we found conserved during the apoptosis of invertebrate and mammalian cells.

Functional Consequences

Why do mitochondria fragment during apoptosis irrespective of the organism in which programmed cell death is studied? The simplest answer is that fragmentation is a simple consequence of mitochondrial dysfunction that occurs in response to an apoptotic stimulus. This could be an event downstream of cytochrome c release in mammals **(128)**, or irrespective of OM permeabilization in lower organisms where activation of the apoptosome does not seem to require the release of proapoptotic proteins from mitochondria. However, multiple evidence indicates that this is not the case, especially in mammals. First, a chemical inhibitor of Drp1 is capable of blocking the release of cytochrome c and, therefore, apoptosis **(129)**; second, cells devoid of BAX and BAK respond to intrinsic stimuli with the same fragmentation observed in their wild type counterparts **(130)**; third, a handful of reports indicate that it is possible to delay or even to block apoptosis by upregulating the fusion machinery that antagonizes mitochondrial fragmentation **(122, 131, 132)**. Thus, fragmentation cannot be solely regarded as a downstream consequence of mitochondrial

dysfunction but is likely to participate more directly in the recruitment of this organelle in the death cascade. Most likely, fragmentation takes part in the release of proapoptotic proteins from the IMS, although the mechanism by which this is exerted is still unknown. One possibility is that sites of fission are somehow favored for the insertion/oligomerization of Bax and Bak, or that the pro-fission proteins can contribute to the activation of multidomain proapoptotics. Alternatively, fission can be coordinated with remodeling of the cristae, as it has been experimentally substantiated by the group of G. Shore **(133)**. This would make more cytochrome c available for the release from mitochondria with high levels of Drp1 on their surface. Finally, it is similarly conceivable that fragmented mitochondria are dysfunctional and therefore activate as-yet unknown mechanisms that lead to the release of proapoptotic proteins from their intermembrane space. In conclusion, mitochondrial fragmentation appears to be intimately linked to mitochondrial apoptosis; however, further studies using genetic models are required to verify the relative position of fission with respect to cytochrome c release and caspase activation, prior to an in-depth analysis of the mechanism(s) by which enhanced fission contributes to the release of proapoptotic proteins from mitochondria during apoptosis.

Conclusions: Which Role for Mitochondrial Shape Changes in Apoptosis Associated with Disease?

Normal membrane dynamics are necessary for mitochondrial function, as substantiated by evidence pointing to a role for mitochondrial shaping proteins in several aspects of cell life and death (Fig. 6.1 and Color Plate 1).

Dysfunction of mitochondria is proposed to be involved in neurodegenerative diseases. Deficiencies in energy supply, free-radical generation, Ca^{2+} buffering, or control of apoptosis could all theoretically contribute to a progressive decline of the central nervous system. Parkinson's disease, for example, illustrates well how different genes finally impinge directly or indirectly on mitochondrial function, causing fatal dysfunction of dopaminergic neurons **(134)**. Several proteins that are encoded by genes mutated in neurodegenerative diseases are involved in mitochondrial dynamics, which suggests that this is particularly important for the integrity of the nervous system. In neurodegenerative diseases other than Parkinson's disease, deregulation of mitochondrial dynamics seems to be a recurrent theme. Mutations in human MFN2 cause Charcot-Marie-Tooth disease **(135)**, while mutations in the Opa1 gene are associated with autosomal dominant optic atrophy (ADOA) affecting mainly retinal ganglion cells and causing progressive blindness due to the loss of these neurons **(136, 137)**. In this disease, for example, pathogenic mutations cause mitochondrial fragmentation and do not protect cells from apoptosis **(90, 91)**. We already described the role of OPA1 in influencing apoptosis by controlling

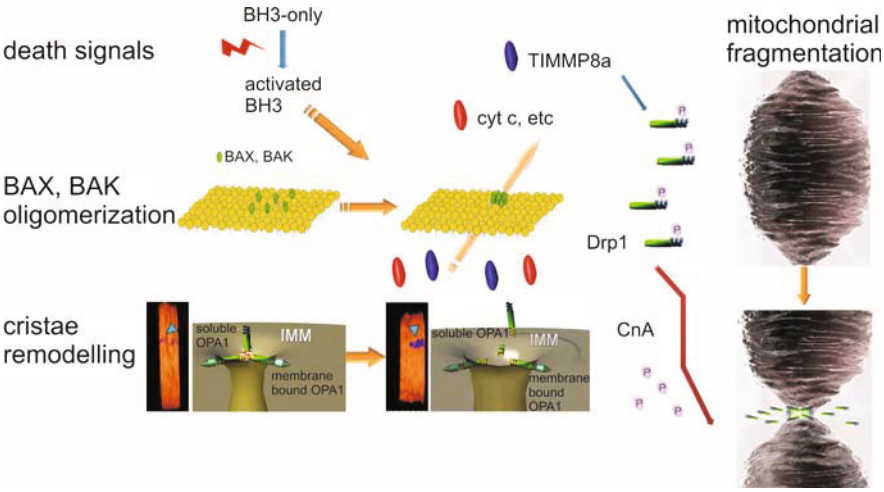

Fig. 6.1 Schematic representation of the amplificatory loop at the mitochondrial level in response to an apoptotic stimulus. Three main interconnected mitochondrial steps are represented: (1) oligomerization of Bax and Bak, which generates a physical pathway for the efflux of proteins across the outer mitochondrial membrane; (2) Opa1-controlled remodeling of the cristae, leading to the redistribution of cytochrome c in the intermembrane space; (3) activation of mechanisms that cause mitochondrial fragmentation, following calcineurin (CnA)-dependent dephosphorylation of Drp1, or interaction of the latter with TIMMP8a, a component of the import machinery of mitochondria that is released together with cytochrome c. (*see* Color Plate 1)

cristae remodeling in cooperation with the rhomboid protease PARL **(90)**. It remains to be clarified whether ADOA is caused by the impairment of the profusion or the antiapoptotic role of OPA1, but we can affirm that steps have been taken in understanding the molecular mechanisms leading to degeneration of the retinal ganglion cells in ADOA. Further work will contribute to our knowledge of mitochondrial function, and an open question is whether one can therapeutically exploit mitochondrial function and dynamics to treat neurodegenerative diseases.

From the other side, IAP proteins (inhibitors of caspase activity) are often upregulated in cancer cells **(138**, **139)**. An event that could be crucial to ensure the adequate activation of caspases, despite the presence of IAPs, for an efficient cell death is the complete release of cytochrome c in the cytosol, a process highly regulated by mitochondrial-shaping proteins, as deeply elucidated in this chapter. Interestingly, MFN1 is also upregulated in nonsmall cell adenocarcinoma of the lung and in other cancer cell lines, further substantiating a role for mitochondrial shaping proteins not only in neurodegeneration but maybe also in transformation **(140)**. Mitochondrial shaping proteins therefore seem to be an appealing target also to modulate and enforce the mitochondrial phase of apoptosis in cancer cells.

We must gain a deeper understanding of how mitochondrial shaping proteins regulate cytochrome c release. In particular, it is unclear if and why egress of cytochrome c should be favored from fragmented mitochondria. This question has crucial implications if one modulated mitochondrial morphological changes in order to influence apoptosis. Inhibition of GTPase activity could have opposite effects on cell viability. If fission mediated by the GTPase DRP-1 is crucial to release cytochrome c, GTPase inhibitors should not have any beneficial effect in driving apoptosis of cancer cells. Conversely, should upregulation of mitochondrial shaping proteins such as MFNs or OPA1 prove to be a hallmark of transformation, one could imagine that targeting them with specific inhibitors could provide a way to maximize cytochrome c release and hence apoptosis of cancer cells. Alternatively, one potential approach would be to enforce DRP-1 translocation to mitochondria by impinging on the pathways that control its translocation to the organelle and the subsequent fission and release of cytochrome c.

In conclusion, modulation of mitochondrial shaping proteins offers an appealing and promising novel strategy to interfere with diseases of increased (neurodegeneration) or reduced (cancer) apoptosis.

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Chapter 7
The Endoplasmic Reticulum Pathway

Michael W. Harr and Clark W. Distelhorst

Abstract Virtually all organisms adapt to stress in order to prolong their survival. At the subcellular level, the endoplasmic reticulum (ER) responds to stress by inducing ER-specific signaling pathways to reestablish homeostasis between protein synthesis and processing, a mechanism called the *unfolded protein response* (UPR). However, when cells endure a persistent irreversible state of ER stress, they undergo apoptosis. Apoptosis initiated at the ER is distinct from mitochondrial or death receptor-mediated apoptosis but may involve or require cross-talk from intrinsic or extrinsic pathways. Two functions of the ER are to facilitate the maturation of newly synthesized proteins and to maintain stores of intracellular calcium. Therefore, apoptosis induced by ER stress is frequently characterized by perturbations in protein processing and transport and/or a loss of calcium homeostasis. Additionally, because the ER and mitochondria reside in close proximity to one another, pro- and antiapoptotic proteins, such as the Bcl-2 family members, localize to the ER to regulate apoptosis in response to stress. This chapter summarizes the key pathways associated with cell survival and apoptotic cell death in the context of ER stress and aberrant calcium signaling.

Keywords Endoplasmic reticulum · Apoptosis · Calcium · Bcl-2

Introduction

Proper functioning of the endoplasmic/sarcoplasmic reticulum (ER) is essential for normal cellular homeostasis. Two primary functions of the ER are to (1) process nascent polypeptide chains so that properly modified proteins can be transported to the Golgi apparatus and (2) maintain internal stores of calcium

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ion. When ER homeostasis is compromised, a program called the *unfolded protein response* (UPR) is activated to prolong cell survival by increasing protein folding, facilitating the degradation of misfolded proteins, and inhibiting protein synthesis. Cells subjected to sustained and irreversible stress undergo programmed cell death, typically by apoptosis.

ER stress can be induced by several physiological processes. For example, the UPR is required for normal differentiation of B cells and pancreatic beta cells, both of which secrete large quantities of immunoglobulin and insulin, respectively **(1)**. Furthermore, abnormal lipid metabolism and pathological conditions, such as obesity and diabetes, can stimulate ER stress. Deficiencies in the UPR are also associated with neurodegenerative diseases and ischemia **(2)**. Biological factors contributing to ER stress in each of these cases may be the loss of calcium homeostasis, insufficient protein glycosylation, or glucose deprivation **(3–6)**.

Agents commonly used to induce ER stress in cell culture are tunicamycin, thapsigargin, and brefeldin A. We refer to each of these throughout the course of this chapter. Briefly, tunicamycin prevents N-linked glycosylation, whereas thapsigargin inhibits the Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase (SERCA), thereby depleting the concentration of ER luminal calcium. Brefeldin A inhibits ER to Golgi transport, thus abolishing protein processing. These agents induce ER stress by blocking an indispensable component of ER function; chronic exposure to any of these results in cell death.

The first part of this review addresses cell survival pathways in response to ER stress, including (1) three signaling pathways of the UPR, (2) the role of ER chaperones during abnormal protein processing, and (3) autophagy. Subsequent sections focus on apoptosis pathways initiated at the ER as well as those regulated by calcium and Bcl-2 family members.

The Unfolded Protein Response

Three signaling components of the UPR have been described to date (Fig. 7.1). Each of these activates specific transcription factors that turn on ER-responsive genes **(7)**. Some of these genes encode chaperones that are expressed in the ER lumen, such as the glucose-related protein Grp78 (also known as immunoglobulin binding protein, BiP). In the unstressed state, Grp78 serves to dock larger ER transmembrane proteins, and dissociation of these interactions activates each arm of the UPR **(1, 8, 9)**.

The first component of the UPR was shown to be mediated by inositol-required protein-1 (IRE-1), a transmembrane serine/threonine kinase that undergoes autophosphorylation in its cytosolic domain in response to ER stress **(10–13)**. Autophosphorylation of IRE-1 stimulates its endoribonuclease activity. Once active, it cleaves an intron out of the X-box binding protein-1 (XBP1) gene and the remaining fragments are ligated together in the 5' to 3' orientation **(14)**.

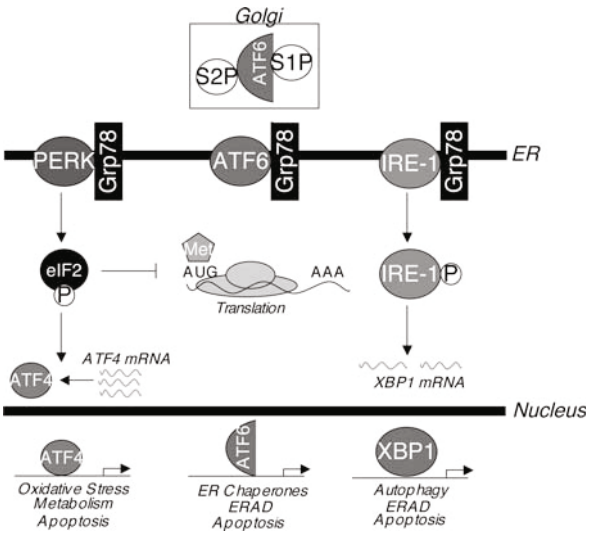

Fig. 7.1 ER stress induces the unfolded protein response. The ER transmembrane proteins IRE-1, ATF6, and PERK are constitutively bound by Grp78. ER stress causes Grp78 to dissociate from these complexes, enabling IRE-1, ATF6, and PERK translocation from the ER membrane. Each proximal UPR signal induces transcription of prosurvival and death genes. It has been suggested that the time and/or duration of stress may be an important determinant of cell fate

This intron is present in mature XBP1 mRNA, and in unstressed cells, the full-length XBP1 gene product functions as a transcription factor to repress ER target genes **(3)**. However, during ER stress, truncated XBP1 transcriptionally activates ER-responsive genes to stimulate protein folding and degradation **(15**, **16)**. Recently, it was shown that the IRE-1-mediated cleavage of XBP1 enhanced cell survival in HEK cells stimulated with tunicamycin or thapsigargin **(17)**. Additionally, cleavage of XBP1 mRNA occurred early after treatment and was inhibited only after persistent ER stress occurred in parallel with cell death, suggesting that IRE-1 signaling partially enables cell survival.

The second component of the UPR induces the activating transcription factor-6 (ATF6). ATF6 is anchored to the ER membrane and docked by Grp78 in the ER lumen **(8)**. ER stress causes ATF6 to dissociate from Grp78, thereby enabling its translocation to the Golgi apparatus, where it becomes a substrate for two Golgi-specific proteases, S1P and S2P **(8**, **18)**. The newly processed ATF6 protein translocates to the nucleus to transcriptionally activate ER-responsive genes **(19**, **20)**. Importantly, ATF6 is a positive transcription factor for Grp78 and XBP1, and induction of these genes serves to amplify the UPR and provide cross-talk between pathways **(7**, **19)**. Another important ATF6-responsive gene is CHOP (also referred to as GADD153), a CCAAT enhancer binding protein transcription factor induced by DNA damage **(21**, **22)**.

Transcriptional activation of CHOP has been implicated in ER stress-induced apoptosis; we discuss it later in this chapter.

The third component of the UPR involves the PKR-like ER kinase PERK. PERK is similar to IRE-1 in that it is autophosphorylated upon activation by ER stress **(23)**. When active, PERK phosphorylates the translation initiation factor eIF2α to globally inhibit protein synthesis **(24)**. This delay in protein synthesis may allow time for proper folding of preexisting proteins and degradation of misfolded proteins in an effort to reestablish ER homeostasis. PERK deficient mice do not survive in response to ER stress, however, this defect in PERK signaling can be overcome by chemical inhibitors of translation, such as cycloheximide **(25)**. The eIF2 translational block may also induce cell survival by repressing translation of the NFκB inhibitor IκB or perhaps by disrupting the interaction between IκB and NFκB **(26, 27)**. Paradoxically, eIF2α phosphorylation accelerates the UPR by specifically inducing translation of the CREB transcription factor, activating transcription factor-4 (ATF4) **(28)**. This occurs because ATF4 mRNA contains a 5' untranslated region comprising several upstream open reading frames that normally function to repress translation of the downstream coding sequence. Yet when eIF2α is phosphorylated during ER stress, ATF4 mRNA is more efficiently translated **(1, 28)**. PERK-mediated upregulation of ATF4 contributes to survival by protecting cells from oxidative stress and nutrient deprivation **(29)**.

ER Chaperones

Under stress conditions, proximal signaling molecules such as IRE-1, ATF6, and PERK are necessary for upregulating the transcription of ER chaperones (e.g., Grp78) to enhance ER protein-folding capacity. As mentioned previously, Grp78 functions to anchor large transmembrane proteins, such as IRE-1, to the ER membrane **(30)**. But during ER stress, Grp78 weakly associates with hydrophobic regions of misfolded proteins **(31, 32)**. Like its homologue, Hsp70, it has a peptide-binding domain and ATP-binding pocket, thus requiring ATP hydrolysis. J-proteins, which are also Hsp family members, promote Grp78 ATPase activity and substrate binding [reviewed in **(31)**]. Although Grp78 has multiple substrates, it may preferentially bind misfolded proteins after UPR induction **(1)**. Another prominent ER chaperone is Grp94, which is a homologue of Hsp90. Expression of Grp94 is only observed in vertebrates and therefore may have evolved in higher eukaryotes to accommodate complex protein folding and assist in the adaptive immune response by promoting antigen presentation **(33, 34)**.

Other key chaperones involved in mediating the UPR are calnexin, and calreticulin. Calnexin and calreticulin are calcium-binding proteins that are responsible for the proper folding of glycosylated proteins. In unstressed cells, proteins are glycosylated at their N-terminus by glycans and subsequently

trimmed by glucosidases **(6, 35)**. Calnexin and calreticulin specifically interact with monoglucosylated proteins for proper folding and transport to the Golgi apparatus **(32)**. During ER stress, when proteins have undergone improper glycosylation or folding, they are either maintained in the calnexin cycle before being transported out of the ER, or subjected to protein degradation by a process called ER-associated degradation (ERAD) **(32, 36)**. ERAD is a mechanism by which misfolded or aggregated proteins are polyubiquitinated and retrotranslocated to the cytosol, where they are targeted for degradation by the proteasome **(37, 38)**. Notably, Grp78 is required for ERAD due to its ability to promote the transfer of misfolded proteins, lacking the appropriate number of sugar molecules, to a family of ER mannosidase-like enzymes called EDEMs **(39, 40)**. As shown in Fig. 7.1, ERAD is a direct downstream effector function of IRE-1 signaling, because EDEM2 is a transcriptional target of the XBP1 cleavage product **(41)**.

ER Stress and Autophagy

Macroautophagy (hereafter referred to as autophagy) is a process by which organelles, macromolecules, and proteins are phagocytosed in order to compensate for intracellular stress that can arise from conditions such as nutrient deprivation, hypoxia, or abnormal cell growth **(42, 43)**. It is characterized by the formation of double-membrane vesicles, or autophagosomes, that fuse with lysosomal components to facilitate the degradation of cellular constituents **(44)**. Autophagy, which may be thought of as a mechanism of cell survival, may also lead to cell death depending on the stimulus and duration of stress **(45)**. Importantly, it has been implicated in ER stress pathways and is likely an important process for maintaining cell survival or accelerating programmed, nonapoptotic, cell death during sustained ER damage **(46–48)**.

Autophagy is mediated, in part, by several evolutionarily conserved Atg genes originally identified in yeast **(49)**. Many of these, including the putative tumor suppressor gene Beclin-1 (Atg6), are expressed in mammalian cells and required for autophagy in some tissues **(50)**. In yeast, Atg8 is upregulated by the XBP1 orthologue Hac-1, implying that the IRE-1 signaling pathway mediates autophagy in response to ER stress (Fig. 7.1) **(48)**. In one study, poly-glutamine aggregation induced ER stress and autophagy, which correlated with increased Atg12 mRNA. Further, loss of Atg5 and mutations in PERK and eIF2α inhibited the autophagic response while preserving poly-glutamine aggregation and apoptosis **(51)**. It was also reported that a mutant form of the protein dysferlin, which plays a role in muscular dystrophy, was shown to aggregate in the ER and stimulate autophagy, whereas the wild-type protein was degraded by ERAD **(52)**. Consistent with other studies, a non-phosphorylatable mutant of eIF2α blocked the induction of autophagy, suggesting that the PERK component of the UPR is required for autophagy in a mammalian system.

However, the mechanism by which the UPR triggers autophagy may depend on the type of ER stress-inducing agent. For example, thapsigargin and tunicamycin stimulated ER stress and autophagy even in the absence of PERK and ATF6, but not in IRE-1-deficient cells **(46)**. In light of these observations, it is possible that PERK mediates the autophagic response to ER protein aggregates, whereas IRE-1 signaling does so when protein glycosylation and/or calcium homeostasis are compromised. Nevertheless, these data suggest that autophagy antagonizes apoptosis in response to ER stress, providing evidence that it is a mechanism of survival in this context.

Apoptosis Pathways

If persistent stress occurs, the affected cells die by apoptosis. All three components of the UPR induce apoptosis by activating the transcription factor CHOP (Fig. 7.1) **(22, 28, 53, 54)**. The role of CHOP in ER stress-induced apoptosis was exemplified by early studies in which mouse embryonic fibroblasts derived from $CHOP^{-/-}$ mice were resistant to apoptosis induced by tunicamycin, thapsigargin, and the calcium ionophore A23187. In this study, renal cell viability was elevated in $CHOP^{-/-}$ mice and the percentage of apoptotic cells was markedly decreased **(55)**. The mechanism behind CHOP-dependent apoptosis in response to stress has not been fully elucidated, although CHOP has been shown to repress transcription of the antiapoptotic protein Bcl-2 **(56)**. Additionally, CHOP transcriptionally activates two genes, GADD34 and ERO1α, that enhance protein synthesis and oxidation of ER proteins, respectively **(57)**. Like cells derived from $CHOP^{-/-}$ mice, mutations in GADD34 increase resistance to tunicamycin-induced apoptosis.

Alternatively, activation of IRE-1 can induce apoptosis by a signal transduction pathway that is independent of XBP1 cleavage. Activated IRE-1 recruits TNF-receptor associated factor 2 (TRAF2), and association of these molecules activates JNK signaling **(58)**. JNK activation and signaling induces apoptosis in a number of cell types, including thymocytes, cardiac myocytes, and neurons **(59–61)**. Moreover, the MAP kinase ASK1 was found in complex with TRAF2 **(62)**. While IRE-1-mediated activation of JNK induced ER stress in neurons with poly-glutamine aggregation, $ASK1^{-/-}$ neurons were resistant to ER stress and cell death, indicating the importance of ASK1 in this pathway **(63)**. Together, these data suggest that IRE-1, when in complex with TRAF2, activates ASK1 in response to ER stress and that these events are required for apoptosis mediated by JNK signaling.

Also found in complex with IRE-1 and TRAF2 is caspase-12 **(64)**. Caspase-12 is an ER-localized protease containing a caspase activation recruitment domain (CARD) that is activated in mammalian cells by inducers of ER stress **(65)**. The importance of caspase-12 in the initiation of apoptosis induced by ER stress was first identified by Junying Yuan and colleagues in a model of Alzheimer's

disease **(66)**. Potent inducers of ER stress, such as thapsigargin and tunicamycin, activated caspase-12, while other apoptosis-inducing agents, including Fas, TNF, and cycloheximide, did not. Accordingly, the loss of caspase-12 conferred resistance to apoptosis induced by thapsigargin and tunicamycin, whereas knockout cells were unaffected by Fas, TNF, cycloheximide, or staurosporine. Moreover, thapsigargin upregulated the expression of caspase-12 in HEK293 T cells, and its activation facilitated the translocation and subsequent activation of caspase-7 **(67)**. Interestingly, caspase-12 was shown to activate caspase-9 *in vitro*, providing evidence for a mechanism of caspase-9 activation that is independent of cytochrome c **(68, 69)**. Caspase-12 may also be activated by m-calpain as a consequence of cytosolic calcium elevation after ER stress, thus providing more than one mechanism for its activation **(70)**.

In spite of the fact that cytochrome c may not be required for the activation of caspase-9 by caspase-12 in response to ER stress-inducing agents, several studies have shown that mitochondria contribute to the apoptotic process. For example, Bax/Bak$^{-/-}$ cells are resistant to apoptosis induced by thapsigargin and tunicamycin **(71)**. However, it is possible that Bak functions, in part, at the ER since reconstitution of ER-targeted Bak in double-knockout cells restores caspase-12 activation **(72)**. Nevertheless, cytochrome c release was observed following ER stress induced by brefeldin A and tunicamycin, and mitochondrial membrane permeabilization was required for apoptosis induced by both of these agents **(73, 74)**. In the latter study published by Guido Kroemer's group, Bax oligomerization was detected in mitochondria and subsequently inhibited by stable transfection of Bcl-xL or vMIA (inhibitors of mitochondrial membrane permeabilization).

Calcium Signaling and Apoptosis

Calcium homeostasis is of critical importance for maintaining normal cellular function. Alterations in calcium homeostasis can contribute to ER stress, thus perturbing protein folding and processing. Under physiological conditions, the calcium concentration in the ER lumen is maintained in the millimolar range, while the concentration in the cytosol is much lower (50–100 nM) **(75)**.

Much of our understanding of calcium regulation has evolved from studies in lymphocytes, where two prominent calcium channels, inositol 1,4,5-trisphosphate (IP_3) and ryanodine receptors, are responsible for mobilizing calcium out of the ER **(76, 77)**. These calcium channels are also expressed in cardiac and brain tissue, which require calcium flux for the excitation and action potential of muscle and neuronal cells, respectively. IP_3 receptors release ER luminal calcium after hydrolysis of phosphatidyl-inositol-4,5-bisphosphate (PIP_2), which occurs in response to lymphocyte activation **(77)**. Also, in T cells, ryanodine receptors release calcium in response to cyclic ADP ribose, a second messenger generated by T-cell receptor activation **(78)**. In immature thymocytes, calcium efflux can result in

cell survival or cell death, depending on the avidity of agonist stimulation **(79–83)**. During this stage of development, the majority of CD4/CD8-positive thymocytes die by negative selection in order to eliminate potentially autoreactive T cells from differentiating and migrating to secondary lymphoid organs. Our laboratory has recently proposed that the effector function (e.g., cell survival or cell death) may be determined by the frequency and amplitude of calcium oscillations **(84, 85)** (summarized in Fig. 7.2).

The concept that calcium is an important signaling molecule involved in apoptosis came from early studies using glucocorticoid-treated thymocytes **(86–88)**. These reports showed that glucocorticoid hormones elevate cytosolic calcium levels, thereby inducing the biochemical characteristics of apoptosis, such as endonuclease activity and DNA fragmentation. It was later determined that glucocorticoids induce apoptosis in thymocytes, in part by depleting ER calcium stores **(89, 90)**. Additionally, type III IP_3 receptor was shown to be upregulated by dexamethasone and localized to the plasma membrane **(91)**. In Jurkat T cells, antisense mediated knockdown of type I IP_3 receptor protected cells from apoptosis induced by dexamethasone **(92)**. Together, these findings suggested an important role for IP_3 receptor-mediated calcium elevation in models of glucocorticoid-induced apoptosis; however, recent findings in our laboratory have called into question whether or not IP_3 receptor upregulation is necessary for the observed effects of dexamethasone on intracellular calcium stores in lymphocytes **(93)**.

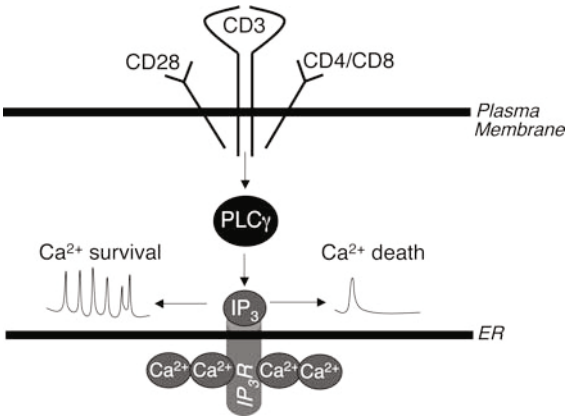

Fig. 7.2 Distinct patterns of IP_3-mediated calcium signaling determine cell fate. In T cells, activation of the CD3 component of the T-cell receptor and co-stimulatory receptors (CD4, CD8, and CD28) generates IP_3 by phospholipase C-mediated hydrolysis of PIP_2. IP_3 ligand binds to the IP_3 receptor to stimulate ER calcium release. During strong T-cell receptor activation, calcium is released in the form of a single transient calcium wave (right) and cells undergo apoptosis. In contrast, during weak T-cell receptor activation, calcium is released in the form of repetitive calcium oscillations or spikes. Repetitive calcium signals encode a cell survival pathway by inducing calcineurin and NFAT nuclear localization

The concentration of ER luminal calcium may also be an important factor in determining the apoptotic response **(94)**. For example, the overexpression of calreticulin, resulting in an increased calcium pool, was associated with enhanced cytochrome c release and apoptosis after treatment with thapsigargin and staurosporine. In contrast, the loss of calreticulin was associated with resistance to both agents.

It is now evident that several cytotoxic agents increase cytosolic calcium levels **(95, 96)**. Among these are thapsigargin, glucocorticoids, hydrogen peroxide, cisplatin, and staurosporine (Fig. 7.3). It is important to note that although these agents disrupt calcium homeostasis, they do not necessarily induce an ER-stress specific pathway, as is the case for thapsigargin, tunicamycin, and brefeldin A. Yet it is likely that apoptosis induced by these agents involves cross-talk between the ER and mitochondrial pathways. For example, ceramide and staurosporine generate mitochondrial calcium waves in an IP_3-dependent fashion **(97)**. Because both organelles are in close proximity to one another **(96, 98)**, calcium released out of the ER can be pumped into mitochondria by the calcium uniporter channel **(99–101)**. Increases in the mitochondrial calcium concentration stimulate cytochrome c release, leading to the formation of the apoptosome and the activation of caspase-3 **(102–104)**.

Further studies imply that apoptosis mediated by cytosolic calcium elevation can be amplified by two forms of positive feedback [reviewed in **(96)**].

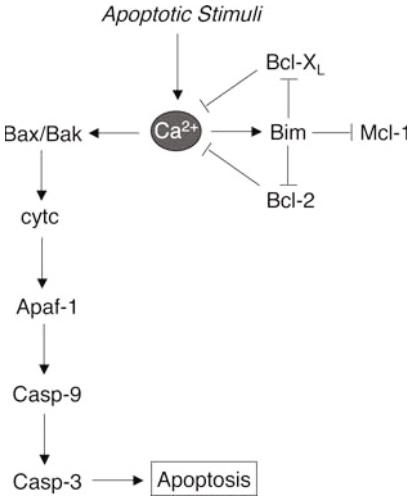

Fig. 7.3 Calcium-dependent apoptosis is regulated by ER and mitochondrial pathways. Apoptotic stimuli (e.g., thapsigargin, glucocorticoids, hydrogen peroxide, cisplatin, and staurosporine) enhance cytoplasmic calcium levels. The antiapoptotic proteins Bcl-2 and Bcl-xL inhibit apoptosis by blocking IP_3 receptor channel activity or decreasing ER luminal calcium. The BH3-only protein Bim can bind and sequester Bcl-2, Bcl-xL, and Mcl-1, thus promoting Bax and Bak oligomerization. Once activated, Bax and Bak induce cytochrome c release from the mitochondria, stimulating formation of the apoptosome (cytochrome c, Apaf-1, and caspase-9) and inducing apoptosis

First, type I IP_3 receptor was shown to be a substrate for caspase-3 when apoptosis was induced by staurosporine or Fas in Jurkat cells, but not in caspase-3-deficient MCF7 cells **(105)**. In addition, transfection of DT40 B cells (deficient in all three IP_3 receptor subtypes) with a form of IP_3 receptor that is resistant to caspase-3 cleavage decreased the rate of staurosporine-induced apoptosis **(106)**. These data suggest that the proteolytic cleavage of IP_3 receptors by caspase-3 decreases ER calcium by generating a "leaky" calcium channel, which, in turn, leads to apoptosis **(105, 107)**. The second type of positive feedback may be elicited by cytochrome c. Saul Snyder and colleagues have shown that cytochrome c interacts with IP_3 receptors after being translocated to the ER membrane during apoptosis to sustain high levels of cytosolic calcium **(108, 109)**.

Regulation of Calcium Signaling by Bcl-2

Over the past two decades, significant contributions have been made in the field of apoptosis after Bcl-2 was discovered to be an antiapoptotic oncogene that localized to mitochondria. Although often overlooked, Bcl-2 is also present at the ER, where it functions to inhibit proapoptotic signals **(110–112)**. When Bcl-2 is targeted to the ER membrane, it exhibits antiapoptotic activity against agents that decrease the mitochondrial membrane potential **(113)**. As shown in Fig. 7.3, Bcl-2 inhibits cytosolic calcium elevation and apoptosis induced by several stimuli, including growth factor withdrawal, thapsigargin, hydrogen peroxide, staurosporine, and ceramide **(95)**. By inhibiting calcium efflux out of the ER, Bcl-2 and Bcl-xL also prevent its redistribution and uptake by mitochondria, while the overexpression of Mcl-1 decreases mitochondrial calcium levels without affecting ER luminal calcium **(95, 114–116)**. A dual role for Bcl-2 at the ER and mitochondria was illustrated by a study in which a small molecule inhibitor of Bcl-2 (HA14-1) induced calcium elevation and cytochrome c release in AML cells **(117)**. The response to HA14-1 was prevented after chelating extracellular calcium with EGTA or inhibiting the mitochondrial uniporter. In addition, HA14-1 failed to induce cytochrome c release in mitochondrial preparations in the absence of calcium, suggesting that ER calcium mobilization was required for cytochrome c release and apoptosis.

Bcl-2 inhibits ER calcium signaling by at least two independent mechanisms. Recent studies in T cells have shown that Bcl-2 inhibits proapoptotic calcium signals by directly interacting with IP_3 receptors while preserving ER luminal calcium **(118)**. Intriguingly, Bcl-2 did not have an inhibitory effect on prosurvival calcium signals induced by weak T-cell receptor stimulation **(85)**. Additional evidence that Bcl-2 inhibits IP_3 receptor function was demonstrated by experiments showing that purified Bcl-2 protein decreased IP_3-mediated channel opening in an artificial lipid bilayer system **(118)**. This interaction was also

reported in MCF7 cells, where Bcl-2 overexpression diminished ATP and IP_3-induced cytosolic calcium elevation **(119)**. In another study, Bcl-2 decreased the level of carbachol-induced mitochondrial and subsarcolemmal calcium in dystrophic myotubes **(120)**. Finally, Kevin Foskett and colleagues determined that, like Bcl-2, Bcl-xL interacts with IP_3 receptors, not to inhibit, but to enhance calcium signals generated by low concentrations of IP_3 **(121, 122)**.

Bcl-2 may modulate IP_3 receptor activity by altering its phosphorylation status, albeit indirectly. Early work has shown that Bcl-2 interacts with calcineurin and, further, that calcineurin interacts with IP_3 receptors **(123–125)**. Melvin Billingsley and colleagues have proposed a mechanism by which Bcl-2 binds calcineurin in neuronal cells and mobilizes calcineurin to IP_3 receptors after ischemic injury **(123, 126, 127)**. Because calcineurin is a potent phosphatase, it is reasonable that under these conditions it could dephosphorylate IP_3 receptors, thereby decreasing their calcium channel activity **(124, 128, 129)**. Unpublished work from our laboratory has shown that IP_3 receptor phosphorylation was decreased in Bcl-2-overexpressing cells, perhaps supporting a role for a calcineurin-dependent pathway.

In contrast with the findings reviewed above, it has been proposed that Bcl-2 inhibits calcium signaling by decreasing the calcium concentration in the ER lumen. Initial studies showed that Bcl-2 overexpression inhibited ATP-induced calcium elevation by leaking calcium out of the ER and Golgi and that the depletion of luminal calcium was attributed to enhanced membrane permeability **(130, 131)**. Second, cells deficient in Bax and Bak have decreased levels of ER luminal calcium and increased phosphorylation of type I IP_3 receptor, both of which could be prevented by knockdown of type I IP_3 receptor or Bcl-2, respectively **(132)**. Cells deficient in Bax and Bak showed increased binding between Bcl-2 and type I IP_3 receptor, suggesting that this interaction facilitates calcium leak rather than channel inhibition. Other investigators have proposed an alternative explanation for Bcl-2's effect on luminal calcium. For example, Bcl-2 has been shown to interact with SERCA and decrease the expression of both SERCA and calreticulin, thereby reducing the ER calcium pool **(133–135)**. Conversely, Kuo et al. have reported that Bcl-2 not only interacts with SERCA, but also upregulates SERCA mRNA to increase the calcium pool **(136)**. Such differences in experimental findings may be attributable to cell type, in which the localization and/or abundance of Bcl-2 family members vary. In spite of these differences, it is apparent that Bcl-2, whether by inhibiting IP_3 receptor channel activity or by depleting ER luminal calcium, functions as an antiapoptotic molecule on the ER membrane.

Regulation of Calcium Signaling by Bax and Bak

Bax and Bak are multidomain (BH1-3) proapoptotic proteins that localize to the ER and mitochondria. Their functional importance has been demonstrated by multiple studies using $Bax^{-/-}$ and $Bak^{-/-}$ cells that are markedly resistant to ER

stress and apoptosis-inducing agents, including, but not limited to, thapsigargin, tunicamycin, and brefeldin A **(71**, **137)**. As discussed above, Bax and Bak double-knockout cells have reduced ER luminal calcium compared to wild-type cells **(132**, **137)**. Thus, cytosolic calcium elevation resulting from agonist stimulation in Bax- and Bak-deficient cells is decreased, as is mitochondrial calcium uptake **(137)**. Staurosporine-induced calcium elevation was inhibited in a Bax-deficient prostate cancer cell line, but ER calcium mobilization and mitochondrial uptake were restored after Bax was ectopically expressed **(138)**. Consistent with these findings, the overexpression of Bax or Bak caused both proteins to localize to the ER and mitochondria, and both induced ER calcium mobilization followed by cytochrome c release, a process that was inhibited by Bcl-2 **(139)**. In a study by Craig Thompson's group, ER-targeted Bak stimulated ER calcium release followed by activation of casapse-12, suggesting the involvement of Bak in the ER stress pathway **(72)**. Interestingly, Bak targeted to mitochondria induced cleavage of caspase-7 while having no effect on ER calcium or caspase-12. Together, these data indicate the importance of Bak and Bax in maintaining calcium homeostasis and mediating the response to a variety of apoptotic stimuli.

BH3-Only Proteins

Like other Bcl-2 family members, BH3-only proteins have been extensively studied at the mitochondria. In general, these proteins promote apoptosis by stimulating the oligomerization of Bax and Bak at the outer mitochondrial membrane **(140**, **141)**. It should be noted there is some controversy as to how BH3-only proteins activate Bax and Bak, and another model suggests they do so indirectly by sequestering Bcl-2 and Bcl-xL **(142)**. Although their mechanism of action is not completely understood, it is recognized that several BH3-only proteins, including Bim, Bik, PUMA, and NOXA, also localize to the ER and contribute to apoptosis by regulating ER resident proteins.

Bim is a recently identified BH3-only protein that binds and inhibits Bcl-xL, Bcl-2, and Mcl-1 **(143**, **144)** (Fig. 7.3). Morishima et al. have reported that Bim translocated to the ER after treatment with tunicamycin from the dynein motor complex **(145)**. In this study, the overexpression of ER-targeted Bim was strongly associated with apoptosis, while the transfection of Bcl-xL inhibited Bim translocation and caspase-12 activation, providing substantial evidence that Bim is linked to the ER stress pathway. Bim phosphorylation may also contribute to the apoptotic response at the ER. JNK, which is involved in the ER stress pathway via the phosphorylation of ASK1, phosphorylates Bim in response to UV radiation, causing its dissociation from the dynein motor complex, although the cellular localization of Bim was not determined in this context **(146)**. This study suggests that the phosphorylation of Bim by JNK stimulates apoptosis. However, other investigators provide evidence that Bim phosphorylation by JNK promotes its degradation, thus decreasing the sensitivity to apoptosis-inducing agents **(147)**. It should be noted that both studies reported

two distinct JNK phosphorylation sites, which may account for the difference in apoptotic response. Yet the latter model is supported by data from Andreas Strasser and colleagues demonstrating that inducers of ER stress dephosphorylate Bim, making it resistant to proteasome-mediated degradation. The authors also include evidence that Bim is upregulated at the transcriptional level by CHOP, providing further rationale for its role in the ER stress pathway **(148)**.

Bik is a BH3-only protein first shown to interact with Bcl-2, Bcl-xL, and the Bcl-2 viral homologue E1B-19 **(149, 150)**. Its transcription is induced by the viral oncogene E1A, and hence the tumor suppressor p53, a target of oncogene activation **(151)**. The induction of Bik was associated with a decrease in ER luminal calcium, which was inhibited by the siRNA-mediated knockdown of Bik, implying that it contributes to ER calcium homeostasis **(152)**. According to this study, Bik may also require the presence of Bax and/or Bak at the ER, since p53 activation induced Bak translocation to the ER membrane. Evidence for this model is supported by the observation that Bik fails to induce ER calcium release in $Bax^{-/-}$ and $Bak^{-/-}$ cells **(152)**. Furthermore, it is likely that Bik functions in parallel with the mitochondrial pathway since its induction on the ER stimulates cytochrome c release when the ER light membrane is reconstituted with cytosolic components *in vitro* **(153)**.

The BH3-only proteins NOXA and PUMA are also upregulated by p53 and have been implicated in the ER stress pathway **(154, 155)**. Evidence suggests that NOXA may cooperate with Bik to enhance cytochrome c release **(156)**. Thus, it is possible that NOXA and Bik act synergistically to induce Bax activation and cytochrome c release in response to ER or genotoxic stress **(156)**. Additionally, PUMA was shown to be upregulated by tunicamycin, even in p53 null cells, suggesting that PUMA may be upregulated by tunicamycin in a p53-independent manner **(155, 157)**. More recently, PUMA was upregulated in a mouse model of ALS that showed characteristic features of ER stress, and $PUMA^{-/-}$ mice exhibited enhanced cell survival and slower progression of ALS disease **(158)**. Collectively, these data indicate the importance of BH3-only proteins in mediating apoptosis from the ER. Because apoptosis induced by these molecules is generally inhibited by the elevated expression of Bcl-2, Bcl-xL, and Mcl-1, it is likely that the relative abundance of proapoptotic versus antiapoptotic proteins at the ER and mitochondria is a key determinant in the apoptotic process.

Conclusion

The molecular events that define a particular ER pathway are dynamically evolving. Because ER homeostasis is absolutely required for life, eukaryotic cells have developed a number of mechanisms to survive during times of stress. For example, the IRE-1 signaling pathway, first discovered by Peter Walter and colleagues nearly 20 years ago, has recently been shown to induce autophagy in

ER-stressed cells, demonstrating a link between two ancient processes that may function cooperatively to facilitate survival. Yet even such "survival pathways" are at times equivocal considering that IRE-1 signaling can induce apoptosis by upregulating CHOP and that autophagy can result in nonapoptotic programmed cell death. Thus, even after the depth of knowledge generated in the past decade, we can reasonably conclude that the factors governing life-and-death decisions during ER stress are not mutually exclusive.

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Chapter 8
Reactive Oxygen Species in Cell Fate Decisions

Han-Ming Shen and Shazib Pervaiz

Abstract Redox homeostasis is a function of the balance between the intracellular generation of reactive oxygen species (ROS) and the cellular antioxidant defense systems. Therefore, the degree of oxidative stress is a direct outcome of the rate at which the cells' metabolic processes fuel ROS production and the efficiency with which the antioxidant machinery is able to deal with the impending accumulation. Invariably, the rate of ROS production outweighs the rate of detoxification, thereby leading to excessive accumulation of ROS, manifested as oxidative stress. Although the cellular processes involved in ROS generation, the antioxidant defense mechanisms, and the chemistry of the various redox reactions have all been well described for years, the last couple of decades have seen an enormous increase in activity around the biological relevance of ROS during physiological as well as pathological settings. These studies have highlighted the direct involvement of ROS generated from intracellular sources, such as the NADPH oxidase and/or mitochondrial electron transport chain, in a variety of models of cell death signaling. Many novel targets of ROS have been identified, and their biological relevance is beginning to be unraveled. Whiles a large body of evidence has corroborated the association between elevated ROS and cell death signaling, there is also an emerging notion that at relatively low levels, ROS function as signaling molecules to promote cellular proliferation and growth. This chapter aims to review the current state of our understanding of the cellular sources of ROS as well as the involvement of ROS in cell fate signaling.

Keywords Reactive oxygen species · Mitochondria · TNFα family death receptors · JNK · NF-κB

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Introduction

Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and the function of the antioxidant defense mechanisms **(1, 2)**. ROS refer to a group of oxygen-centered free radicals that are produced during the metabolic process of the aerobic organisms and are closely involved in various physiological and pathological processes. The main ROS species include superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\cdot}OH$). Among them, $O_2^{\cdot-}$ is usually the original radical generated at various cellular sites with relative weak reactivity. With the presence of superoxide dismutase (SOD), $O_2^{\cdot-}$ could be readily converted to H_2O_2, which is relatively more stable and cell-permeable. In the presence of transit metals such as iron and cupper, H_2O_2 and $O_2^{\cdot-}$ undergo a Haber-Weiss reaction to give rise to the highly reactive $^{\cdot}OH$ that is capable of inducing various forms of oxidative damage via reaction with the molecular targets, including DNA, proteins, and lipids **(1)**. On the one hand, there are other forms of ROS with significant biological functions including hypochlorous acid (HOCl) generated from H_2O_2 by myeloperoxidase, and peroxynitrite (OONO-) formed from a reaction between $O_2^{\cdot-}$ and nitric oxide ($NO^{\cdot}$). On the other hand, aside from their damaging activity, intracellular ROS have also been identified as important signaling molecules involved in a variety of signaling pathways controlling the cellular responses to various environmental stimuli **(3)**.

At present, the diverse activities of intracellular ROS in initiating and/or amplifying death signals or in the regulation of apoptosis have been well studied and extensively reviewed **(4–7)**. In this chapter, we attempt to summarize some of the recent developments related to the following topics: (1) the production of ROS in apoptotic cells; (2) the role of ROS in death receptor-mediated apoptosis (extrinsic pathway); (3) the role of ROS in mitochondria-mediated apoptosis (intrinsic pathway); (4) redox regulation of the caspase cascade; (5) ROS on nuclear transcription factor-kappa B (NF-κB) and the mitogen-activated protein kinases (MAPK) pathways; and (6) the conflicting roles of ROS in apoptosis regulation.

Cellular Sources of ROS

The oxidative stress status frequently observed in apoptotic cells could be the result of enhanced ROS production and/or an impaired antioxidant system. There is compelling evidence demonstrating an increase in ROS production upon exposure to a variety of apoptosis stimuli, such as death receptor ligands, DNA damage agents, UV, ionizing radiation, etc. The intracellular sources of ROS include (1) NADPH oxidase (NOX), (2) the mitochondrial electron transport chain (ETC), (3) cytochrome p450, (4) xanthine oxidase, and (5) lipoxygenase and cyclooxygenase. Summarized ahead are two of the major intracellular sources of ROS with clear relevance to apoptosis.

NADPH Oxidase (NOX) and ROS

NADPH oxidase (NOX) is a membrane-bound enzyme complex consisting of the catalytic subunit gp91phox together with the regulatory subunits p22phox, p47phox, p40phox, p67phox, and the small GTPase Rac **(8)**. In the presence of NADPH, NOX mediates the production of $O_2^{\cdot -}$ upon stimulation. Its function in phagocytes as a key mechanism for killing invaded microorganisms has been well documented via the production of $O_2^{\cdot -}$, a process well defined as a respiratory burst **(8)**. At present, there is substantial evidence suggesting that in nonphagocytes, NOX1-derived $O_2^{\cdot -}$ is an important mechanism in apoptotic cell death mediated by various apoptotic stimuli, including Fas ligand (FasL) **(9)**, TNFα **(10)**, DNA damage agents **(11)**, and growth factor deprivation **(12)**. A recent study by Kim et al. has illustrated the molecular mechanism in TNFα-mediated activation of NOX1 by demonstrating the signaling events involving TNFR1, TNF receptor-associated death domain protein (TRADD), and RIP1 **(10)**, a process associated with TNFα-induced necrotic cell death. It remains to be further tested whether such a signaling pathway is also involved in TNFα-induced apoptotic cell death.

Mitochondrial ETC as the Source of ROS

The implication of mitochondria in ROS-mediated apoptosis is twofold: (1) Mitochondria are the major source of intracellular ROS production in most cells and ROS are mostly byproducts of aerobic metabolism via the mitochondrial ETC; and (2) mitochondria have been well established as the key regulator for apoptosis, controlling the intrinsic apoptosis pathway via the release of critical proapoptotic proteins such as cytochrome c and SMAC/Diablo. Recent observations have highlighted the critical relationship among mitochondria, oxidative stress, and apoptosis **(6, 13)**.

Although endowed with a heavy presence of antioxidant enzymes, mitochondria remain as the most powerful source of ROS, where the steady-state generation of $O_2^{\cdot -}$ is five- to 10-fold higher than in the cytosol or nucleus. It is known that $O_2^{\cdot -}$ is generated due to the incomplete coupling of electrons and H^+ with oxygen in the ETC, in particular at the site of Complex I and III of the mitochondrial ETC; a substantial amount of oxygen (about to 0.2–0.3%) consumed by the cell is converted to $O_2^{\cdot -}$. Most $O_2^{\cdot -}$ is produced at the matrix side of the inner mitochondrial membrane but is quickly converted to H_2O_2 by the mitochondrial SOD2 (MnSOD). H_2O_2 can, in turn, react with $O_2^{\cdot -}$ in a Haber-Weiss reaction or engage in a Fenton reaction in the presence of Fe^{+2} or Cu^{+2} to generate the highly reactive and damaging $^{\cdot}OH$ radical. There is also evidence for direct H_2O_2 production from the mitochondrial outer membrane by the action of the flavo-enzyme monoamine oxidase (MAO). Furthermore, stimulation of nitric oxide (NO) by the mitochondrial nitric oxide synthase

(mtNOS) has been implicated in the generation of ROS via its inhibitory effect on Complex I, or by affecting mitochondrial respiration through S-nitrosation of cysteine residues of Complex IV **(14)**. H_2O_2 is then degraded to H_2O by catalase and/or glutathione peroxidase or diffuses through the mitochondrial membrane to reach other intracellular locations. One critical question remains to be examined: How do various apoptotic signals usually acting on the cell membrane (such as cell death ligands) or on nuclear DNA (such as DNA damage agents) relay the signals to the mitochondrial respiratory chain to promote ROS production? One possibility is that enhanced ROS production is the result of the caspase cascade, as the 75-kDa subunit of respiratory Complex I in the mitochondria has been identified as a critical substrate for caspase-3; and caspase-mediated cleavage of this subunit is able to lead to the disruption of electron transport, the production of ROS, and the loss of mitochondrial structural integrity **(15)**. On the other hand, in tumor necrosis factor α (TNFα)-induced apoptosis, some of the key signaling molecules such as TNF receptor-associated factors (TRAF) and receptor interacting protein 1 (RIP1), as well as their downstream effector kinases such as c-Jun N-terminal kinase (JNK), have been implicated in TNFα-mediated ROS production from mitochondria **(16–18)**, although currently little is known about the direct effect of those signaling molecules such as JNK on the mitochondrial respiratory chain.

Mitochondria as Targets of ROS in Apoptosis

The involvement of ROS in mitochondria-dependent apoptosis is evident by the inhibitory effect of antioxidants and ROS scavengers on apoptosis signaling downstream of mitochondria. Indeed, excessive increases in ROS production induce mitochondrial outer membrane permeabilization (MOMP) and mitochondrial matrix swelling and as a result induce the translocation of death-amplifying proteins such as cytochrome c and AIF to the cytosol **(6)**. These effects have been demonstrated by ROS generated from sources within or outside the mitochondria. For example, the drug-induced mitochondrial generation of H_2O_2 was shown to trigger MOMP and cytosolic acidification and amplified apoptotic signaling in human tumor cells **(19)**. Furthermore, this increase in intracellular H_2O_2 was shown to trigger oligomerization and translocation of Bax to the mitochondria, which triggered a second increase in mitochondrial H_2O_2 production and facilitated the egress of cytochrome c to the cytosol. Similar effects of ROS on Bax activation (oligomerization) and of active-Bax on the secondary generation of ROS from mitochondria have been demonstrated in other model systems, thus linking the proapoptotic Bcl-2 family of proteins to ROS-mediated mitochondrial permeabilization. Along similar lines, the probability of electrons leaking out to oxygen to generate $O_2^{\cdot -}$ is significantly enhanced in an oxidatively stressed environment, thus setting up a self-amplifying loop of *ROS-induced ROS production*.

The precise mechanisms by which ROS generated from within or outside the mitochondria result in the induction of mitochondrial permeability transition or opening of the inner membrane permeability transition pore (PTP) remain somewhat unclear. The effect of ROS on PTP-mediated cytochrome c release has been associated with supersaturation of the mitochondrial Ca^{++} buffering capacity, resulting in excessive accumulation of Ca^{++} in the mitochondrial matrix, a stimulus for PTP opening **(20)**. In addition, recent studies have also demonstrated that the inner mitochondrial adenine nucleotide translocase (ANT) was a target of ROS generated upon exposure to anticancer drugs. In a separate study, mitochondrial voltage-gated anion channel 1 (VDAC1) has been implicated as a target of ROS ($O_2^{\cdot -}$ in particular) and the release of cytochrome c. A direct effect of toxicants, such as high concentrations of arsenic trioxide, on the PTP opening has been linked to increased ROS production at the level of Complex I and oxidative modification of ANT (SH-groups) **(21)**. A strong link between ROS and mitochondrial apoptosis, in particular mitochondrial permeability increase and release of cytochrome c, is the oxidation of the mitochondrial lipid cardiolipin before cytochrome c release. Cardiolipin is specifically expressed in the IMM and serves to hold cytochrome c within the intermembranous space via electrostatic as well as hydrophobic interactions **(22)**; breaking these interactions is a prerequisite for the egress of cytochrome c. Recent evidence strongly suggests that oxidative modification of cardiolipin by mitochondrial ROS during apoptosis facilitates its release of cytochrome c from the mitochondria, as oxidized cardiolipin associates poorly/weakly with cytochrome c **(23)**. Cardiolipin oxidation has been linked to glutamate-induced cytochrome c release as well as p53-mediated apoptosis **(24, 25)**. Indeed, a more convincing piece of evidence has been the observation that H_2O_2-mediated peroxidation of cardiolipin, facilitated by the cardiolipin-cytochrome c complex, precedes cytochrome c release during apoptosis **(26)**. Interestingly, the antiapoptotic activity of the mitochondrial antioxidant proteins glutaredoxin, peroxiredoxin, and glutathione peroxidase 4 have been linked to their ability to protect cardiolipin oxidation.

Another source of ROS during apoptosis is p66Shc, a 66-kDa isoform of the growth factor adapter Shc, present in mitochondria and shown to regulate life span in mammals **(27)** via its ability to induce oxidative stress. p66Shc is a redox enzyme, which readily oxidizes cytochrome c and generates intramitochondrial ROS, resulting in PTP opening, cytochrome c release, and apoptosis **(28)**. Monomeric p66Shc can interact with cytochrome c to generate H_2O_2 in the mitochondria and trigger mitochondrial permeabilization, while redox-defective mutants of p66Shc fail to induce mitochondrial ROS and intrinsic apoptosis. The precise mechanisms by which various apoptotic stimuli promote mitochondria-derived ROS production via p66Shc are not well understood. A very recent study has identified the role of protein kinase C (PKC) in relaying the cell death signals to p66Shc **(29)**. Taken together, the production of ROS from mitochondria occurs via multiple mechanisms and at multiple sites, depending on the nature of cell type and stimuli, and is a key effector in mitochondria-dependent apoptotic signaling.

Regulatory Role of ROS in Death Receptor-Mediated Apoptosis

ROS in TNFR1-Mediated Apoptosis

TNFα is a pleiotropic inflammatory cytokine with a wide range of functions that are known to be executed via two cell surface receptors: TNF receptor 1 (TNFR1) and receptor 2 (TNFR2). Among them, TNFR1 is the main receptor mediating the biological function of TNFα **(30**, **31)**. The binding of TNFα to TNFR1 triggers a series of intracellular events, including the recruitment of TRADD and the formation of two signaling complexes **(32)**: (1) the plasma membrane-bound complex (complex I) consisting of TNFR1, TRADD, RIP1, and TRAF2, leading to the rapid activation of nuclear transcription factor-kappa B (NF-κB) and the mitogen-activated protein kinases (MAPK) pathways; and (2) the cytoplasmic complex (complex II) containing TRADD, RIP, Fas-associated death domain protein (FADD), and caspase-8, essential for TNFα-induced apoptosis through a caspase cascade. In most cells, the combination of the above signaling pathways determines the diverse biological activities of TNFα, including cell growth and cell death.

So far, the redox regulation of TNFR1-induced apoptosis has been extensively studied and reviewed **(33**, **34)**. There are two opposing hypotheses regarding the role of ROS in TNFR1-inducced apoptosis. The first hypothesis supports the proapoptotic function of ROS based on the following two lines of evidence: (1) stimulation with TNFα resulted in a rapid increase in intracellular ROS levels; and (2) various antioxidants, such as N-acetylcysteine (NAC), butylated hydroxyanisole (BHA), and increased expression of SOD and thioredoxin, were capable of protecting against TNF cytotoxicity.

On the other hand, some other reports have demonstrated the inhibitory effect of ROS on TNFα-induced cell death, based on observations that the prooxidant status offers protection against TNFα-induced apoptosis **(35**, **36)**. There are a number of critical points in understanding such conflicting roles of ROS in TNFα-mediated cell death. First, the level and duration of ROS production upon TNFα exposure may be important. Unfortunately, the actual ROS levels were rarely quantified in most of the studies discussed above, probably due to the technical difficulty in such quantitative measurement. Second, the source of ROS may also contribute to the conflicting role of ROS in TNFR1-mediated cell death. For instance, ROS generated from the Rac1-NOX system protect, whereas mitochondria-derived ROS promote TNF-induced apoptosis **(37)**. Third, ROS have opposite effects on TNFα-induced apoptosis and nonapoptotic/necrotic cell death, suggesting that part of the discrepancy in the literature is possibly due to the lack of accurate measurement of these two forms of cell death. Last, but not least, the biological function of ROS production should be evaluated in the context of other signaling pathways activated downstream of TNFR1. It is clear now that ROS serve as the key mediator in the cross-talks between various signaling pathways downstream of TNFR1, in particular JNK and NF-κB activation. These topics are discussed in detail in the following sections.

Involvement of ROS in CD95-Mediated Apoptosis

The Fas (CD95/Apo1) is probably the most well-characterized death receptor in apoptosis **(38, 39)**. Following receptor ligation with Fas ligand (FasL), some key signaling molecules such as FADD are recruited to the receptor to initiate the formation of the death-inducing signaling complex (DISC), followed by activation of initiator caspases such as caspase-8.

Similar to the role of ROS in the TNFR1-initiated cell death pathway, there is substantial evidence demonstrating the proapoptotic role of ROS and oxidative stress in CD95-induced cell death. First, Fas ligation has been found to promote intracellular ROS production **(40, 41)**. Second, various antioxidants such as NAC, SOD or its mimics, and catalase were able to block Fas-induced apoptosis **(41, 42)**. As for the intracellular source of ROS generated in cells with an activated Fas-mediated signaling, both the mitochondrial electron transport chain **(41, 43)** and NOX **(9)** have been implicated. Fas-mediated NADPH oxidase activation and ROS generation may involve ceramide and PKCzeta **(9)**, while relatively little is known about how the signals are replayed from the Fas receptor to the mitochondria to promote ROS formation from the mitochondrial respiratory chain.

A number of attempts have been made to elucidate the underlying mechanisms responsible for the proapoptotic role of ROS in Fas-mediated cell death. One possibility is that ROS are able to upregulate the expression of Fas at the cell surface **(43, 44)**, although the exact mechanism for such upregulation is still elusive. Another possibility is that ROS are capable of promoting apoptosome formation and the activation of caspases-3 and -9 and by oxidizing Apaf-1 **(41)**. While the proapoptotic function of ROS in Fas-mediated apoptosis is evident, there are intriguing findings showing the antiapoptotic function of ROS in the Fas-induced death pathway. Such conflicting effects of ROS on Fas-mediated apoptosis are discussed in the subsequent section of this chapter.

ROS and TRAIL-Mediated Apoptosis

TNF-related apoptosis-inducing ligand (TRAIL) is a newly discovered member of the TNF superfamily with a unique property of selectively inducing apoptosis in a variety of tumor cells and transformed cells, but not in most normal cells **(45)**. Therefore, TRAIL is considered a promising cancer therapeutic agent and is currently in Phase II clinical trials. TRAIL-induced apoptosis is well known to be executed via two death receptors (DR4 and DR5). Similar to TNFR1 and Fas, there is evidence supporting a proapoptotic role of ROS in TRAIL-induced apoptosis, including a substantial amount of ROS accumulation in TRAIL-treated cancer cells and the protective effects of antioxidants on TRAIL-induced apoptosis **(46)**. At present, mitochondria are believed to be main source of intracellular ROS production in TRAIL-treated cells, based on

the observation that the uncoupling of the mitochondrial respiratory chain by Bcl-2 or carbonyl cyanide m-chlorophenylhydrazone (CCCP) is able to sensitize TRAIL-induced apoptosis **(46, 47)**. So far, there is no indication whether TRAIL promotes ROS production via other mechanisms such as the activation of NOX.

One important understanding regarding the proapoptotic function of ROS in TRAIL-induced apoptosis is the regulatory role of ROS in the expression of DR4 and DR5. At present, TRAIL resistance is the main limiting factor hindering its application in clinical oncology; various approaches have been developed to overcome this resistance. In fact, many of the sensitizing agents studied have one common mechanism: the promotion of DR4/5 expression via ROS **(48, 49)**. Our recent work on andrographolide, a diterpenoid lactone isolated from a traditional herbal medicine *Andrographis paniculata,* has demonstrated that andrographolide is able to greatly sensitize TRAIL-induced apoptosis in TRAIL-resistant cancer cells via sequential events including ROS production, JNK activation, p53 phosphorylation and stabilization, and DR4 upregulation (data to be published). Therefore, understanding the role of ROS in the TRAIL cell death signaling pathway has important implications in TRAIL-based therapy in clinical oncology, in particular in developing sensitizing agents to overcome TRAIL resistance.

Redox Regulation of JNK and NF-κB

Redox Regulation of JNK During Apoptotic Signaling

JNK or stress-activated protein kinase (SAPK) is an important subgroup of the MAPK family and is readily activated in response to various environmental stress factors to exert diverse cellular functions including cell proliferation, differentiation, and cell death **(50)**. The activation pathway of JNK has been well elucidated via the typical MAPKKK-MAPKK-MAPK module. JNK is phosphorylated and activated by two MAPKKs (JNNK1/MKK4/SEK1 and JNKK2/MKK7), which possess dual specificity on Thr183 and Tyr185. Upstream of JNKK1 and JNKK2, several MAPKKKs have been identified, including MEK kinase 1 (MEKK1) and apoptosis signal-regulating kinase (ASK1). The upstream signaling events upstream of MAPKKKs are relatively less well understood.

At present, signaling pathways controlling ROS-induced JNK activation have been well discussed **(51)**. The first and probably most important pathway involves ASK1. The discovery by Saitoh et al. that thioredoxin is the internal inhibitor of ASK1 has directly linked ROS and oxidative stress with ASK1 activation **(52)**. The activity of ASK1 depends on the redox status of thioredoxin, and only the reduced form of thioredoxin, but not the oxidized form, is capable of binding to ASK1 and blocking its kinase activity. In the presence of

ROS, the oxidized thioredoxin dissociates from ASK1 and therefore activates ASK1 by oligomerization and phosphorylation. Therefore, it is believed that the thioredoxin-ASK1 system serves as the molecular switch that converts the redox signal to kinase activation.

A number of other signaling molecules have also been identified in ROS-mediated JNK activation. They include the following:

(1) Src kinase. It has been found that genetic disruption of Src or inhibition of Src kinase activity leads to specific suppression of JNK activation by H_2O_2, but not on ERK and p38 **(53)**.
(2) Glutathione S-transferase Pi (GSTp). The monomeric form of GSTp binds to the c-terminal of JNK and suppresses its kinase activity, while H_2O_2 treatment caused GSTp oligomerization and dissociation of the GSTp-JNK complex, leading to JNK activation **(54)**.
(3) MAPK phosphatases. ROS contribute to the persistent JNK activation by the inhibition of JNK-inactivating phosphatases. Such inhibition is achieved by converting the catalytic cysteine to sulfenic acid **(55)**.

Some of the signaling pathways described above are independent of the MAPKKK-MAPKK module, indicating the complex nature of JNK activation in the context of oxidative stress.

After establishing the signaling pathway in ROS-mediated JNK activation, the next theme is to understand the proapoptotic function of JNK in ROS-induced cell death. At present, there is substantial evidence supporting the notion that JNK serves as an important proapoptotic mechanism in cells under oxidative stress. First, as discussed above, JNK is readily activated by ROS via various signaling pathways. Second, the suppression of JNK by either genetic or pharmacological approaches offers significant protection against apoptosis induced by ROS. For instance, $ASK1^{-/-}$ cells are resistant to H_2O_2-induced apoptosis **(56)**. Similar resistance was also found in cells with an over-expression of the dominant-negative mutant of JNK **(57)**. A specific JNK inhibitor (SP600125) has also frequently been used to effectively block H_2O_2-induced apoptosis.

In addition to apoptosis, ROS and oxidative stress are able to induce non-apoptotic/necrotic cell death **(58, 59)**. The emerging question is how JNK activation is able to contribute to two opposing forms of cell death induced by ROS and oxidative stress. A possible clue is provided by the downstream target(s) of JNK. In apoptosis, mitochondria appear to be the main site of action for JNK, based on a study using primary murine embryonic fibroblasts with deletion of both JNK1 and JNK2 **(60)**. At mitochondria, JNK is able to directly interact and regulate the function of Bcl-2 family members. For instance, JNK can phosphorylate and inhibit the antiapoptotic function of Bcl-2 **(61)** and myeloid cell leukemia 1 (Mcl-1) **(62)**, or enhance the function of proapoptotic Bcl-2 family members such as Bax, Bim, and Bmf **(63, 64)**. On the contrary, in ROS-mediated necrosis, JNK may act on a different group of target molecules. One such target is poly(ADP-ribose) polymerase-1 (PARP-1).

Recent work in our laboratory has revealed that ROS-mediated JNK contributes to the sustained activation of PARP-1, leading to depletion of ATP and necrosis **(10)**. Future work is needed to elucidate the molecular mechanisms underlying the regulatory functions of JNK in cell death, including both apoptosis and necrosis.

Regulation of NF-kB During ROS-Mediated Apoptosis

NF-κB consists of a family of five proteins: RelA/p65, RelB, c-Rel, p100, and p105, which are characterized by the presence of a Rel homology domain (RHD) required for specific DNA binding, dimerization, and nuclear localization. It has been well established that NF-κB is one of the key regulatory mechanisms involved in controlling the transcription of a number of important genes that play critical roles in immune function, inflammation, cell proliferation, and cell death **(65)**.

Among various signaling pathways controlling NF-κB activation, one highly debatable topic is the redox regulation of this important transcription factor. Earlier work demonstrated that ROS are able to activate NF-κB based on the following observations **(66)**: First, exogenous ROS such as H_2O_2 are capable of activating NF-κB or ROS are involved in NF-κB activation induced by other stimuli such as TNFα, LPS, IL-1, PMA, and ionizing radiation. Second, various antioxidants such as NAC and pyrrolidine dithiocarbamate (PDTC) were able to block NF-κB activation induced by the above-mentioned stimulation. However, accumulating evidence in recent years has challenged the earlier notion that NF-κB is activated via a redox mechanism. One good example for understanding the complex nature of the redox regulation of NF-κB is the TNFα signaling pathway, a topic that has been extensively reviewed and discussed **(67, 68)**. Despite the earlier findings suggesting a promoting function of ROS in TNFα-induced NF-κB activation, a number of recent studies have challenged such a notion and have provided evidence that ROS do not activate NF-κB but, on the contrary, suppress NF-κB activation triggered by TNFα. At present, the mechanisms underlying the inhibitory effects of ROS on NF-κB have not been well elucidated. One possibility is via the oxidation of certain cysteine residues in the NF-κB protein as well as in the IKK complex **(69, 70)**. Convincing evidence from the work by Hayakawa et al. has reinforced the earlier findings by demonstrating that NAC and PDTC, two antioxidants often used to block NF-κB activation, suppress the TNF-mediated NF-κB pathway via mechanisms independent of their antioxidant activity. Moreover, endogenous ROS produced through Rac-NOX failed to activate NF-κB signaling but, instead, reduced the magnitude of its activation **(71)**. Taken together, the emerging consensus is that ROS are unlikely to be a general modulator in NF-κB activation, and the exact

effect of ROS on NF-κB (activation or inhibition) is highly stimulus- and cell type-specific.

NF-κB normally acts as a cell survival mechanism in response to stress factors and cytokines such as TNFα. At present, it is not known whether the activation of NF-κB by ROS restrains ROS-induced apoptosis or whether, to the contrary, the suppression of NF-κB by ROS contributes to ROS-mediated cell death. Due to their established importance in controlling the cellular responses to stress factors, either topic warrants further investigation, especially in cancer cell biology.

ROS as Messengers Between NF-κB and JNK in TNFα-Induced Cell Death

As described earlier, downstream of TNFR1, there are two distinct signaling pathways: NF-κB and JNK. Generally, NF-κB plays a prosurvival function, while JNK promotes TNFα-induced cell death **(30)**. One major work of progress in understanding the molecular mechanism in TNFα-induced cell death is the elucidation of the interaction between these two signaling pathways. In normal cells, TNFα induces an earlier and transient JNK activation, while in cells that are deficient in NF-κB activation, JNK activation is enhanced and sustained, suggesting that NF-κB functions as a potent JNK inhibitor. More importantly, the antiapoptotic function of NF-κB is, at least in part, achieved through its ability to suppress the prolonged activation of JNK **(72, 73)**.

In the search for key mediators of the interplay between NF-κB and JNK, a number of candidates have been proposed, including XIAP, GADD45b, A20, ferritin heavy chain (FHC), and MnSOD **(72–74)**, all of which are the target molecules of NF-κB. It thus appears to be logical that the activation of NF-κB leads to the transcriptional upregulation of these target genes and then the suppression of JNK and subsequent cell death. At present, there is accumulating evidence supporting the critical role of ROS as the key player relaying the cross-talk between NF-κB and JNK **(34, 75, 76)**. The signaling mission of ROS in the cross-talk between JNK and NF-κB could be summarized as the following:

(1) TNFα promotes intracellular ROS production, which is responsible for sustained JNK activation via the ASK1-JNKK2-JNK module or via the suppression of MAPK phosphatases, as discussed earlier.
(2) ROS are capable of inhibiting TNFα-mediated NF-κB activation. As described earlier, the inhibitory effect is achieved via the direct oxidation of critical cysteine residues in the NF-κB protein or IKK.
(3) NF-κB regulates the expression of several key antioxidant enzymes or proteins, such as MnSOD and FHC to eliminate ROS, thus serving as a negative-feedback loop to suppress ROS-mediated JNK activation.

(4) NF-κB may also indirectly block ROS production from mitochondria via suppression of the caspase cascade, based on earlier findings that activated caspases directly act on the respiratory complexes of mitochondria to disrupt mitochondrial function and to enhance the generation of ROS **(15)**. The main antiapoptotic function of NF-κB is based on its transcriptional regulation of many antiapoptotic genes such as *XIAP* and *c-FLIP*, which have a direct inhibitory effect on caspase activation **(30)**.
(5) Activated JNK is capable of promoting ROS production from mitochondria, thus forming a positive-feedback loop between JNK and ROS **(17)**.
(6) ROS accumulation and sustained JNK activation under the condition of NF-κB suppression have been implicated in both forms of cell death (apoptotic and nonapoptotic/necrotic) mediated by TNFR1 **(17, 18)**.

As discussed above, the signaling function of ROS in the cross-talk between NF-κB and JNK is mainly established in the TNFR1 signaling pathway. It remains to be determined whether ROS play a similar role in cell death induced by other cellular stresses that are capable of activating NF-κB and JNK simultaneously. In contrast to the inhibitory function in TNFα-treated cells, NF-κB positively regulates UV-induced JNK activation and promotes UV-induced apoptosis **(77)**, suggesting the nature of the cross-talk between NF-κB and JNK is stimulus-specific. However, most of these effects of ROS downstream of TNFα signaling have been reported in systems using mouse embryonic fibroblasts (MEF); it is important to investigate whether the intermediary role of ROS between JNK and NF-κB could be extended to other cell types, in particular cancer cells. In this regard, there is evidence to link an increase in ROS production and JNK activation to enhanced cell susceptibility to apoptosis in oncogenically transformed NIH 3T3 cells **(78)**. Moreover, through c-FLIP knockdown using the RNA interference technique, a very recent study proved the mediating role of ROS in the cross-talk between NF-κB and JNK in a number of human cancer cells such as cervical cancer HeLa, colon carcinoma HCT116, and lung carcinoma A549 cells **(79)**. Given the importance of ROS accumulation and sustained JNK activation in determining cell death (both apoptosis and necrosis) to cellular stress, selective induction of ROS accumulation and JNK activation will be an alternative strategy to promote the desired cell death in tumor cells.

Understanding the critical role of ROS in the cross-talk between NF-κB and JNK and cell death could have potential implications in developing better and more effective therapies and also provides thoughts for reviewing the current clinical practices in light of the diverse roles of ROS in the biology of disease.

The Other Faces of ROS

Pro-life and Proliferative Activity of ROS

Evidence is accumulating that depending upon the cellular levels of ROS, the effect of ROS on cell fate could be highly varied. To that end, the observations linking low levels of $O_2^{\cdot -}$ and H_2O_2 to proliferative signaling have resulted in a paradigm shift in that ROS are not exclusively associated with cell and tissue damage/death. Mild elevation in intracellular $O_2^{\cdot -}$ or H_2O_2 stimulates growth responses in a variety of cell types **(80–82)** via activation of early growth-related genes such as *c-fos* and *c-jun* **(82)**, alterations in the activities of protein kinases, oxidative inactivation of phosphatases, and activation of transcription factors **(83)**. Of note, strong evidence for the "pro-life" activity of ROS also comes in the form of NADPH-dependent generation of ROS upon growth factor stimulation or cytokine receptor activation, such as platelet-derived growth factor (PDGF), tumor necrosis factor-alpha (TNF-α), IL1β, IL6, IL3, fibroblast growth factor (FGF-2), transforming growth factor-alpha (TGF-α), and insulin **(3, 83)**. Similarly, T-cell activation during an immune response is strongly augmented in the presence of $O_2^{\cdot -}$ or at relatively low concentrations of H_2O_2 **(84)** via an ROS-dependent increase in IL2 promoter activity and transcription and surface expression of the IL2 receptor (IL2R) **(3)**. Furthermore, intracellular ROS positively influence ion channel activities, such as IP3 receptor-mediated intracellular Ca^{+2} mobilization, and Na^+/Ca^{+2} and Na^+/H^+ (NHE) membrane transport pumps **(82, 83)**. Interestingly, an increase in the activity of these transport channels is observed during growth stimulation, and an active NHE pump has been shown to influence cell division by maintaining an alkaline intracellular milieu **(85)**. These direct effects of ROS on survival signaling are corroborated by the inhibitory effect of ROS scavengers, such as SOD or catalase, on cell proliferation and survival in some systems **(82)**. Collectively, these data implicate intracellular ROS as major effector molecules impacting diverse signaling circuits and metabolic processes. However, it should be pointed out that the pro-life role of ROS is only observed under a mild prooxidant state, as the effects could be completely reversed if the concentration of ROS in the cells is high enough to induce oxidative stress and trigger death signaling.

Deregulation of the intracellular redox status has been implicated in a number of hereditary disorders with a high predilection for neoplasia. Therefore, it is now well accepted that certain types of cancer cells produce substantial amounts of ROS, thereby rendering the intracellular milieu "prooxidant" **(80, 81)**. The link between a prooxidant milieu and carcinogenesis is further supported by the ability of $O_2^{\cdot -}$ and other oxygen radicals to promote cellular transformation, together with experimental data reinforcing the tumor suppressor activity of the $O_2^{\cdot -}$ scavenger MnSOD **(86–89)**. This is corroborated by

the lower expression of MnSOD as well as its relatively poor induction upon oxidant challenge in tumor cells. The obvious consequence of the deficiency in the enzymatic activity of MnSOD is a buildup of $O_2^{\cdot -}$ anion, which has been linked to the high levels of DNA oxidation in some forms of cancer. These observations are supported by the effects the reexpression of MnSOD has on promoting differentiation and reversing the invasive phenotype **(87–90)**. More convincing evidence for linking a prooxidant intracellular milieu with the acquisition or progression of carcinogenesis comes from studies implicating cell survival networks, such as the PI3Kinase-Akt pathway, in increased intracellular ROS generation **(91)**. Along similar lines, the direct involvement of intracellular $O_2^{\cdot -}$ in proliferation induced by the oncoprotein $p21^{Ras}$ has been demonstrated in lung fibroblast cells **(92)** and has been shown to be a function of the Ras-dependent activation of the GTPase Rac1. Indeed, cancer cells constitutively expressing the activated form of Ras (RasV12) maintained significantly elevated levels of $O_2^{\cdot -}$, which correlated with resistance to drug-induced apoptosis. Interestingly, both the elevated levels of $O_2^{\cdot -}$ as well as the resistance to apoptosis were suppressed upon expression of RacN17, a dominant-negative form of Rac1 **(93)**. In addition, the identification of the nonphagocytic mitogenic oxidase Nox1 and its ability to stimulate growth via $O_2^{\cdot -}$ further lends credence to the prooxidant theory of carcinogenesis **(94)**.

Apoptosis Inhibition: $O_2^{\cdot -}$ or H_2O_2?

The association between intracellular ROS and several death stimuli, such as TNF-α, C2 ceramide, dexamethasone, UV irradiation, and chemotherapeutic drugs, supports the notion that ROS are deleterious to cells and tissues **(95)**. However, efficient apoptotic signaling has been observed under hypoxic conditions, which brings into question the obligatory role of ROS in death signaling **(96, 97)**. In fact, there is strong evidence to link the prooxidant state to the inhibition of apoptosis in tumor cells **(93, 94, 98–101)** and, contrarily, to support the proapoptotic effect of a reduced intracellular environment. The underlying mechanisms that allow ROS to support survival signaling on the one hand and facilitate death execution on the other are beginning to be elucidated. For example, a direct or indirect inhibitory effect of low levels of intracellular ROS on the apoptotic pathway has been demonstrated in a variety of model systems. Could this explain, at least in part, resistance to chemotherapy in tumor cells? Indeed, recent findings including our own observations have demonstrated that a mild increase in intracellular ROS inhibited apoptosis in tumor cells, irrespective of the trigger **(93, 98, 99, 101–106)**. These results suggest that the role of intracellular $O_2^{\cdot -}$ in regulating cell sensitivity to apoptosis appears to be common to a variety of apoptotic stimuli such as receptors, drugs, and viruses. In this regard, the apoptosis-inhibitory effect of elevated intracellular $O_2^{\cdot -}$ was demonstrated in a human melanoma cell line (M14)

tailored to overexpress or repress the cytosolic $O_2^{\cdot-}$ scavenger Cu/Zn SOD **(101)**. The overexpression of Cu/Zn SOD resulted in a significant decrease in the intracellular level of $O_2^{\cdot-}$ with a concomitant increase in cell sensitivity to anticancer agents. In contrast, repression of the Cu/Zn SOD protein or exposure to the SOD inhibitor diethyldithiocarbamate (DDC) blocked apoptosis by maintaining a slightly elevated intracellular $O_2^{\cdot-}$ **(98)**. Similar results have been reported by Lin et al. in a model of virus-induced apoptosis where elevated intracellular $O_2^{\cdot-}$ served as a survival signal and lowering $O_2^{\cdot-}$ facilitated death execution **(107)**. As a result, it has become clear that the biological effects attributed to ROS might differ depending upon the intracellular level and the actual species involved.

These data demonstrating the death inhibitory activity of intracellular $O_2^{\cdot-}$ have challenged the dogmatic view of ROS as deleterious to cell growth and survival. Indeed, the direct involvement of $O_2^{\cdot-}$ in death signaling has been questioned by findings implicating intracellular H_2O_2 and its metabolite $^{\bullet}OH$ radical in ROS-induced cell death **(108)**. In this regard, an increase in the intracellular $O_2^{\cdot-}$ concentration in the absence of cytotoxic levels of H_2O_2 does not kill cells but contrarily inhibits activation of the apoptotic pathway **(104, 105, 109)**. H_2O_2 is a weak oxidizing and reducing agent and, despite its poor reactivity, can trigger apoptosis or necrosis depending upon its concentration **(99, 109)**. Interestingly, apoptotic concentrations of H_2O_2 ($<$500 μM in most systems) result in a decrease in intracellular $O_2^{\cdot-}$, an increase in the ratio of reduced to oxidized glutathione (GSH:GSSG), and a significant drop in intracellular pH (pH_c), thereby creating a reduced intracellular milieu **(109)**. In contrast, necrotic concentrations of H_2O_2 result in an increase in the ratio of oxidized to reduced glutathione (GSSG:GSH), consistent with the definition of oxidative stress. The intracellular generation of H_2O_2 is also a common feature upon exposure of tumor cells to anticancer drugs, which is predominantly a function of the mitochondrial respiratory chain **(19)**. It thus appears that a permissive intracellular environment for apoptotic signaling is a function of a tight balance between intracellular $O_2^{\cdot-}$ and H_2O_2 and that a decrease in intracellular $O_2^{\cdot-}$ or a reciprocal increase in H_2O_2 facilitates apoptotic execution **(110)**.

Considering the contrasting effects of intracellular $O_2^{\cdot-}$ and H_2O_2 on cell death/survival signaling, the precise intracellular targets of these ROS species are not completely understood. The inhibitory effect of $O_2^{\cdot-}$ on cell death signaling could be linked to its ability to inactivate caspase proteases, mediators of apoptotic signaling, via oxidative modification **(101, 111–114)**. In contrast, apoptotic concentrations of H_2O_2 strongly induce enzymatic activities of caspases-3 and -9 **(115)**, although it is not clear whether this activation is a direct result of H_2O_2 or mediated through H_2O_2-induced release of mitochondrial death factors. The latter scenario appears more plausible considering the observation that the antiapoptotic activity of the mitochondrial protein Bcl-2 is linked to its ability to inhibit mitochondrial H_2O_2 production **(116)**. An alternative mechanism, recently reported with drug-induced apoptosis, is the

inhibitory activity of H_2O_2 on the ATP-dependent Na^+/H^+ antiporter that regulates cytosolic pH in the physiological range, thereby leading to marked acidification of the intracellular milieu **(117)**. To that end, the involvement of H_2O_2 in drug-induced translocation of Bax to the mitochondria was demonstrated to be via a drop in cytosolic pH **(118)**.

Superoxide as the Oncogenic Signal of Bcl-2

The regulatory role of intracellular prooxidant environment on cell death signaling was further corroborated in a model of Bcl-2-mediated death inhibition. *Bcl-2* is the first in a family of genes (classified as the Bcl-2 family) consisting of pro- and antiapoptotic members that serve as checkpoints or inducers of apoptosis signal transduction **(119)**. Expression of the prototype antiapoptotic protein Bcl-2 prevents apoptosis in response to a host of death stimuli by blocking the release of death amplification factors from the mitochondria **(120–122)**. Of note, Bcl-2 is localized to the membranes of the mitochondria, the ER, and the nuclear envelope, all of which are sites of intracellular ROS generation **(102)**. Historically, Bcl-2 has been described as an antioxidant due to its ability to suppress lipid peroxidation induced by H_2O_2 **(102)**. However, recent data have challenged this notion by demonstrating that Bcl-2 did not by itself function as an antioxidant, but instead the overexpression of Bcl-2 was associated with a prooxidant intracellular milieu that reinforced the cellular antioxidant defense systems. Along similar lines, the death-inhibiting activity of Bcl-2 was linked to its ability to generate a slightly prooxidant intracellular environment in human leukemia cells, which could be alleviated by the NADPH oxidase inhibitors **(106)**. More recently, the prooxidant activity of Bcl-2 was linked to its ability to increase mitochondrial oxygen consumption and cytochrome c oxidase (COX) activity in human tumor cells **(123)**. These reports provide strong evidence linking oncogene-mediated cell survival and proliferation to a prooxidant intracellular milieu and suggest an association between prooxidant state and tumor promotion/progression. More importantly, these findings present excellent targets to design compounds for the effective execution of tumor cells.

Intracellular pH as a Potential Target of ROS

A decrease in intracellular $O_2^{\cdot-}$ or an increase in H_2O_2 culminates in shifting the cytosolic pH (pH_c) to a significantly acidic level, a phenomenon commonly observed during receptor- or drug-induced apoptosis **(106, 124, 125)**. To that end, a possible mechanism implicating mitochondria ETC-derived H_2O_2 (upon exposure to anticancer drugs) in cytosolic

acidification has been demonstrated. This acidification of the intracellular milieu could be a function of H_2O_2-mediated activation of PARP leading to depletion of cellular NADH and ATP stores and culminating in the inhibition of critical ATP-driven pH regulators, such as the Na+/H+ transporter (NHE) **(19)**.

Recent observations have highlighted the critical role of cytosolic pH (pH_c) and tumor microenvironment in cell migration and invasion **(126)**. An alkaline cytosolic milieu is an invariable finding in tumor cells (7.12–7.65 vs. 6.99–7.20 in normal tissues), which has been attributed to the increase in the ability of tumor cells to secrete H+ ions, an activity that increases with tumor aggressiveness **(127, 128)**. The mechanism behind this reverse-pH gradient (acidic extracellular milieu and alkaline pH_c) involves oncogene-dependent activation/stimulation of NHE1 **(129)**. Interestingly, recent evidence appears to directly implicate NHE1 as a downstream target of intracellular ROS such that an increase in intracellular $O_2^{\cdot-}$ stimulated NHE1 promoter activity and gene expression, whereas exposure to apoptotic concentrations of H_2O_2 had an opposite effect on both the promoter activity as well as the expression of the exchanger **(130)**. Furthermore, the divergent effect of intracellular $O_2^{\cdot-}$ and H_2O_2 on NHE1 expression correlated with their respective effects on cell survival, thereby strongly suggesting a role for NHE1 in redox-mediated cell fate decisions. These observations present novel intracellular targets for the development and design of effective anticancer drugs.

Concluding Remarks

Intracellular ROS play a critical role in cell fate signaling. There is ample evidence to link the increased generation of ROS with a variety of cell death triggers. Not only are the intracellular sources of ROS varied under physiological as well as pathological disease states, but unraveling new cellular targets of ROS provides novel opportunities for designing specific therapies against a host of disease states involving altered redox status. One interesting revelation has been the association of a low prooxidant state, invariably found in transformed cells, with cell proliferation and growth and the inhibition of apoptotic machinery. This in itself provides further avenues to explore in terms of biomarkers and selective targets for more effective drug design. The future bodes well for this field, as cell metabolism is being linked to a variety of pathological states, and we can therefore expect a surge of new discoveries in the near future.

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Chapter 9
The Integration of Metabolism and Cell Death

Jonathan L. Coloff, Yuxing Zhao, and Jeffrey C. Rathmell

Abstract Metabolism is a fundamental cellular process necessary for energy production and biosynthesis. While the biochemistry of basic metabolic pathways has been known for some time, how the metabolic state of a cell regulates fate decisions has remained poorly understood. In recent years, cellular metabolism has been shown to play an integral role in the regulation of apoptosis, in particular via interaction with the Bcl-2 family of proteins. This chapter focuses on the inherent relationships between metabolism and apoptosis, as well as several more intricate mechanisms of how apoptotic and metabolic machinery interact to determine cell fate under a variety of conditions, including a potential for the metabolic regulation of apoptosis in cancer.

Keywords Apoptosis · Metabolism · Glucose · Akt · p53 · Autophagy · Cell Death

A Shared Platform: The Mitochondria

Metabolism is a fundamental cellular process necessary for energy production and biosynthesis. While the biochemistry of basic metabolic pathways has been known for some time, how the metabolic state of a cell regulates fate decisions has remained poorly understood. In recent years, cellular metabolism has been shown to play an integral role in the regulation of apoptosis, in particular via interaction with the Bcl-2 family of proteins. This chapter focuses on the inherent relationships between metabolism and apoptosis, as well as several more intricate mechanisms of how apoptotic and metabolic machinery interact to determine cell fate under a variety of conditions, including a potential for the metabolic regulation of apoptosis in cancer.

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The first and most obvious relation between metabolism and apoptosis is their shared platform, the mitochondria. It has long been appreciated that mitochondria act as the powerhouse of the cell, utilizing metabolites from the cytoplasm to fuel the TCA cycle, β-oxidation, and oxidative phosphorylation to generate the ATP that is necessary for life. As different cell types have vastly different metabolic demands, these are highly regulated and dynamic processes. Rapidly proliferating cells require large amounts of energy and macromolecular precursors to duplicate all of their components and eventually themselves. On the other end of the spectrum are quiescent cells, which require minimal ATP to maintain ion pumps and other essential cellular functions that maintain cell integrity. These vastly different needs require the integration of complex extracellular signals in the form of growth factors and cytokines that determine how and where energy and metabolites are made or acquired. Ultimately, mitochondria play an integral role in these decisions.

Mitochondria also play an essential role in the intrinsic apoptotic pathway. In this pathway, mitochondria are a major site of action of the Bcl-2 family of proteins **(1)**. These proteins, consisting of pro- and antiapoptotic members, regulate mitochondrial outer membrane permeabilization (MOMP), which in turn leads to the release of the proapoptotic effector cytochrome c, and thus the activation of caspases and execution of apoptosis. Regulation of the Bcl-2 family and MOMP is accomplished by sensing extracellular signals and stresses that control their activity and ultimately the decision of life or death. Because of their shared dependence on mitochondria, apoptosis and metabolism are inherently linked, and mitochondrial physiology is critical for the appropriate function of both of these processes. Disrupted mitochondrial metabolism, the loss of mitochondrial membrane potential, and the deregulated exchange of metabolites between the mitochondria and the cytoplasm can lead to MOMP, cytochrome c release, and induction of apoptosis.

Apoptosis Is an Energy-Dependent Process

Apoptosis is an energy-dependent process that neatly disposes of doomed cells. This is in contrast to the energy-independent necrotic cell death, where energy to carry out the most basic of cellular functions—maintenance of ionic gradients—becomes limiting, and the cell quite simply falls apart and releases its contents into the microenvironment, resulting in an inflammatory response. A fundamental distinction between apoptosis and necrosis, therefore, is energy dependence. In the absence of sufficient ATP, apoptosis cannot occur. A key ATP-dependent regulatory point in the apoptotic cascade is the formation of the apoptosome. The apoptosome is a protein complex formed after MOMP that consists of Apaf-1, cytochrome c, and the initiator caspase-9. Once formed, this complex activates the executioner caspase-3 to carry out apoptotic cell death. Importantly, ATP binding and hydrolysis by Apaf-1 are required to facilitate the conformational changes necessary for apoptosome activation **(2, 3)**.

During severe metabolic stress, in which ATP is too limiting for apoptosis, some apoptotic stresses will lead to necrosis **(4)**. The specific metabolic program of a cell may also influence the cell death pathway. For example, certain cell types, such as activated lymphocytes **(5)** or cancer cells **(6)**, depend on glycolysis for the majority of their ATP production instead of the more ATP-efficient mitochondrial oxidative phosphorylation. This metabolic state can also arise under conditions that inhibit a cell's ability to perform oxidative phosphorylation, such as mitochondrial DNA damage and/or hypoxia. If these cells are subjected to apoptotic stress in the form of DNA alkylating agents, the DNA repair pathway and poly(ADP-ribose) polymerase (PARP) are activated. PARP can utilize large amounts of NAD+ in its attempt to repair the DNA and may lead to depletion of cellular NAD+. In highly glycolytic cells, this can severely interrupt cellular metabolism and lead to ATP depletion, as NAD+ is an essential glycolytic cofactor. Therefore, under these circumstances, cells will undergo necrotic cell death despite an abundance of glucose in the extracellular environment **(7)**. The dependence of apoptosis on ATP, therefore, can be a significant regulatory point in cell death.

The PI3K/Akt Pathway

While many cell signaling pathways are known to be activated by growth factors, one particular pathway has been highlighted for its role in promoting cell metabolism and preventing cell death—the PI3K/Akt pathway. Akt has been shown to promote glucose metabolism by trafficking glucose transporters to the cell surface to enhance glucose uptake. This has been most clearly analyzed in the insulin receptor signaling pathway, in which Akt2 phosphorylates AS160 to promote Glut4 translocation to the cell surface **(8)**. Akt1 and Akt2 can also regulate trafficking of Glut1 to the cell surface [**(9–12)**] and prevent internalization of cell surface Glut1 **(11)**. In addition to promoting glucose uptake, Akt can promote glucose metabolism through glycolysis by promoting hexokinase (HK) mitochondrial localization **(13–15)** and by phosphorylating and activating PFK2 **(16)**, which leads to the allosteric activation of PFK1 via fructose-2,6-bisphosphate, thus promoting glycolytic flux. Akt has also been shown to promote glucose flux through alternative glucose metabolic pathways including the pentose phosphate pathway (PPP) **(17)**. Importantly, the activation of Akt appears to promote a highly glycolytic metabolic program that promotes glucose utilization in favor of other fuels. In particular, Akt can inhibit the mitochondrial lipid transporter CPT1, thus limiting the transport of lipids into the mitochondrial matrix for mitochondrial β-oxidation **(18**, **19)**.

In addition to playing a major role in the growth factor-dependent regulation of metabolism, the PI3K/Akt pathway also has well-defined antiapoptotic functions that regulate Bcl-2 family proteins (Fig. 9.1). Akt can phosphorylate the proapoptotic BH3-only protein Bad to promote Bad sequestration in the

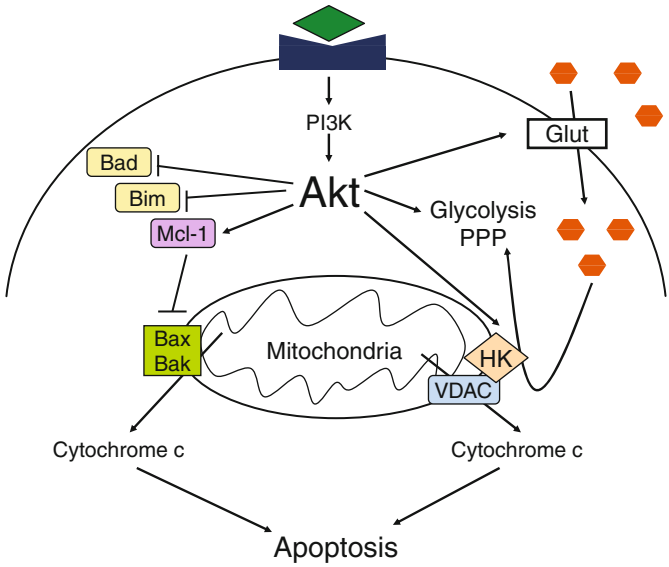

Fig. 9.1 The PI3K/Akt pathway regulates metabolism and apoptosis. Akt inhibits apoptotic cell death by suppressing the activation and induction of the BH3-only proteins Bad and Bim and stabilizes the antiapoptotic Bcl-2 family member Mcl-1. Akt also promotes the surface localization of glucose transporters as well as the mitochondrial localization of hexokinases to promote glucose uptake and promotes the expression and activity of several glycolytic genes

cytosol by 14-3-3 proteins **(20**, **21)**. This prevents Bad from translocating to the mitochondria, where it can exert is proapoptotic role via interaction with and inhibition of Bcl-2 **(1)**. Akt also inhibits the transcriptional induction of the BH3-only protein Bim by phosphorylation and inhibition of FoxO family transcription factors **(22**–**24)**. In addition to inhibiting the induction or activity of proapoptotic BH3-only proteins, Akt also can regulate the proteolytic turnover of the antiapoptotic Bcl-2 family member Mcl-1. In particular, Akt phosphorylation of glycogen synthase kinase 3 (GSK3) inhibits the ability of GSK3 to phosphorylate Mcl-1 **(25**, **26)**, which otherwise promotes Mcl-1 ubiquitination by the E3 ligases Mule **(27)** and β-TRCP **(28)**.

While these well-described and well-accepted functions of the PI3K/Akt pathway are seemingly distinct, recent evidence has suggested that they are instead intricately linked. In particular, it appears that Akt's regulation of glucose metabolism is essential for its ability to promote cell survival. In cytokine-dependent hematopoietic cells, the expression of a constitutively active form of Akt can maintain glucose metabolism and prevent cell death upon cytokine withdrawal **(17**, **29)**. Akt is completely dependent on glucose to accomplish its antiapoptotic effects, however, as glucose deprivation or limitation leads to Bax activation and cytochrome c release from the mitochondria **(13**, **17**, **29)**. This dependence of Akt on glucose may be primarily energetic

rather than specific for immediate products in glucose metabolism, as the pharmacologic activation of β-oxidation as a fuel source for ATP generation can render Akt largely independent of glucose in some cell systems **(19)**.

In addition to relying on glucose as an energy source, Akt-expressing cells may also require glucose to regulate the key glycolytic enzyme, hexokinase (HK). Akt promotes both HK activity and mitochondrial localization **(13, 17)**. The mechanism of HK regulation by Akt is not entirely certain, but Akt has recently been shown to phosphorylate HK2 to promote mitochondrial targeting **(30)**. At the mitochondria, HK can interact with the voltage-dependent anion channel (VDAC), which is located in the outer mitochondrial membrane and participates in the translocation of mitochondrial metabolites and ATP into and out of the mitochondria **(31)**.

The HK/VDAC interaction may regulate the Bcl-2 family directly, by inhibiting Bax recruitment to VDAC **(32)**, or indirectly, via regulating the open/closed state of VDAC. In the indirect model, disruption of HK/VDAC associations promotes closure of VDAC, which can lead to cytochrome c release and apoptosis. In this model, it has been proposed that the HK/VDAC interaction is dependent primarily on Akt-regulated glucose uptake **(13)** and the presence of intracellular glucose or glucose-6 phosphate to bind HK **(15)**. Glucose hydrolysis is not necessary in this situation, as a nonhydrolysable glucose analogue, 2-deoxyglucose, can fully replace glucose to allow Akt to inhibit apoptosis **(14)**. In addition, the indirect model for Akt dependence on HK may be independent of Bax- and Bak-dependent mitochondrial disruption. Instead, VDAC closure could lead to cytochrome c release and caspase activation through loss of the outer mitochondrial membrane integrity **(14)**. While the precise mechanism by which Akt may regulate HK and depend on HK targeting to mitochondria relative to the role of glucose hydrolysis **(17)** remains uncertain, it is likely to contribute to Akt-mediated survival.

Nutrient Deprivation and Cell Death

One cause of metabolic stress that can lead to cell death is nutrient deprivation. While nutrient limitation in extreme cases can lead to necrosis, an apoptotic response has been shown to occur in many instances. Oxygen is a critical element to allow oxidative phosphorylation and electron transport, and while oxygen limitation leads to an adaptive transcriptional response **(33)**, complete anoxia causes cell death. Through mechanisms that are not yet clear, the antiapoptotic Bcl-2 family protein Mcl-1 is degraded in anoxia, allowing the activation of the proapoptotic proteins Bak and Bax **(34)**. Glucose limitation has also been shown to induce Bax activation and apoptotic cell death **(35, 36)**. Glucose withdrawal has been shown to induce apoptotic cell death in diverse cell types, including neurons and hematopoietic cells. In both cases, Bcl-2 and Bcl-xL, respectively, have been shown to inhibit glucose withdrawal-induced

cell death **(29, 37)**. In addition, morpholino knockdown of the glucose transporter Glut1 in developing zebra fish causes neuronal apoptosis that can be prevented by simultaneous knockdown of the proapoptotic BH3-only protein Bad **(38)**. Together these findings indicate that a nutrient stress response pathway, initiated upon glucose deprivation, leads to Bcl-2 family-mediated apoptosis rather than metabolic catastrophe and necrosis.

The precise mechanism that leads to Bax activation after glucose withdrawal has remained elusive, but several recent studies have begun to shed light on this pathway of Bcl-2 family regulation. For example, a recent study by Alves et al. **(39)** showed that the proapoptotic BH3-only protein Noxa is upregulated upon T-cell activation and that its expression appears to be determinant in apoptosis in limiting glucose conditions. In the same study, Mcl-1 is shown to be sensitive to glucose levels, with reduced glucose availability leading to reduced Mcl-1 expression. This demonstrates quite clearly that Noxa and Mcl-1 are sensitive to glucose limitation, and the balance of these pro- and antiapoptotic Bcl-2 family members can regulate cell death under situations of glucose limitation in T cells.

Another example of nutrient limitation and apoptosis has been described in the *Xenopus* oocyte extract system. This system has been used as a model for apoptosis because extracts made from *Xenopus* oocytes undergo spontaneous apoptosis, with loss of mitochondrial integrity and release of cytochrome c, after four to six hours of incubation at room temperature **(40)**. It was unclear, however, how this apoptotic process was initiated. Recently, it was shown that depletion of NADPH within the extract due to decreased metabolic flux through the pentose phosphate pathway (PPP) may be the cause of apoptosis **(41)**. If glucose-6 phosphate or other metabolites that can lead to NADPH generation via the PPP are added to the extracts, apoptosis is significantly delayed or blocked. While NADPH is known to protect cells from redox stress, this does not appear to be the mechanism of protection from apoptosis in the *Xenopus* system. Rather, NADPH appears to regulate caspase-2 phosphorylation by CaMKII to suppress caspase-2 activation, which could promote mitochondrial release of cytochrome c and apoptosis. It is unclear how broadly this pathway may apply to other cell types, but caspase-2 is also regulated by heat shock proteins **(42)** and may serve as a general metabolic or stress sensor and initiator caspase.

Nutrient limitation can also lead to the self-digestive process of autophagy. While initially thought of as a cell death mechanism, autophagy is now generally considered to be a survival mechanism in times of nutrient deprivation. In cells in which apoptosis is prevented, such as Bax- and Bak-deficient or Bcl-2- or Bcl-xL-overexpressing cells, autophagy can be induced in times of metabolic stress **(43)**. Autophagy allows cells to break down intracellular components and utilize their constituents as metabolic fuel. This can generate sufficient ATP to maintain ion pumps and prevent necrosis. Furthermore, in Bax- and Bak-deficient cells, autophagy can provide sufficient nutrients to keep cells alive for months **(44)**, which clearly demonstrates a role for autophagy as a metabolic survival mechanism. In addition, this pathway has been shown to be critical for

lymphocyte survival **(45, 46)** and may play a key role in tumor cell survival *in vivo* **(47, 48)**. The relationship between autophagy and apoptosis remains somewhat unclear, but autophagy undoubtedly has an important role in the metabolic control of cell death.

Metabolic Regulation of Apoptotic Machinery

In contrast to nutrient deprivation, increased metabolism can protect cells from apoptosis. Glut1 and HK1 are strongly upregulated in lymphocyte activation **(5, 49)** and are often overexpressed in cancer **(50–52)**, leading to elevated glucose uptake and metabolism **(17, 53)**. Recent evidence has pointed to this increase in glucose metabolism as being antiapoptotic itself. The overexpression of Glut1 and HK in cytokine-dependent hematopoietic precursor cells increases glucose uptake and glycolysis, can largely maintain glucose metabolism after cytokine withdrawal, and confers a significant survival advantage over control cells **(53)**. Similarly, increased glucose uptake has been shown to be antiapoptotic in neurons **(54)**, cardiomyocytes **(55)**, and primary T lymphocytes **(53)**, suggesting that this metabolic pathway may represent a generalized survival mechanism.

Further investigation of how increased Glut1 expression and glucose uptake protect from cell death has revealed that increased glucose metabolism initiates an antiapoptotic glucose-sensitive signaling pathway (Fig. 9.2). This pathway culminates in the stabilization of the antiapoptotic Bcl-2 family member Mcl-1 to prevent Bim-mediated toxicity and activation of Bax **(53)**. This protection of

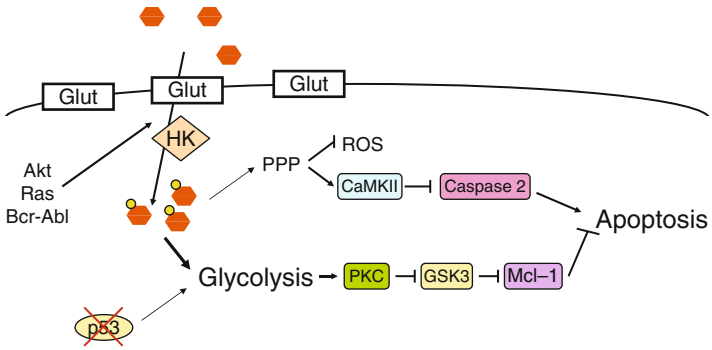

Fig. 9.2 Glucose metabolism can inhibit apoptotic cell death. Increased glucose metabolism can inhibit cell death by multiple pathways. Increased glycolytic flux initiates a signaling pathway that culminates in the stabilization of Mcl-1 and the inhibition of apoptosis. Also, glucose flux through the pentose phosphate pathway can inhibit reactive oxygen species accumulation and inhibit the activation of the initiator caspase-2. This may contribute to evasion of apoptosis in cancer cells, as oncogenes such as Akt, Ras, and Bcr-Abl can promote glucose metabolism, as can loss or inactivation of the tumor suppressor p53

Mcl-1 appears to be mediated by PKC-dependent inhibition of GSK3, preventing its degradation in the proteasome, as described earlier **(26)**. Increased glucose has been appreciated for some years to alter intracellular lipids in a way that can activate or affect the localization of PKCs **(56, 57)**. In this case, increased glucose uptake leads to altered PKC localization to promote the phosphorylation and inhibition of GSK3. Especially when considered in contrast to the finding that the loss of glucose metabolism leads to a loss of Mcl-1 **(39)**, this nutrient-regulated signaling pathway represents a direct mechanism by which the status of cellular glucose metabolism may impact the Bcl-2 family of proteins to adjust the threshold for apoptosis.

Another example of increased metabolism inhibiting cell death is the prevention of caspase-independent cell death (CICD) by the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). It is well known that caspases are the executioners of apoptosis, cleaving a variety of protein substrates leading to apoptotic cell death. If caspases are inhibited pharmacologically or by the expression of viral caspase inhibitors after mitochondrial disruption and cytochrome c release, however, some cells may still survive despite the mitochondrial damage. Survival, however, depends on the upregulation of GAPDH. This role for GAPDH was found in a screen to identify genes capable of preventing CICD in Jurkat cells **(58)**. While GAPDH's day job in the glycolytic pathway producing ATP is an important part of its inhibition of CICD, GAPDH also appears to moonlight in the nucleus as a transcriptional activator of autophagy. Importantly, both glycolytic and autophagy-inducing functions of GAPDH are necessary to prevent CICD, possibly for energy production after mitochondrial disruption, and to stimulate the autophagic clearance of damaged mitochondria from the cytoplasm.

Regulation of Metabolism by Apoptotic Proteins

In addition to the metabolic regulation of apoptotic proteins, some apoptotic proteins can, in turn, regulate metabolism. Given the shared platform of the mitochondria, this reciprocal regulation is not altogether surprising. The best-described example is the metabolic function of the proapoptotic BH3-only protein Bad. The apoptotic functions of Bad are regulated through phosphorylation by kinases including PKA and Akt. When cells are withdrawn from growth factors, Bad is dephosphorylated and localizes to the mitochondria, where it binds Bcl-2 and Bcl-xL to promote apoptosis. It is now clear, however, that Bad can reside in a large protein complex containing glucokinase, also known as hexokinase IV **(59)**. When associated with this complex, phosphorylated Bad promotes glucokinase activity, thus promoting glucose uptake and metabolism. When Bad becomes dephosphorylated, or when Bad is deleted genetically, glucokinase activity decreases and cells become deficient in their ability to maintain glucose homeostasis. Importantly, the involvement of Bad in

this complex has now been shown to play a role in regulating glucose-stimulated insulin secretion in pancreatic β cells, expanding Bad's role in the regulation of metabolism from the cellular level to that of an entire organism **(60)**. It will be interesting to see whether other BH3-only proteins regulate metabolism in a similar manner, and whether cell-permeable BH3 peptides may provide therapeutic options in the treatment of diabetes, as has been suggested **(60)**.

It has also been shown that during their execution of apoptosis, active caspases can inhibit cell metabolism by acting at the mitochondria. After integration of apoptotic stimuli and MOMP, caspase activation leads to the cleavage of a wide variety of intracellular proteins that cause apoptotic cell morphology and ensure that cell death remains immunologically silent. One such caspase substrate is the p75 subunit of Complex I in the electron transport chain **(61)**. Thus, after MOMP, caspase-3 can return to the mitochondria to disrupt the electron transport chain, leading to enhanced reactive oxygen species (ROS) production that may function as a feedback activation loop enhancing the apoptotic cascade **(62)**. While the expression of a noncleavable p75 mutant was not able to prevent cell death, it did maintain ATP levels and reduce ROS after mitochondrial membrane permeabilization **(61)**. The importance of this pathway in regulating mitochondrial function in response to apoptotic stimuli is not entirely certain but may be critical to ensure that damaged mitochondria are fully disabled to prevent cells from inappropriately recovering from apoptotic stimuli.

New Metabolic Roles for Old Proteins: p53

The tumor suppressor p53 has long been known as a key regulator of the cell cycle and apoptosis, and recent work has identified p53 target genes that function in metabolic pathways that may contribute to p53's ability to regulate cell death **(63, 64)**. Under low levels of DNA damage or oncogenic cell stress, p53 is induced and causes cell cycle arrest, allowing the cell a chance to repair itself before reentering the cell cycle. If the levels of stress are too great for the cell to overcome, however, p53 promotes transcription of several proapoptotic proteins including the Bcl-2 family members Bax and Puma to induce apoptosis **(65, 66)**.

In addition to the regulation of Bcl-2 family proteins that may affect cell death, p53 has also been shown to control the expression of genes that can regulate cell metabolism to impact cell survival. An example of a newly discovered metabolic function of p53 is its transcriptional regulation of the synthesis of the cytochrome c oxidase 2 (SCO2) gene **(63)**. SCO2 is a key member of the cytochrome c oxidase (COX) system, which is involved in mitochondrial oxidative phosphorylation and oxygen consumption in the mitochondria. Disruption of the p53-dependent transcription of SCO2, such as that which occurs in p53-deficient cells, decreases electron transport and causes cells to switch to a

highly glycolytic metabolic phenotype, which may initiate the antiapoptotic nutrient signaling pathway described above. p53 also regulates TIGAR (tp53-induced glycolysis and apoptosis regulator) under low levels of cell stress **(64)**. TIGAR's amino acid sequence shows homology to the bisphosphatase domain of the dual-function enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2), which functions to regulate glycolytic flux. In a manner opposite that previously discussed for the Akt-dependent regulation of PFK2, TIGAR reduces glycolytic flux by allosterically inhibiting PFK1. This suppression of glycolysis can increase glucose availability for the pentose phosphate pathway (PPP), which, as previously described, can have antiapoptotic effects via the neutralization of ROS. While much remains unknown about TIGAR's role in regulating apoptosis, it appears to be one example of p53's ability to alter metabolism in response to low levels of cell stress as a cell-protective mechanism. Further research on the role of TIGAR as a p53 target will likely advance our understanding of how metabolic pathways can affect cell survival.

The Warburg Effect and Cancer: Bringing It All Together

Studies on the biology of cancer have provided a clear example of how metabolism and cell death may intersect to affect a human disease. Otto Warburg conducted a series of seminal experiments in the 1920s investigating the metabolic properties of cancer cells in which he observed that cancer cells have greatly increased rates of glucose uptake, glycolysis, and lactate production, even in the presence of oxygen, relative to their normal counterparts **(67)**. Since Warburg's discovery, this metabolic phenotype of aerobic glycolysis has been repeatedly observed in many types of cancer. Recently, this metabolic phenotype has been taken advantage of in the clinic to image tumors in human patients via ^{18}F-2-deoxyglucose positron emission tomography (PET) imaging. In this clinical test, radioactively labeled glucose tracers are used to identify cells with high glucose uptake, such as cancer cells.

The factors that lead to aerobic glycolysis in cancer are now becoming apparent. One such cause of this metabolic phenotype is hypoxia in solid tumors and activation of the HIF transcription factor, which induces a variety of glycolytic genes **(33)**. In addition to hypoxia, however, oncogenes and tumor suppressors have been shown to directly promote aerobic glycolysis. In particular, the PI3K/Akt pathway appears to play a prominent role in the development of this metabolic phenotype **(68)**. The PI3K/Akt pathway is frequently activated in cancer due to mutation, amplification of PI3K subunits, or loss of the tumor suppressor and lipid phosphatase PTEN, and has been shown to be sufficient to promote aerobic glycolysis in cancer cells **(69)**. Other oncogenes, including Ras and Bcl-Abl, have also been shown to promote aerobic glycolysis **(70)** and surface trafficking of the Glut1 glucose transporter **(71, 72)**. It is

possible, however, that the metabolic effects of these oncogenes can be attributed to activation of PI3K/Akt, since their ability to activate PI3K is required for their oncogenecity in certain circumstances **(73**, **74)**. In addition, the loss of p53 expression or activity in many cancers may also promote aerobic glycolysis due to loss of the p53-dependent expression of SCO2 **(63)**, which would likely reduce mitochondrial respiration and further force a highly glycolytic phenotype in cancer cells.

If the activation of PI3K/Akt and/or the loss of p53 is responsible for the cancer metabolic phenotype, the Warburg effect may play a prominent role in the ability of cancer cells to evade apoptosis. Increased activation of Akt can promote glucose uptake and may bypass normal mechanisms that would lead to nutrient limitation in cancer. The increased glycolytic flux and decreased mitochondrial metabolism may then inhibit apoptotic cell death induced by a variety of cell stresses due to direct regulation of Bcl-2 family members **(53)**. The metabolic and cell survival effects of Akt are directly linked, as cells with active Akt require glucose metabolism to promote cell survival **(13**, **17**, **29)** and are unable to efficiently utilize other fuel sources **(18**, **19)** or activate autophagy when glucose is limiting **(75)**. Cancer cells may therefore suffer from a metabolic addiction, in which they not only require glucose to generate ATP and macromolecular precursors to support their rapidly proliferating lifestyle, but also in order to evade apoptotic death under the many stressful conditions that they encounter throughout the body. Thus, a promising area for future cancer therapies may be to identify pathways and targets for metabolic inhibition. Ultimately, metabolic changes induced by a variety of stimuli, such as those that occur during lymphocyte activation or oncogenesis, demonstrate the impact that metabolism can have on cell death pathways and represent an important area for future study.

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Chapter 10
Transcriptional Regulation of Apoptosis

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Abstract Apoptosis is tightly regulated at the transcriptional level through a number of transcription factors. In response to different stimuli, these transcription factors bind to specific DNA sequences in the promoters of the genes that are involved in apoptosis regulation to stimulate or suppress their expression, leading to apoptosis initiation. Transcriptional regulation of apoptosis is essential for tissue homeostasis and normal development and serves as a barrier against tumorigenesis.

Keywords Apoptosis · Transcription factors · Bcl-2 family · Death receptors

Introduction

Apoptotic cell death is tightly regulated at the protein level through several different mechanisms, such as direct protein-protein interactions (e.g., interactions among the Bcl-2 family members), subcellular distribution (e.g., cytochrome c release), proteolytic cleavage (e.g., caspase activation), and posttranslational modifications (e.g., Bad phosphorylation). On the other hand, the core apoptotic machinery, including Bcl-2 family members, death receptors and ligands, caspases and other genes, are also regulated at the mRNA level. The expression of these genes collectively determines the balance between proapoptotic and antiapoptotic activities and makes the ultimate decision of whether a cell will live or die. The transcriptional regulation of apoptosis is important in tissue homeostasis and development, and its deregulation contributes to pathogenesis in many human diseases.

Transcription factors are the central players in the regulation of apoptosis. In response to different apoptotic stimuli, they are activated and bind to specific

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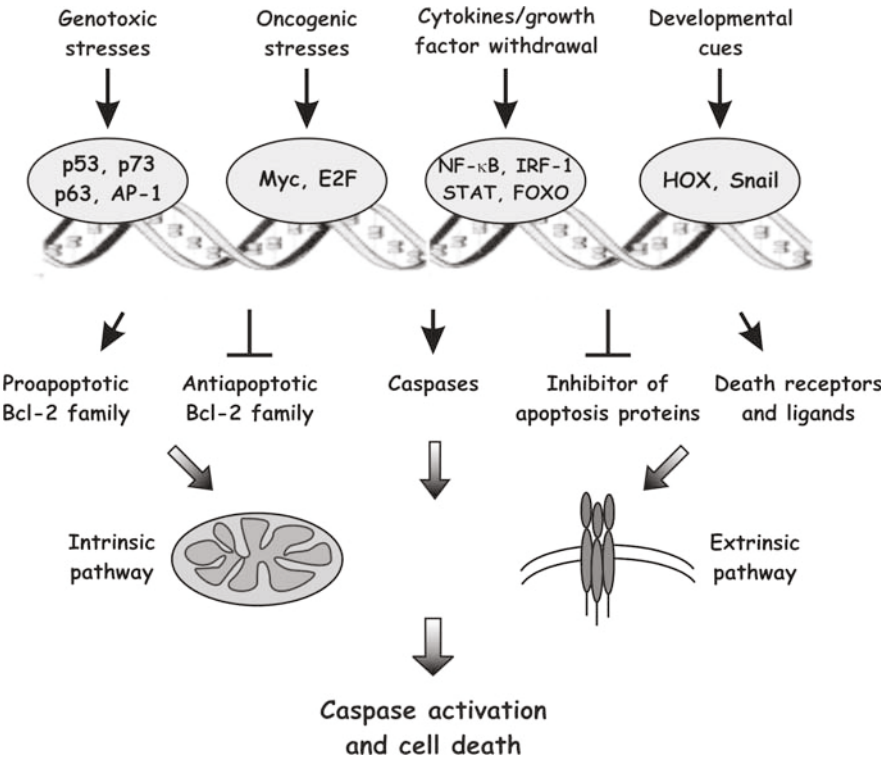

Fig. 10.1 Transcriptional regulation of apoptosis. Different stimuli activate various transcription factors leading to the activation or repression of many genes involved in the induction of apoptosis. These include proteins involved in the mitochondrial, or intrinsic, pathway and the death receptor, or extrinsic, pathway. Caspases can also be activated directly by these transcription factors, or as a result of activation of both pathways, leading to cell death

DNA sequences in the promoters of their target genes to promote or inhibit their expression (Fig. 10.1). For example, DNA damage leads to the stabilization of p53, which in turn activates the expression of many proapoptotic genes, leading to cell death under certain conditions. The aberrant expression of oncogenes, such as c-Myc and E2F1, induces apoptosis. Proinflammatory cytokines or growth factors modulate apoptosis through the actions of NF-κB, IRF, STAT, or the FOXO family of transcription factors. The HOX and Snail families of proteins are critical regulators of developmental apoptosis. Aberrations in some of these transcription factors, such as the inactivation of p53 through genetic alterations or the overexpression of c-Myc, play a critical role in tumor initiation or progression.

The transcriptional regulation of a gene involves highly complicated networks. The expression of a single gene can be controlled by many different transcription factors, and a single transcription factor can regulate the expression

of hundreds of genes. It used to be very difficult to pinpoint how apoptosis is regulated at the transcriptional level. The advent of powerful techniques such as DNA microarray and serial analysis of gene expression (SAGE) has made it possible to systematically identify most, if not all, downstream targets of a particular transcription factor. Methods such as gene targeting, RNA interference, and chromatin immunoprecipitation (ChIP) have further allowed unambiguous determinations of the roles of transcription factors and their downstream targets in apoptosis regulation.

This chapter summarizes recent knowledge of two major apoptotic pathways that rely on differential gene expression for their regulation, focusing on the transcriptional factors and their downstream targets (Table 10.1).

Regulation of Genotoxic Stress-Induced Apoptosis by the p53 Family and AP-1

Apoptotic Response to Genotoxic Stress

The cells in the human body continuously encounter DNA damage generated from both endogenous and environmental sources. Though mild DNA damage can be repaired, severe DNA damage often results in apoptosis, especially in rapidly dividing tissues. The proper repair of DNA damage and the removal of cells containing permanently damaged DNA are believed to be important mechanisms of tumor suppression. DNA damage leads to the activation of a number of transcriptional factors, and the p53 and Activator Protein 1 (AP-1) families are among the best studied.

The p53 Family Proteins

The p53 family of proteins, including p53, p73, and p63, are sequence-specific transcription factors **(1**, **2)**. Albeit with different affinities, all three proteins can bind to the same p53-responsive elements [p53 RE, two copies of a 10-bp motif 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3', separated by a 0- to13-bp spacer]. p73 and p63 are more structurally related compared to p53, and they can bind to DNA sequences somewhat different from the p53 RE, which might explain their target specificities. The p53 family of proteins can also function as transcriptional repressors, but the underlying mechanisms are not well understood.

Apoptosis Mediated by p53

p53 is the founding member of the p53 family of proteins. *p53* mutations are frequently found in human tumors and in the germ line of Li-Fraumeni cancer

Table 10.1 Transcription factors and their downstream targets in apoptosis regulation

Apoptoic stimuli	Transcription factors	Downstream targets	
		Direct	Indirect
Genotoxic stresses	p53	PUMA, Noxa, Bid, Bax, (Bcl-2), (Bcl-xL), Apaf-1, HTR2A, Fas, DR4, DR5, PIDD, REDD1, PERP, Slug, p53AIP1, p53DINP1, Pw1/Peg3, PIG3, PIG8/Ei24, FDRX, Scotin, cathepsin D, and miR34	caspase-1, caspase-6, caspase-8, and PIG 6
	p73	PUMA, Noxa, Bax, p53AIP1, Scotin, FDRX, and DAN	
	p63	FasL and REDD1	
	AP-1	Fas, FasL, (c-FLIP), and Bcl-3	Bim, p53, and p73
Oncogenic stresses	Myc	PUMA, Bax, (Bcl-2), (Bcl-xL), FasL, caspase-8, p53, and E2F1	Bim, Arf, (A1), and (NF-kB)
	E2F	Bim, Noxa, PUMA, Hrk/DP5, (Bcl-2), (Mcl-1), Apaf-1, caspase-2, caspase-3, caspase-7, caspase-8, caspase-9, JMY, ASPP1, ASPP2, p73, SIVA, and Arf	TRAF2, DIP, Galectin-1, and MAP3K5
Cytokines and growth factor withdrawal	NF-κB	Bfl-1, Bcl-2, Bcl-xL, Fas, and FasL	c-FLIP, DR4, DR5, XIAP, cIAP-1, cIAP-2, and DcR1
	IRF-1	TRAIL, caspase-1, and caspase-8	FADD, (survivin), and caspase-7
	STAT	Bid, (Bcl-2), Bcl-xL, survivin, Fas, FasL, caspase-1, and caspase-8	(Bax), Mcl-1, c-FLIP, FLICE, TRAIL, and (caspase-3)
	FOXO	PUMA, FasL, TRAIL, TRADD, and Arf	Bim and (Bcl-xL)
Embryonic development	HOX	Bcl-2, Reaper, p53, c-Myc, and Zac 1	Egl-1 and FLASH
	Snail	(PUMA) and (Egl-1)	(Noxa), (Slurp1), (caspase-2), (caspase-3), (caspase-6), (caspase-7), (caspase-9), and XR11

Targets in ( ) are suppressed by transcription factors.

syndrome patients **(3)**. p53 is required for DNA damage-induced apoptosis in many tissue and cell types such as thymocytes, intestinal progenitors, mouse embryonic fibroblasts (MEFs), and some tumor cells. The overexpression of p53 induces apoptosis in various cell types. The transcriptional activation by p53 appears to be essential for its proapoptotic function *in vitro* and *in vivo*. In response to genotoxic stress, p53 undergoes extensive posttranslational modifications, including phosphorylation and acetylation, which stabilize p53 and enhance its transcriptional activities **(3, 4)**. Though genes involved in virtually every step of apoptosis can be regulated by p53, only a small number of them have been shown to be required for p53-dependent apoptosis **(5)**.

Convincing evidence has demonstrated that p53 mediates DNA damage-induced apoptosis through the intrinsic pathway, particularly through the BH3-only proteins PUMA and Noxa. PUMA and Noxa are activated in a p53-dependent manner following DNA damage **(6–8)**. Much like p53, PUMA is required for DNA damage-induced apoptosis in several tissues, including thymocytes, MEFs, and developing neurons **(5)**. *Noxa*-knockout mice exhibited reduced apoptosis following DNA damage in some cells, albeit at a much lesser extent **(5)**. Interestingly, p53 can directly activate the transcriptional repressor Slug to suppress *PUMA* and prevent radiation-induced apoptosis in hematopoietic stem cells **(9)**. Other proapoptotic Bcl-2 family members, such as Bax and Bid, can also be activated by p53. However, their induction following DNA damage is not strictly dependent on p53 **(10, 11)**.

Several other p53 targets exert their effects on the mitochondria or Bcl-2 family proteins, including the developmentally regulated gene *Pw1/Peg3* **(12)**, the mitochondrial protein p53AIP1, and p53DINP1 **(13, 14)**. In some cells, p53 can suppress the expression of antiapoptotic Bcl-2 family proteins, such as Bcl-2 and Bcl-xL **(15, 16)**. p53 can also facilitate the execution step in apoptosis by enhancing the expression of several genes, including the adapter protein *Apaf-1*, which is critical for the assembly of active apoptosome **(17)**, *caspases-1*,*-6*, *-8*, and the proapoptotic serine protease *HTRA2*, which is capable of inhibiting inhibitor-of-apoptosis proteins (IAPs) **(18)**.

On the other hand, the transcriptional regulation of the extrinsic pathway by p53 appears to augment rather than mediate p53-dependent apoptosis. p53 engages the death receptor pathways by activating the expression of *Fas*, *DR4*, and *DR5* **(19–21)**. The death domain containing protein PIDD and the PMP-22/gas family protein PERP contribute modestly to p53-dependent apoptosis induced by γ-irradiation in thymocytes or neurons **(22, 23)**. However, the induction of these genes following DNA damage generally does not require p53.

Several p53 targets can modulate apoptosis without directly affecting the core machinery of apoptosis. For example, several redox proteins involved in ROS generation are regulated by p53, including p53-induced gene (PIG)3, PIG6, ferredoxin reductase (FDRX), and REDD1 **(24–27)**. p53 activates the expression of Ei24/PIG 8 **(28)**, lysosomal proteinase cathepsin D **(29)**, and an ER and nuclear membrane protein Scotin **(30)**. These genes are generally not required for, but enhance, p53-dependent apoptosis following DNA damage.

Several groups recently showed that a family of noncoding small RNAs, namely microRNA 34 (miR34a, b, and c), is induced by DNA damage in a p53-dependent manner via the p53 REs, and the inhibition of Mir-34a suppressed DNA damage-induced apoptosis in MEF or embryonic stem (ES) cells **(31)**. The proapoptotic targets of miR34 have yet to be identified.

Apoptotic Targets of p73 and p63

Unlike p53, p63 and p73 encode several different transcripts through the use of alternative promoters or alternative splicing, resulting in the transcriptionally competent proteins TAp73 and TAp63 and the inhibitory proteins ΔNp73 and ΔNp63. The overexpression of TAp73 and TAp63 can induce apoptosis in various cell types. p63 and p73 play major roles in the development of neuronal tissues, epithelial stem cell renewal, and epithelial homeostasis **(1**, **2)**. They also appear to selectively enhance the activation of proapoptotic p53 targets in some cells following DNA damage **(32)**.

p73 is induced by a subset of DNA-damaging agents and is involved in sensitivity to the alkylating agent cisplatin **(1**, **2)**. Posttranslational modifications of p73, such as phosphorylation and acetylation, can be induced by DNA damage and enhance p73-induced apoptosis **(33)**. p73 shares several proapoptotic targets with p53, including *PUMA*, *Noxa* **(34)**, *Scotin* **(35)**, *p53AIP1*, *FDRX*, and *BAX* **(35)**, suggesting that the p73 transcriptional program can promote apoptosis through the intrinsic pathway. The secreted protein DAN appears to be a p73-specific target and is involved in retinoic acid- or cisplatin-induced apoptosis in osteoblasts **(36)**.

p63 deficiency was found to abrogate radiation-induced apoptosis in mouse oocytes **(37)**. However, little is known about how p63 is regulated by DNA damage. One study showed that chemotherapeutic agents can induce p63 in hepatocellular carcinoma cells and identified a number of putative p63 targets, including *caspases-1*, *-3*, *-4*, *-5*, *-8,* and *-9*, *CD95*/*FasL*, *TNF-R1*, *Trail-R1*/*DR4*, *Trail-R2*/*DR5*, *BAX*, *Bim*, *Rad9*, *DAP3*, and *Apaf-1* **(38)**. In another study, *Bik*, *PIG11*, *PIG 12*, *PIG1*, *PUMA*, *HMDM2*, *PIG6*, and *Apaf-1* were found to be induced by TAp63 **(39)**. *REDD1* was identified as a proapoptotic target of p63 involved in ROS generation in a separate study **(27)**. Many of these genes have been shown to be regulated by p53 or p73, but it is not clear whether any are required for p63-mediated apoptosis and whether p63 binds to their promoters following DNA damage *in vivo*.

AP-1 Family

The AP-1 family consists of several subfamilies of dimeric basic region-leucine zipper (bZIP) transcription factors, including Jun (c-Jun, JunB, JunD), Fos

(c-Fos, FosB, Fra-1, and Fra-2), Maf (c-Maf, MafB, MafA, MafG/F/K, and Nrl) and ATF (ATF2, LRF1/ATF3, B-ATF, JDP1, and JDP2) **(40)**. All of these proteins are capable of binding to either 12-O-tetradecanoylphorbol-13-Acetate (TPA) response elements (TRE, 5'-TGAG/CTCA-3') or cAMP response element (CRE, 5'-TGACGTCA-3'). The best-characterized AP-1 transcription factor is the heterodimeric complex of c-Jun and c-Fos, which regulates diverse cellular processes, including apoptosis **(40, 41)**.

AP-1 in Apoptosis Regulation

Certain genotoxic stresses, such as short-wavelength UV irradiation and alkylating agents, can upregulate AP-1 **(41)**. The overexpression of c-Jun or c-Fos induces apoptosis in various cell lines **(40, 41)**. MEFs and monoblastic leukemia cells expressing a dominant-negative c-Jun are resistant to apoptosis induced by UV or other genotoxic agents. Several kinases such as JNK1/JNK2 and Polo-like kinase 3 (Plk3) have been implicated in regulating AP-1 activities following genotoxic stresses. The inhibition of c-Jun phosphorylation mediated by these kinases impaired UV-induced apoptosis **(41)**.

Proapoptotic AP-1 targets include those involved in both the intrinsic and extrinsic pathways. c-Jun and c-Fos can directly activate the *FasL* gene through an AP-1 binding site in its promoter **(42)**. *FasL* induction by UV and alkylating agents was reduced in c-Jun-deficient MEFs **(43)**. c-Fos can directly bind to the promoter of the antiapoptotic gene *c-FLIP* to repress its expression, which is involved in TRAIL-induced apoptosis in prostate cancer cells **(44)**. c-Jun also activates the expression of the BH3-only protein Bim in response to neural growth factor withdrawal **(45)**. On the other hand, c-Jun can promote apoptosis indirectly by inhibiting the p53-dependent activation of p21 **(46)**, or by upregulating p73 levels following cisplatin treatment **(47)**.

Under certain circumstances, AP-1 appears to have antiapoptotic functions. Melanoma cells expressing dominant-negative c-Jun are sensitized to UV-induced apoptosis with elevated level of *Fas*, whose promoter contains an AP-1 binding site. Cells deficient in c-Jun exhibited an increased Fas cell surface expression and a higher sensitivity to FasL-mediated apoptosis **(48)**. AP-1 was also found to inhibit apoptosis through the induction of the antiapoptotic Bcl-2 family protein Bcl-3 in T cells **(49)**.

Other Transcription Factors in Genotoxic Stress-Induced Apoptosis

Genotoxic stress can activate other transcription factors, such as NF-κB **(50)** and E2F-1 **(51)**. Elevated E2F-1 expression induces chemosensitization in some cancer cells, but how it regulates apoptosis is still poorly understood. NF-κB overexpression is generally linked to chemoresistance through the induction of

various antiapoptotic proteins **(52, 53)**. Some of their targets are discussed in later sections of this chapter.

Regulation of Oncogenic Stress-Induced Apoptosis by E2F and MYC

Oncogenic Stress-Induced Apoptosis

A major step in cellular transformation leading to cancer is the activation of oncogenes that promote cellular proliferation. In normal cells, the aberrant expression of oncogenes represents a stress condition that often triggers cellular responses such as senescence or apoptosis. For example, the activation of the oncogenes *Ras* and *cyclin D1* leads to p53-dependent responses via Arf induction. The elimination of cells with oncogenic stress by apoptosis is an effective safeguard mechanism against oncogenic transformation. Two classical oncogenes, *E2F* and *Myc*, are often involved in oncogenic stress-induced apoptosis. Both are transcription factors with a variety of downstream targets, including those involved in cell cycle progression and apoptosis. The activation of these oncogenes through mechanisms such as the inactivation of the E2F inhibitor pRb and the genomic amplification of *c-Myc* are frequently observed in tumor cells.

Apoptosis Mediated by E2F

E2F transcription factors, including E2F 1, 2, and 3, lead a dual life. Under a nonstressed state, E2F proteins are needed for cell cycle progression. Once its inhibitory binding partner, pRb, becomes hyperphosphorylated, E2F can activate the transcription of many genes needed for cells to initiate the S phase. On the other hand, E2F can be activated by oncogenic or genotoxic stress, leading to apoptosis **(54)**. The overexpression of E2F family members alone in cultured cells or transgenic mice can cause apoptosis [reviewed in **(55)**]. E2F1 overexpression also sensitized cells to apoptosis induced by chemotherapeutic drugs or γ-irradiation **(55)**. *E2F1*-knockout mice have increased tumor incidence and are more resistant to a variety of apoptotic stimuli **(56)**. However, apoptotic response to several DNA-damaging agents, including adriamycin, UV, and γ-irradiation, is intact in E2F-deficient cells **(56)**, indicating that the apoptotic effect of E2F is cell type- and stimuli-specific.

The proapoptotic activities of E2F can be modulated by posttranslational modifications. For example, E2F1 can be phosphorylated by ATM, ATR, or Chk2 in response to stress, leading to increased protein stability and activation **(54)**. Acetylation appears to be the key to regulating E2F's functional duality: Nonacetylated forms lead to cellular survival after certain stresses, whereas

acetylated E2F mediates apoptosis induction **(57)**. Interestingly, apoptosis induced by E2F3 was found to be dependent upon E2F1, suggesting a hierarchal relationship among E2F family members in apoptosis induction **(54)**.

E2F functions upstream of other transcription factors, in particular the p53 family, to induce apoptosis in some cells by the upregulation of the p53 activator Arf. However, the role of Arf in E2F-mediated apoptosis is controversial, as other studies have shown that p53-mediated apoptosis in response to E2F1 overexpression or Rb inactivation does not require Arf **(54, 56)**. An alternative mechanism is that E2F1 transactivates proapoptotic co-factors for p53, such as JMY, a junction-mediating and regulatory protein, ASPP1, and ASPP2 **(55)**. In addition to p53, p73 is also involved in E2F-mediated apoptosis. The regulation of *p73* expression by direct binding of E2F1 to its promoter was found to be dependent upon E2F1 acetylation **(56)**. The knockdown or dominant-negative forms of p73 significantly inhibited E2F1-induced apoptosis **(55)**.

E2F can also induce p53-independent apoptosis in cultured cells or mice. Most strikingly, the loss of apoptosis in *p53*-knockout cells was found to rely on pRb binding to E2F1 **(55)**. Several less-characterized proteins are implicated in E2F-mediated and p53-independent apoptosis. DIP, a mitochondrial protein induced by E2F1, can promote apoptosis through a partially caspase-independent mechanism **(58)**. DIP suppression resulted in increased cell survival following E2F1 activation. Galectin-1 is another E2F1 target, and its knockdown inhibited E2F1-mediated apoptosis **(59)**. The kinase MAP3K5/ASK1 has also been implicated in apoptosis in response to E2F overexpression **(60)**. However, additional evidence is necessary to verify the functional roles of these genes in E2F1-mediated apoptosis.

E2F can directly activate a number of genes involved in the intrinsic apoptotic pathway, such as *Apaf-1* and *caspases-2*, *-3*, *-7*, and *-9* **(61, 62)**. Recent studies showed that E2F1 can bind the promoters of *Noxa*, *PUMA*, *Bim*, and *HRK/DP5* to induce the transcription of these BH3-only proteins **(63)**. The knockdown of *Noxa* or *PUMA* impaired E2F1-mediated apoptosis, while suppressing E2F1 decreased DNA damage-induced *PUMA* expression. Some studies showed that the ability for E2F to induce apoptosis depended on its DNA binding domain, but not its transactivation domain **(55)**, suggesting that transcriptional repression by E2F is also involved in apoptosis. This is supported by the observation that E2F suppresses several antiapoptotic genes, including *Bcl-2* and *Mcl-1* **(64, 65)**. This repression depends upon the DNA binding domain of E2F1 and was found to be independent of p53.

E2F can also induce the expression of a few genes in the extrinsic apoptotic pathway. One of these is *caspase-8* **(62)**. By turning on caspase-8, E2F1 can sensitize cells to apoptosis following treatment of TNF-α and other death-inducing ligands. SIVA, a proapoptotic protein expressed in a variety of cells, can be induced by E2F and interact with members of the TNF receptor family and Bcl-2 family members, therefore linking the intrinsic and extrinsic pathways **(66)**. E2F can also inhibit TRAF2, leading to the sensitization of cells to apoptosis by inhibiting NF-κB signaling **(67)**.

Apoptosis Induced by Myc

Similar to E2F, Myc is necessary for cell cycle progression under normal conditions, but initiates apoptosis when aberrantly expressed. Myc overexpression has been detected in approximately 70% of human tumors **(68)**. Myc is a basic helix-loop-helix leucine-zipper (bHLH-zip) transcription factor that binds to E-box CAYGTG motifs in the promoters of many genes. Myc forms a heterodimer with Max, another bHLH-zip protein to activate transcription **(68)**. Myc/Max can also repress transcription by binding and disrupting other transcriptional activators, such as Miz-1. Whether Myc induces apoptosis via the activation or repression of transcription remains unclear, as attempts in making a Myc mutant defective in transactivation have been unsuccessful **(68)**. Over 1,000 Myc target genes have been identified by a number of different methods (listed in http://www.myc-cancer-gene.org), showing the Herculean task of determining which are bona fide genes regulated by Myc. ChIP analysis has revealed even larger numbers of potential targets. While most studies rely on Myc overexpression, a small handful using cells lacking c-Myc have determined if Myc is essential for their expression **(68)**.

Similar to E2F, Myc can induce *Arf* expression, thereby activating the p53 pathway **(68)**. The importance of both Arf and p53 has been demonstrated *in vivo* using a mouse model for Myc-induced apoptosis, the Eμ-myc transgenic mouse. The knockdown of *Arf* and *p53* in this model led to a reduction in Myc-induced apoptosis **(69)**. The *p53* promoter contains an E-box, which may mediate a direct effect of Myc on p53 transcription. Studies have shown that the indirect induction of p53 in response to Myc activation is caused by the transcriptional upregulation of *Arf*, which relieves the inhibition of Mdm2 inhibition on p53 **(68)**. How *Arf* is upregulated by Myc still remains a mystery. Some studies have suggested that Myc relieves the repression on *Arf* transcription by other transcription factors, such as Bmi-1, Twist, Tbx2, and Tbx3 **(68)**. Myc can also induce E2F1, which can regulate *Arf* expression **(70)**.

Myc transcriptionally activates a number of genes involved in the intrinsic pathway. Bax is a major regulator of Myc-induced apoptosis **(71)**. E-box domains were located in the *BAX* promoter and were shown to directly bound by c-Myc/Max heterodimers. The *BAX* promoter could not be activated in the Myc-null cells. Another activator of Myc-induced apoptosis may be *PUMA* **(72)**, whose promoter was shown to contain a binding site for Myc via ChIP analysis **(73)**. However, this may be dependent upon cell type, as it was only seen in human cells but not in MEFs. The third proapoptotic gene suggested to be regulated by Myc is *Bim*. Using the Eμ-myc transgenic mouse model, *Bim* was found to be induced by Myc **(74)**. The loss of *Bim* resulted in reduced apoptosis following cytokine deprivation or antigen receptor cross-linking in lymphocytes.

Myc has also been suggested to repress antiapoptotic genes in the intrinsic pathway, such as *Bcl-2* and *Bcl-xL* **(64, 75)**. In studies using Eμ-myc transgenic cells, primary hematopoietic cells, and mouse fibroblasts, Myc was shown to

reduce levels of these two genes. How Myc represses these genes is still uncertain, although evidence using promoter-reporter assays suggests that Myc can directly inhibit the transcription of *Bcl-xL* **(68)**. But it is certain that these two targets of Myc are important, as the Bcl-2/Bcl-xL pathway is frequently disrupted in lymphomas from Eμ-myc transgenic mice **(76)**.

The activation of the extrinsic pathway by Myc has also been demonstrated *in vitro*. The activation of Myc can sensitize cells to TNF-α- or Fas-induced death **(77, 78)**. This may be due to the binding of Myc to the *FasL* promoter. The overexpression of Myc was shown to induce FasL expression, and the loss of Myc and Max inhibited expression from its promoter **(79, 80)**. An additional target of Myc that could sensitize the cells to TNF-α is NF-κB. The expression of Myc was shown to interfere with RelA/p65 transcription but not the nuclear translocation of NF-κB. The activation of Myc reduced the expression of NF-κB-inducible gene *A1*, while restoring *A1* induction exogenously suppressed Myc-induced TNF-α sensitization **(81)**. Thus, Myc was able to inhibit NF-κB, leading to an indirect reduction of the antiapoptotic Bcl-2 family member A1.

Regulation of Cytokine- and Growth Factor Withdrawal-Induced Apoptosis by NF-κB, IRF-1, STAT, and FOXO

Cytokine- and Growth Factor Withdrawal-Induced Apoptosis

Cytokines are soluble hormone-like proteins that mediate signaling between cells and act via cell surface cytokine receptors. Cytokines allow the communication and coordination among different immune cells, or between immune cells and other types of cells in the body, which ensures the timely activation or elimination of the host defense. Cytokines can activate a number of transcription factors to promote the survival or apoptosis of B or T cells. Growth factor withdrawal triggers apoptosis in multiple tissues via irreversible damage to the mitochondria and a crisis in energy metabolism. The most extensively studied transcription factors involved in cytokine- or growth factor withdrawal-mediated apoptosis include those in the NF-κB, STAT, IRF, and FOXO families.

NF-κB and Apoptosis

NF-κB is a dimeric transcription factor complex formed by members of a highly conserved family of proteins that share the Rel homology domain (RHD). In mammals, there are five members of this family: RelA, RelB, c-Rel, NF-κB1, and NF-κB2 **(82)**. In unstimulated cells, NF-κB complexes are usually sequestered in the cytoplasm through their interaction with members of the inhibitor-of-κB (IκB) family. Upon stimulation by proinflammatory cytokines, such as TNF-α

and interleukin-1β, IκB is phosphorylated by IκB kinase, which results in the ubiquitination and proteasomal degradation of IκB, allowing the nuclear translocation of NF-κB complexes. Nuclear NF-κB dimers in turn bind to promoters, thus activating or suppressing target gene transcription **(82)**.

A large body of evidence has demonstrated an antiapoptotic role of NF-κB **(83)**. For example, NF-κB is the major cellular factor responsible for resistance to TNF-α-induced cell death *in vitro* and *in vivo* **(82)**. NF-κB can regulate the expression of several antiapoptotic proteins involved in the intrinsic pathway of apoptosis, such as the antiapoptotic *Bcl-2* family members *Bcl-2*, *Bcl-xL*, and *Bfl-1* **(84–86)**. NF-κB can also induce the expression of inhibitor-of-apoptosis proteins (IAPs) including XIAP, cIAP1, and cIAP2, which bind to and inhibit caspases **(87, 88)**. The regulation of these proteins by NF-κB is tissue-specific **(82)**.

NF-κB can also exert its antiapoptotic function through the extrinsic pathway. DcR1, a receptor for TNF-related apoptosis-inducing ligand (TRAIL), was identified as a transcriptional target of NF-κB and antagonizes TRAIL-mediated apoptosis **(89)**. c-FLIP, which is a caspase-8 inhibitor, can be upregulated by NF-κB to promote resistance to FasL or TNF **(90)**. The restoration of c-FLIP expression in NF-κB-null cells inhibited TNF- or FasL-induced cell death **(90)**.

NF-κB can also promote apoptosis under certain conditions by activating the expression of proapoptotic genes. For example, DR4 and DR5 can be regulated by the c-Rel subunit of NF-κB **(91)**. Both the dominant-negative mutant of the inhibitory protein IκBα and the transactivation-deficient mutant of c-Rel can reduce the expression of these death receptors **(91)**. However, it is unclear whether DR4 and DR5 are direct transcriptional targets of NF-κB.

Apoptosis Mediated by IRF-1

The family of interferon regulatory factor (IRF) transcription factors is important in the regulation of interferon-inducible genes in response to viral infections. IRF consists of at least nine members identified in humans and mice. Among the IRF members, IRF-1 appears to play a key role in activating the expression of a variety of genes in response to stimuli such as IFN-α, -β, and -γ, prolactin, TNF, leukemia inhibitory factor (LIF), interleukin-1 (IL-1), and IL-6 **(92)**. The antiproliferative activities of IRF-1 *in vivo* and *in vitro* can be attributed in part to apoptosis induction, while the inactivation of IRF-1 accelerates transformation.

The induction of caspases is implicated in IRF-1-mediated apoptosis. Several studies showed that the transcriptional activation of *caspase-7* plays an important role in IRF-1-mediated apoptosis induced by IFN-β or -γ **(93, 94)**. *Caspase-1* can be directly activated by IRF-1 and the mutation of the IRF-1 binding site abolished IFN-γ-induced *caspase-1* promoter activity **(95)**. *Caspase-8* was identified as another direct target of IRF-1. Treatment with

IFN-γ in human breast tumor cells induces *caspase-8* expression through IRF-1-mediated transactivation **(96)**. IRF-1 can also promote apoptosis through the extrinsic pathway. For example, IRF-1 induced the expression of *Fas-associated death domain* (*FADD*), and cells expressing dominant-negative FADD are resistant to IRF-1-induced apoptosis **(97)**. *TRAIL* is another direct target of IRF-1, and the knockdown of *IRF-1* by siRNA inhibited IFN-α-induced TRAIL production and cell death **(98)**. ChIP analysis showed that IFN-α induces the direct, time-dependent binding of IRF-1 to the *TRAIL* promoter. Furthermore, the ectopic expression of IRF-1 resulted in the downregulation of the antiapoptotic protein survivin and p53-independent apoptosis **(99)**.

STAT and Apoptosis

The STAT family consists of seven members: STAT1-4, 5A, 5B, and 6, which can be activated by Janus protein kinases (JAKs). In response to cytokines, STAT is phosphorylated by JAK at tyrosine residues upon binding to the receptor, causing STAT to be released from the receptor and undergo homo- or heterodimerization, leading to nuclear translocation and binding to the promoters of target genes **(100)**. By modulating the expression of target genes, STAT regulates a broad range of biological processes, including cell growth, apoptosis, differentiation, survival, and development **(101)**.

STAT1 promotes apoptosis by regulating the genes involved in both the intrinsic and extrinsic pathways **(101)**. ChIP followed by microarray (ChIP– chip) analysis has identified *Bid* as a candidate target of STAT1 in interferon-induced apoptosis **(102)**. STAT1 can bind to *Bcl-xL* promoter to activate its transcription and suppress apoptosis **(103)**. However, restoring STAT1 expression in *STAT1*-knockout cells resulted in decreases in *Bcl-xL* and *Bcl-2* promoter activity following IFN-γ treatment **(104)**, indicating that STAT1 can either activate or suppress the expression of *Bcl-xL*. *Fas* and *FasL* are both identified as direct target genes of STAT1 in cardiac cells **(105)**. Cell death receptors and their ligands, such as TRAIL, can be induced by STAT1 in response to IFN-γ treatment **(106)**. STAT1 also regulates the expression of *caspases-1* and *-8* directly following IFN-γ treatment or ischemia in cardiomyocytes **(107, 108)**.

STAT3 activation can either promote or inhibit apoptosis, but more evidence supports its antiapoptotic function **(109)**. Several studies showed that the inhibition of STAT3 activation results in the downregulation of *Bcl-2*, *Bcl-xL,* and *Mcl-1* **(110–112)**. The inhibition of STAT3 leads to the upregulation of *BAX* **(113)** and the suppression of *survivin* **(111)**. STAT3 contributes to the suppression of *Fas* transcription and Fas-mediated apoptosis **(48)**. A constitutively activated form of STAT3 (STAT3-C) upregulated the expression of c-FLIP and led to decreased activities of FLICE and caspase-3 **(114)**.

Studies of *STAT-5a* and *-5b* double-knockout mice have demonstrated an antiapoptotic role of STAT5 in fetal myeloid progenitors by inducing the

expression of *Bcl-xL* through direct binding to its promoter. In erythroleukemia cells and chronic myelogenous leukemia cells, the activation of STAT5 is associated with the suppression of apoptosis through Bcl-xL **(115)**.

Apoptosis Mediated by FOXO

The forkhead transcription factor O family, also known as FOXO, plays an important role in apoptosis induced by cytokine/growth factor withdrawal. The family includes FOXO1 (FKHR), FOXO3A (FKHRL1), FOXO4 (AFX), and FOXO6 **(116)**. In the presence of growth factors, FOXO proteins can be phosphorylated by numerous kinases such as Akt and JNK. The phosphorylation of FOXO masks its nuclear localization signal and interferes with its ability to transactivate genes **(117)**.

FOXO can mediate apoptosis via the intrinsic and extrinsic pathways. The first FOXO target gene identified was *FasL* **(118)**. It has been suggested that FOXO-mediated apoptosis in Jurkat T cells relies on Fas-receptor signaling, involving the direct transcriptional activation of *TRAIL* and *TRADD* **(119, 120)**. In addition, *TRADD* induction by FOXO1 was shown to occur in apoptosis induced by chemotherapeutic agents, suggesting a role of FOXO in genotoxic stress-induced apoptosis. FOXO3A mediates the induction of the BH3-only proteins PUMA and Bim following cytokine/serum withdrawal **(121, 122)**. For PUMA, this regulation correlated with a reduction of activated Akt, thus allowing FOXO3A to remain nuclear and bind to the promoter of *PUMA*. The induction of *Bim*, on the other hand, was shown to occur after the transfection of a constitutively active mutant of FOXO3 in BaF3 and cytotoxic T2 cells. IL-3 and IL-2 deprivation was also shown to induce *Bim* in BaF3 and T cells, respectively. However, it is still not known whether any FOXO member directly binds to the *Bim* promoter **(122, 123)**. FOXO4 can downregulate *Bcl-xL* indirectly by inducing the expression of Bcl-6, a transrepressor that negatively regulates the *Bcl-xL* promoter to promote apoptosis **(124)**.

Another proapoptotic target of FOXO is *Arf* **(125)**. A recent study showed that FOXO proteins activate p19/Arf expression and that inactivation of FOXO proteins leads to accelerated lymphomagenesis in the Eμ-myc model. ChIP analysis showed that FOXO directly binds to the promoter of *Arf*. FOXO's ability to induce *Arf* downstream of Myc suggests a link between the FOXO and p53 pathways.

Regulation of Developmental Apoptosis by HOX and Snail

Apoptosis in Development

In addition to being a stress-evoked response, apoptosis is also an important process that an organism relies on to eliminate unwanted cells during normal

development. Genetic and molecular studies have identified numerous transcription factors involved in developmental apoptosis, among which the HOX and Snail families are the best-characterized and conserved apoptosis regulators in such diverse species as *C. elegans* and *H. sapiens*.

Apoptotic Targets of HOX

The HOX family is a group of transcription factors that play an essential role in cell fate determination and body plan during animal development **(126)**. The mammalian HOX family consists of 39 members that are encoded by four gene clusters located on four different chromosomes. All HOX proteins contain a signature homeo domain that binds to TAAT-like DNA sequences **(126)**. Mutations in the HOX genes result in abnormal body patterning and programmed cell death in several organisms. Some HOX downstream target genes are directly involved in apoptosis regulation **(126)**.

In the nematode *C. elegans*, the BH3-only protein Egl-1 is required for the programmed cell death of somatic cells **(127)**. Similar to their mammalian BH3-only counterparts, Egl-1 initiates cell death by physically interacting with the Bcl-2 homologue CED-9 **(127)**. The HOX gene *MAB-5,* which is required for the programmed cell death of the P11 lineages, interacts with the Pbx homologue CEH-20 to activate the transcription of *Egl-1* **(128)**.

In *Drosophila*, apoptosis occurring during embryonic development is often dependent on three proapoptotic genes, *reaper* (*rpr*), *head involution defective* (*hid*), and *grim*. These genes function by neutralizing DIAP1 to activate caspases **(129)**. The HOX gene *deformed* (*Dfd*) is expressed in the maxillary and mandibular segments and is necessary for the morphological features of the head segments by activating the expression of *rpr* **(130)**. The HOX family member Abdominal A controls the proliferation of neuroblasts by determining the timing of cell death **(131)**, while Abdominal B induces cell death at the boundaries between abdominal segments through rpr **(130)**. In *Xenopus*, the CED-4 homologue *FLICE-associated huge protein* (*FLASH*) is transcriptionally activated by HOXb4 in the notochord of embryos to promote apoptosis **(132)**.

In mammals, *DLX-7*, a divergent homeobox gene, is required for the survival of mouse hematopoietic cells by maintaining c-Myc expression in order to block mitochondrial-dependent apoptosis **(133)**. The HOX family members HOXA5 and human pituitary homeobox 1 (hPitx1) have been shown to regulate *p53* transcription in human breast cancer cells, and the inhibition of p53 significantly attenuated apoptosis induced by hPitx1 **(134, 135)**. HOXc8 regulates the *zinc-finger protein regulator of apoptosis* (*Zac1*) to induce apoptosis in the developing mouse brain **(126)**. The HOX protein Cdx1 can activate *Bcl-2* transcription through a consensus Cdx-binding site in colon cancer cells and rat intestinal IEC-6 cells, which is independent of p53 **(136)**, and the same site also mediates *Bcl-2* activation by A-myb in lymphoma cells **(137)**.

Snail in Apoptosis Regulation

Members of the Snail family are zinc-finger-containing transcriptional repressors **(138)**. Several subgroups have been identified, including Snail 1, Slug/Snail 2, Snail 3/Smuc, and Scratch. The mammalian members share a carboxyl-terminal region containing several C2H2-type zinc fingers that bind to a subset of E box (CAGGTG) sites, and an amino-terminal SNAG domain, which is essential for nuclear localization and transcriptional repression. The SNAG domain is not present in some lower organisms such as *C. elegans* **(138)**. Slug/Snail proteins function at different stages of development in diverse species and are important suppressors of apoptosis in several cell types, such as neurons and hematopoietic progenitor cells **(139)**.

In *C. elegans*, the Snail family member CES-1 directly suppresses the expression of *Egl-1* **(140)**. During development, the *ces-1*-mediated repression of *Egl-1* is relieved by CES-2, a βZIP transcription factor, leading to apoptosis in neurosecretory motor (NSM) "sister" cells. In *Xenopus*, Slug represses the transcription of *caspases-2*, *-3*, *-6*, *-7*, and *-9* and induces the expression of the antiapoptotic Bcl-2 family member *XR11*, thereby inhibiting apoptosis **(139)**. In mammalian cells, Slug specifically antagonizes p53-mediated induction of *PUMA* to inhibit irradiation-induced apoptosis in murine myeloid progenitors **(9)**. Slug can also suppress *Noxa* and a secreted protease, Secreted Ly6/Plaur domain-containing 1 (Slurp1), to prevent apoptosis in murine epidermis **(141)**.

Conclusions

Apoptotic machinery is tightly regulated at the transcriptional level through a number of transcription factors. In response to different stimuli, these transcription factors bind to specific DNA sequences in the promoters of target genes to stimulate or suppress their expression, unleashing apoptotic programs. This evolutionarily conserved regulatory mechanism is essential for tissue homeostasis, for normal development, and as a barrier against tumorigenesis.

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Chapter 11
Clearance of Apoptotic Cells – Mechanisms and Consequences

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Abstract Cells undergo apoptosis in development, tissue homeostasis, and disease and are subsequently cleared by professional and nonprofessional phagocytes in a multistep process. In this chapter, we first break down the clearance process into its components and then show that there is now overwhelming evidence that phagocyte function is profoundly altered following apoptotic cell uptake as well as mounting evidence that clearance defects are responsible for chronic inflammatory disease and contribute to autoimmunity. Finally, we illustrate some examples of the contribution of apoptotic cell clearance to host-pathogen and host-tumor interactions. This establishes the important potential of harnessing apoptotic cell-induced immune activation to manipulate the immune response for therapeutic gain.

Keywords Apoptosis · Phagocytosis · Macrophages · Inflammation · Pathogens

Cell Death and Clearance

Apoptotic cells are rapidly cleared in all metazoa (including organisms that do not possess defined populations of macrophages) in a process akin to macropinocytosis, coined *efferocytosis* (taken from the Latin *effero*, meaning to take to the grave) **(1)**. Most studies have focused on the uptake into professional phagocytes such as macrophages and dendritic cells, but uptake can also occur into fibroblasts, endothelial, epithelial, smooth muscle, and stromal cells and likely many more cell types; in essence, on the removal of dying cells by near neighbors, often of the same cell type as the cell that is dying. From earliest embryologic development, cells need to be removed, which can be because they have fulfilled their function and are no

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longer needed or because they have become damaged or senescent. The effective clearance of apoptotic cells followed by the replenishment of cells and tissues is essential for development, homeostasis, and response to injury. During development, organogenesis requires repeated remodeling and cell turnover occurs at a staggering rate. Even tissues with low turnover rates in adults show extensive turnover during organogenesis, exemplified by the developing mammalian brain, where up to 50% of cells are deleted **(2)**. The deletion of unwanted cells is also critical for the development and maintenance of the innate and adaptive immune system. A trillion neutrophils are eliminated each day mostly from the blood by the liver and spleen but also by the *in situ* phagocytosis of apoptotic neutrophils that have migrated into tissues and replaced in a process that leaves no obvious trace **(3)**. Only 5% of developing thymocytes are exported as mature T cells; the vast majority undergo apoptosis, a process known as negative selection, which allows for the removal of self-reactive and potentially autoimmune lymphocytes **(4)**. In the whole animal, this process is remarkably efficient and usually goes unnoticed. In fact, it has been suggested that the detection of apoptotic cells in tissues should lead one to at least question the presence of a local apoptotic cell clearance defect. Physiological changes associated with growth, age, or pregnancy can generate additional large numbers of apoptotic cells. One striking example is the involuting mammary gland, where mammary epithelial cells clear dying cells and restore the organ to prepregnancy conditions **(5)**. Finally, tissue injury and the ensuing inflammation are invariably associated with cell death and apoptosis of tissue cells or infiltrating cells of the immune system and have been described in numerous experimental models and human diseases **(6, 7)**.

The Clearance Process: Migration Toward Dying Cells

As mentioned, many different mesenchymal and epithelial cell types have the capacity to engulf apoptotic cells **(8)** and may play major roles in their clearance under normal circumstances *in vivo*. The substantive numbers of additional apoptotic cells generated in tissue injury or inflammation, however, are usually associated with the accumulation of mononuclear phagocytes. This has led to the assumption that apoptotic cells release attraction signals that direct phagocytes to their location. In support of this attractive hypothesis, Lauber et al. showed *in vitro* that apoptotic cells secrete a chemotactic signal that induces the attraction of monocytic cells in a caspase-3-dependent fashion **(9)**. They identified the phospholipid lysophosphatidylcholine (LPC) and showed that it was released from apoptotic cells due to caspase-3-mediated activation of the calcium-independent phospholipase A2. Whether LPC is a real attraction signal in inflamed solid organ tissue remains unclear, considering that LPC is likely to be taken up rapidly by neighboring cells. Another possibility is specific monocyte chemoattractant chemokines, such as MCP-1, which have been shown to be secreted by local cells during apoptosis of the corpus luteum **(10)**. However, whether the chemokine is

directly produced by the apoptosing cells or in response to them is not yet clear. An intriguing new study by Koizumi et al. **(11)** suggests that different nucleotides released from dying cells in the brain may act as attractants for microglia and also to enhance microglial phagocytic activity.

In addition to chemical attraction signals, changes in membrane composition characteristic of cells undergoing apoptosis may initiate electric signals that attract phagocytes. Electrical fields play an important role in wound healing and drive the migration of endothelial cells and neutrophils toward the wound center **(12)**. The partial loss of membrane asymmetry in apoptosis generates a surface charge in apoptotic cells that has been shown to induce endothelial sprouting toward the dying cell **(13)**. Furthermore, modified beads that mimic the surface charge of apoptotic cells have been used as simplified targets for phagocytosis assays and have shown similar characteristics as apoptotic cells. Much work has to be done to elucidate the specific mechanisms that direct professional and nonprofessional phagocytes toward dying cells before they lose membrane integrity, but the large number of cells that are effectively cleared makes it inconceivable that such mechanisms do not exist.

The Clearance Process: Recognition of Dying Cells

The second part of the uptake process is the recognition of dying cells by phagocytes; this is facilitated by changes in the composition of the apoptotic cell membrane. Surprisingly, less experimental attention has been given to these surface changes than to the recognition receptors or the soluble bridge molecules, which act secondarily as ligands for phagocyte receptors and mediate the clearance. Among the surface changes, the most universal and best characterized the is loss of phospholipid asymmetry and the translocation of phosphatidylserine (PS) to the outer leaflet of the lipid bilayer, which occurs very early in the apoptotic process **(14**, **15)**. This involves the activation of a nonspecific bidirectional phospholipid flip-flop along with the inhibition of an aminophospholipid translocase that normally confines and returns PS to the inner leaflet **(16**, **17)**. A novel family of P-type ATPases **(18)**, with the yeast analogues Dnf1p, Dnf2p, and Drs2p **(19)**, has been proposed as APLT candidates. Other molecules in addition to PS that are normally confined to the cytosolic side of the cell membrane appear on the cell surface. These include Annexin I, which co-localizes with PS **(20)**, the endoplasmic reticulum protein calreticulin, whose levels increase on the cell surface during programmed cell death by unknown mechanisms, and exposure of DNA **(21)**. Autophagy, which is commonly observed during programmed cell deaths, contributes to dead cell clearance by generating energy-dependent signals necessary for PS exposure and LPC production **(22)**. There are also poorly described modifications of existing surface molecules by oxidation [e.g., phospholipids, **(23**, **24)**] and alterations in sugar chains and surface charge **(25**, **26)**. These result in the generation of sites

resembling oxidized lipoprotein particles, thrombospondin-binding sites, sites capable of binding lectins, the complement proteins C1q and C3b, as well as various collectin-binding sites **(27–29)**.

These surface changes can then either interact directly with receptors on the macrophage surface or bind serum proteins that serve as links between the phagocyte and its apoptotic meal. These extracellular bridge molecules (opsonins) enhance the susceptibility of apoptotic cells to phagocytosis and provide additional recognition (binding site-receptor) arrangements. The bridge molecules milk-fat-globule-EGF-factor 8 (MFG-E8) and the structurally related developmental endothelial locus-1 (DEL-1) **(30)**, growth arrest-specific 6 (Gas6), β2 glycoprotein I (β2GPI), and serum protein S all bind to PS on the apoptotic cell surface. MFG-E8 can then be recognized by $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins through its RGD motif **(31, 32)**, Gas6 by receptor tyrosine kinases of the Axl, Sky, and Mer family **(33)**, and β2-GPI to the β2-GPI-receptor **(34)**.

Early studies proposed a trimolecular complex that involved thrombospondin binding to the apoptotic cell surface followed by interaction of the bound TSP with both the vitronectin receptor ($\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin) and the scavenger receptor CD36 on the phagocyte **(35, 36)**. Each of the three components has also been implicated in other pathways and receptor systems that also participate in apoptotic cell uptake. Thrombospondin itself binds to the ingestion receptor LRP **(37)**, CD36 can interact with anionic phospholipids, and the α_v integrins are candidate receptors for stimulation by MFG-E8 (see above). The possibility that TSP can act as a glue to hold everything together is intriguing, but evidence for defects in apoptotic cell clearance in the double TSP1 and TSP2 knockout mice have so far not been reported. The invocation of TSP in the recognition complex and in the uptake may have additional importance with regards to the consequences of apoptotic cell recognition as it is a major activator of the antiinflammatory mediator TGFβ (see ahead). Other bridge molecules are linked to the recognition of altered sugars and/or lipids on the apoptotic cell surface and include the members of the collectin family surfactant proteins-A and -D (SP-A and SP-D), mannose-binding lectin (MBL), and the collectin-like first component of the classical complement cascade C1q **(29, 38)**. The collectin family of molecules are then recognized through the interactions of their collagenous tails with calreticulin (CRT), which in turn signals for uptake by the phagocyte through the LDL-receptor-related protein (LRP-1/CD91) **(39)**. Pentraxins seem to bind to late apoptotic cells; i.e., C-reactive protein (CRP) has been shown to recognize apoptotic cells **(40)** and to enhance their uptake. On the apoptotic cell surface, CRP may bind to and modulate C1q effects, or to oxidized phosphatidylcholine **(41)** or perhaps to surface DNA, and may also induce the activation of its complement **(42)**. Serum amyloid P component (SAP) also binds to apoptotic cells and can promote their uptake **(40, 43)**. Both SAP and CRP may act in part through binding to Fcγ receptors on the macrophage **(43)**. By contrast, the long pentraxin PTX3 also recognizes apoptotic cells but acts in a negative role by inhibiting apoptotic cell uptake by dendritic cells **(44)**. However, it is important to point out that it remains unclear which role,

if any, antibodies play in apoptotic cell removal, either directly through Fcγ receptors or following the activation of complement.

Many other phagocyte receptors are thought to bind directly to structures exposed on apoptotic cells, including a number of scavenger receptors in addition to CD36 such as the class A scavenger receptor (SR-A) **(45)**, the lectin-like oxidized low-density lipoprotein particle receptor (LOX-1) **(46)**, and macrosialin CD68 **(47)**. Integrin family members in addition to the aforementioned $\alpha v\beta 3$ and $\alpha v\beta 5$ are thought to bind directly to C3b/bi binding sites on the apoptotic cell and include $\alpha m\beta 2$ and $\alpha x\beta 2$ integrins, also known as complement receptor 3 and 4 (CR3 and CR4) **(48)**. Altered ICAM3 on the apoptotic cell surface has been suggested to facilitate apoptotic cell binding to the lipopolysaccharide receptor CD14 **(49)**. ATP-binding cassette transporters have been implicated in the transbilayer redistribution of PS in apoptotic cells and undoubtedly play a role in the recognition of apoptotic cells. ABCA1 was thought to be the mammalian orthologue of CED-7 that contributes to apoptotic cell clearance in *C. elegans;* earlier work suggests that ABCA1 promotes recognition and subsequent engulfment **(50)**. A recent study by Jehle et al. suggests that ABCA7 rather than ABCA1 may be the mammalian orthologue of CED-7 and provides evidence for its role in the clearance of apoptotic cells through interaction with LRP **(51)** (Fig. 11.1).

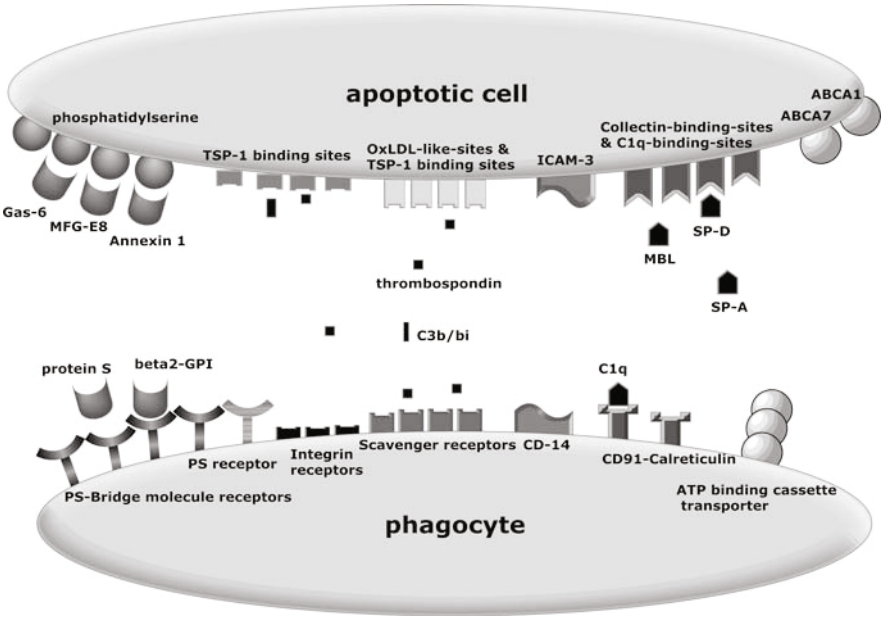

Fig. 11.1 Overview of the large number of bridge molecules and phagocyte receptors as well as apoptotic cell surface alterations that contribute to recognition and clearance. Not shown are as-yet unidentified glycoproteins on the surface of apoptotic cells as well as unidentified lectins on the phagocyte surface that are known to contribute to the recognition process

Given the prominence of PS in the membrane changes occurring during apoptosis and the many studies showing blockade of apoptotic cell uptake by PS and/or by PS-binding proteins, it still remains likely that a specific PS receptor (or receptors) exists. This would presumably be able to directly bind to PS exposed on the apoptotic cell and, from early work on phosphatidylserine inhibition, to be able to recognize the PS head group stereospecifically **(52)**. However, a previously implicated candidate receptor for PS (PSR) is now not thought to act as a surface receptor **(53, 54)**. This molecule appears to be primarily localized in the cell nucleus, and its possible role in apoptotic cell uptake remains to be elucidated.

The assumption that this large number of receptors and bridge molecules only exists to provide redundancy to ensure that the clearance of apoptotic cells is accomplished without secondary necrosis is almost certainly too simplistic. The so-called tether and tickle model by Hoffmann et al. brings some structure into the large number of eat-me signals, bridge molecules, and corresponding phagocyte receptors **(55)**. They show that a number of the "eat-me" signals mediate tethering rather than direct signaling for engulfment and that the engagement of PS appears one of the key processes required for internalization **(56)**. It will be important to further elucidate the role of individual receptors and the expected interactions between them in the actual processes of phagocytosis and intracellular processing.

Discrimination among living, dying, and dead cells is an essential requirement for the appropriate clearance of apoptotic cells. In this context, many of the ligands identified as candidates for recognition of apoptotic cells are also found on viable cells, particular following activation (i.e., PS or calreticulin). One possibility to account for the discrimination is that apoptotic cell recognition and subsequent engulfment require not only the exposure of eat-me signals but also the redistribution into patches. Many of the ligand interactions are likely of low affinity, requiring oligomerization (increase in avidity) for optimal stimulation. This could lead to the generation of a recognition "synapse" between the apoptotic cell surface and the phagocyte **(57)**. There is also evidence that inhibitors ("don't-eat-me" signals) play a role in this discrimination; i.e., the platelet endothelial cell adhesion molecule (PECAM/CD31) interacts homotypically with CD31 on potential phagocytes to facilitate the detachment of living cells and thereby potentially prevents engulfment **(58)**. Its inability to provide the same signals to apoptotic cells prevents detachment and enables ingestion. The integrin-associated protein (IAP/CD47) is expressed on the surface of many cells and can bind to the signal regulatory protein (SIRP-α), which is a critical immune-inhibitory receptor. This interaction prevents the uptake of target cells expressing CD47 **(57)**. On many cell types, CD47 expression is reduced during apoptosis and redistributed into patches distant from those containing PS or calreticulin **(59)**. Consequently, apoptotic cells (or even viable $CD47^{-/-}$ cells) are unable to stimulate SIRP-α and the downstream SHP-1 and lose their ability to prevent uptake. This raises the intriguing possibility that cells may be constantly targeted for removal and have to prove their viability to

prevent the initiation of the clearance process. The authors suspect that there is an extensive yet undiscovered uptake prevention system for viable cells to match the plethora of ligands, bridge molecules, receptors, and signaling pathways already described for the recognition of apoptotic cells.

The Clearance Process: Engulfment of Dying Cells

Mechanisms and signaling pathways of apoptotic cell uptake can be distinguished from uptake via Fc or C3 receptors **(60)**. Many *in vitro* studies of apoptotic cell uptake have directly contrasted phagocytosis of immune-opsonized cells. Fcγ–receptor-mediated phagocytosis relies on the sequential interaction of IgG-coated particles with receptors, which in turn drives the pseudopod extension from the phagocytes surface and results in a tight-fitting phagosome **(61)**, the so-called zipper mechanism. There is now increasing evidence that apoptotic cell engulfment is different in that the uptake of apoptotic cells initially involves the formation of spacious phagosomes accompanied by membrane ruffling and the associated uptake of a "healthy gulp" of the surrounding fluid **(55, 62)**. The process appears more akin to macropinocytosis than classical phagocytosis mediated by IgG or C3 opsonins but can lead to the ingestion of intact apoptotic cells greater than 20 μm in diameter, much larger than usually attributed to macropinocytosis **(63)**. However, it should be noted that some investigators have observed what appears to be closer apposition of the phagosome membrane with the apoptotic cell surface during uptake **(64)**, and it remains possible that different combinations of ligands may promote different physical modes of uptake. Furthermore, as noted ahead, the signal pathways involved in the uptake of apoptotic cells by either macrophages or nonprofessional phagocytes appear to be unique and very highly conserved evolutionarily.

Genetically tractable organisms such as *C. elegans* that allow the dissection of phagocytosis have shed some light on the signaling pathways underlying the engulfment of apoptotic cells. Seven independent genes have been identified in *C. elegans* that are required for efficient corpse removal. Most of these encode signaling molecules rather than receptors and may reflect the redundancy in receptors but the relative conservation of signaling. Double-mutant analyses have shown that the proteins encoded by these genes function in two partially redundant signaling pathways **(65)**. Interestingly, both of these pathways may play a role in promoting the cytoskeletal reorganization and activation of CED-10, which is homologous to the mammalian small GTPase Rac-1. In the first pathway, the proteins CED-2, CED-5, and CED-12 (mammalian homologues CrkII, Dock180, and ELMO, respectively) function to activate CED-10 (Rac); worms deficient in any of these proteins can be rescued by the overexpression of Ced-10 **(66)**. In the second group, the candidate receptor CED-1 (CD91/LRP) probably recognizes an unknown ligand on the apoptotic cell and signals via its

cytoplasmic tail to the adaptor protein CED-6 (hCED-6/GULP), whereas CED-7 (ABCA1/7) is thought to play a role in membrane dynamics. CED-1, CED-6, and CED-7 are required for actin reorganization around the apoptotic cell corpse, and CED-10 (Rac) acts genetically downstream of these proteins to mediate corpse removal, functionally linking the two engulfment pathways as upstream regulators of Rac activation **(67)**. Ravichandran and colleagues have identified another link between Ced-12 (ELMO) and cytoskeletal rearrangements possibly relevant for apoptotic cell engulfment and digestion by showing that ELMO is an Ezrin-Radixin-Moesin (ERM) binding protein **(68)**. ERM proteins are enriched link integral membrane proteins to the actin cytoskeleton and upon activation can also bind to the phagosomal membrane and facilitate actin assembly necessary for phagosome movement and maturation **(69)**. Ultimately, we still know very little about the engulfment of apoptotic cells, but it is apparent that signals leading to actin polymerization and particle internalization depend on the specific receptors that mediate the process and on additional modifying signals that can be generated by complex particles.

The Clearance Process: Digestion

Little is known about how the final step of apoptotic cell clearance is regulated and, more importantly, how it differs from the processing of classically opsonized or microbial cells, which employ a common underlying route of degradation from phagosomes to lysosomes (Fig. 11.2). It has long been established that intracellular pathogens have developed multiple mechanisms to arrest phagosome development to aid their survival **(70)**. This raises the questions of whether dying cells employ similar mechanisms and whether phagosome maturation is dependent on the nature of the dying cell, the mechanism of engulfment, and/or the receptors involved in recognition. These questions were first addressed by Shiratsuchi et al., who showed that peritoneal macrophages from Toll-like receptor 4 (TLR4)-null mice processed apoptotic thymocytes, Jurkat cells, and opsonized thymocytes at a faster rate than wild-type macrophages **(71)**. These observations are made in the context of no apparent changes in the macrophages' ability to bind or engulf their apoptotic or necrotic targets and suggest that TLR-4 negatively regulates the degradation of engulfed cells in macrophages. On the other hand, Blander et al. suggested that phagocytosis of bacteria but not apoptotic cells is impaired in the absence of TLR signaling **(72)**. The authors deduced that two modes of phagosome maturation exist: first, a constitutive process, as observed for apoptotic cells, that is slow and not influenced by signals received through TLRs and inducible, as observed for bacteria, that is faster and can be further enhanced through TLR signaling. This interesting hypothesis would provide a mechanism for how the organism rapidly and efficiently clears potentially harmful bacteria. On the other hand, it is counterintuitive that apoptotic cells are cleared at a slow constitutive rate

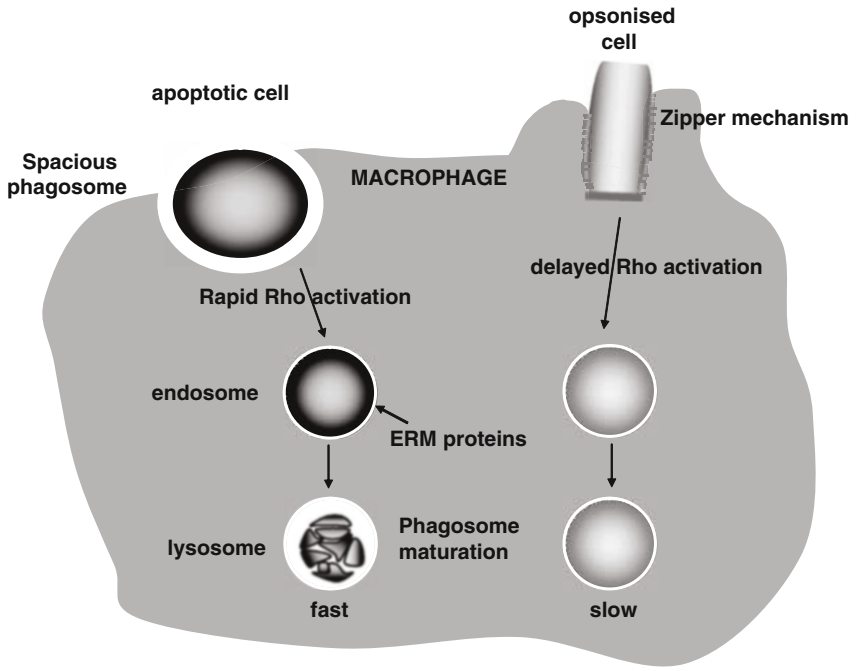

Fig. 11.2 Schematic of our limited understanding of the final step in the clearance process: the digestion of engulfed cells. In macrophages it appears that the rate of phagosome maturation depends on the mode of uptake, whereas in dendritic cells digestion is constitutively slow, which may favor antigen presentation

given the trillions of cells cleared in tissue homeostasis every day **(73)** and the large number of cells that are rapidly cleared in tissue injury without the appearance of multiple apoptotic bodies. Furthermore, a more recent study suggests no influence of TLR signaling (for all particles tested) on phagosome maturation in macrophages **(74)**. Therefore, it still remains unclear whether signals received through receptors not directly involved in apoptotic cell uptake alter phagosome maturation. Molecules and signaling pathways directly engaged in the clearance of apoptotic cells are more likely to be important for this process, and we have recently provided evidence to support this hypothesis: Phagosomes containing apoptotic cells mature more rapidly than those containing opsonized cells in many different phagocytic cell types, including primary macrophages, macrophage cell lines, and fibroblasts, and this effect was independent of the phagocyte species or the ingested target cell **(75)**. We have identified Rho as important for the maturation of phagosomes containing apoptotic cells and shown that its inhibition slowed the acidification rates of phagosomes containing apoptotic cells to those seen with opsonized cells. This is intriguing because the interaction of intact apoptotic cells with macrophages consistently leads to an early activation of Rac, followed subsequently by an

activation of Rho within 30 minutes. The relevance here is that a number of studies have shown that Rho is inhibitory for apoptotic cell uptake **(55**, **76**, **77)**, incidentally showing another difference from FcR-mediated phagocytosis. On the other hand, Rho activation is involved in some of the other processes associated with apoptotic cell-phagocyte interaction, including the production of antiinflammatory mediators such as TGFβ. This suggests that the engulfment of apoptotic cells is dependent on Rac but rapidly initiates Rho activation, which downregulates the ability of macrophages to ingest a second meal of apoptotic targets **(78)** while facilitating the processing and digestion of the ingested cells. Finally, we identified ERM proteins as the downstream targets of Rho kinase and showed that the effects of the dominant-negative inhibition of moesin on acidification were similar to the inhibition of Rho signaling. Our data are consistent with a model in which the Rac and Rho pathways have mutual antagonistic effects, as previously shown for spatiotemporal activation and downstream signaling of Rho and Rac regulating directional cell motility. Consequently, our data in the context of the existing literature suggest that the Rho/Rac balance in a given cell is not only important for apoptotic cell engulfment but also for phagosome maturation and may have direct consequences for antigen presentation. Understanding the processes and regulation of apoptotic cell digestion in phagocytes provides an area for research with profound implications for the innate and adaptive immune response. Additional importance may be derived if one considers the redundancy of the recognition mechanisms, which makes it conceivable that the highly conserved digestive process is the rate-limiting step for the clearance of apoptotic cells.

Inflammatory Mediator Production Following Apoptotic Cell Uptake

Early studies support the notion that apoptotic cell uptake is immunologically neutral. The uptake of apoptotic eosinophils and neutrophils did not cause proinflammatory mediator production, whereas postapoptotic eosinophils or opsonized neutrophils and zymosan particles induce granulocyte–macrophage colony-stimulating factor (GM-CSF), N-acetyl-beta-D-glucosaminidase (NAG), and thromboxane **(79**, **80)**. However, there is now compelling evidence that binding or uptake of apoptotic cells to phagocytes induces the production of TGF-β, and in some model systems IL-10 *in vitro* **(81**, **82)**. These antiinflammatory cytokines have direct autocrine and paracrine effects on proinflammatory cytokine production, as illustrated by the inhibition of LPS-induced TNF-α production **(81)**. More recently, the importance of TGF-β signaling in the consequences of apoptotic cell uptake was examined in more detail: Arachidonic acid release, COX-2, and prostaglandin synthase expression were shown to be dependent on TGF-β production as well as inhibition of thromboxane synthase, sulfidopeptide leukotrienes, nitric oxide synthase, and nitric oxide

(83). Interestingly, apoptotic cell uptake stimulated lipid mediators such as 15-lipoxygenase and 15-hydroxyeicosatetraenoic acid, which enhance uptake **(84)** and, together with resolvins and protectins, dominate the resolution phase of the inflammatory response **(85)**.

In addition to the autocrine and paracrine effects mediated through cytokines and lipid mediators, Ucker and colleagues have described direct effects of apoptotic cells on the proinflammatory transcriptional machinery of macrophages **(86)**. In addition, more direct inhibitory effects of apoptotic cell interaction on NFκB activation have been reported **(87)**, perhaps mediated through Mer **(88)** and not requiring TGF-β. Interestingly, recent work in the model organism *Drosophila* has suggested that the secretory factors decapentaplegic (a TGF-β homologue) and wingless are directly produced by cells undergoing apoptosis and induce signaling cascades for the compensatory proliferation of neighboring cells **(89)**. Nonprofessional phagocytes such as endothelial or epithelial cells that phagocytose neighboring apoptotic cells subsequently produce survival and growth factors such as VEGF and HGF **(90, 91)** that likely contribute to tissue replenishment and the restoration of endothelial and epithelial boundaries. Chronic lung disease including cystic fibrosis and chronic obstructive pulmonary disease (COPD) are characterized by increased numbers of apoptotic cells; this is not just a consequence of the increased induction of apoptosis but also due to impaired clearance by airway epithelial cells [reviewed in **(6)**].

In contrast to the countless reports detailing the antiinflammatory consequences of apoptotic cell uptake, a small number of studies cannot be disregarded that show that very early apoptotic cells can be cleared silently without the release of either pro- or antiinflammatory mediators **(92)** or describe proinflammatory consequences including the release of IL-8 with subsequent neutrophil chemotaxis **(93)** and release of FAS ligand. The recognition mechanism involved in uptake may be critically important for the immunological consequences, as suggested by studies that show phosphatidylserine-dependent ingestion of necrotic cells is immunologically neutral **(94)** and data that suggest a dual function of bridge molecules such as SP-A and SP-D that enhance proinflammatory mediator production when binding to calreticulin/CD91 and inhibit inflammation when binding to SIRP-α **(95)**. Importantly, a series of *in vivo* experiments shows the antiinflammatory effects of apoptotic cell phagocytosis. The deliberate instillation of apoptotic cells into sites of local inflammation in the lungs and peritonea increased the production of TGF-β as well as enhanced the resolution of injury **(96)**. Decreased alveoloar macrophage apoptosis is associated with increased pulmonary inflammation in a murine model of pneumococcal pneumonia **(97)**, and the defective clearance of apoptotic cells in CD44 knockout mice leads to unremitting inflammation following noninfectious lung injury **(98)**. Thus, the innate response to apoptotic cell phagocytosis is dominated by antiinflammatory signals originating from the professional and nonprofessional phagocytes. Considerably less is known about the direct effects of apoptotic cells, but recent observations suggest that changes in surface composition and surface charge may directly influence the restoration of

endothelial layers and angiogenesis **(99)**. An alternative way in which apoptotic cell clearance may mitigate inflammation is by the removal of membranes (or cells) expressing proinflammatory oxidized lipids **(100)**.

Apoptotic cell uptake predominantly initiates mechanisms that contribute to resolution of injury and repair, but this has to be seen in the context of other signals that impinge on the surface receptors of phagocytes. Generally, not all phagocytes within a given population take up apoptotic cells, and those that do frequently take up more than one apoptotic target, which suggests that the activation state and differential receptor expression markedly influence not only phagocytic capacity but also subsequent responses. Necrotic cells and pathogens share many of the ligands of apoptotic cells but usually induce different responses at least partially because they also engage pattern recognition receptors and signaling pathways not activated by apoptotic corpses. On a single-cell level, apoptotic cell uptake activates Rho GTPases **(101)**, which eventually, in turn, as outlined earlier, markedly inhibit phagocytosis **(77)**. This may eventually lead to clearance failure or uptake by phagocytes that initially were not primed for uptake; so far, we can only speculate whether this alters the phagocyte and subsequent immune response in an inflamed focus with ongoing cell death. Macrophage function within complex environments (i.e., inflamed tissue) is notoriously difficult to study, but existing data suggest that macrophage function is not an amalgamate of all the signals received but rather a programmed response induced by the first dominant stimulus the cells are exposed to **(102)**. It is therefore conceivable that apoptotic cell uptake does not immediately switch individual phagocyte function but only does so after a critical number of cells have contributed to an overall change in the microenvironment.

T-Cell Activation Following Apoptotic Cell Uptake

Dendritic cells are the primary antigen-presenting cells for initiating primary immune responses, but macrophages are abundant in inflammatory sites and can act as antigen-presenting cells and perpetuate or terminate immune responses depending on their state of activation **(7, 102)**. After the uptake of necrotic neutrophils, macrophages upregulate co-stimulatory molecules and stimulate significantly higher T-cell proliferation than macrophages that have ingested apoptotic neutrophils **(103)**. The production of IL-12 by macrophages is transcriptionally suppressed following apoptotic cell uptake or treatment with phospatidylserine **(104)**. Immature DCs are capable of extensive phagocytosis, and DC maturation can be inhibited by the engulfment of apoptotic cells with suppressed expression of the co-stimulatory molecule CD86 and, similarly to macrophages, decreased IL-12 production **(105)**. Interestingly, studies conducted in the 1970s show improved allograft survival following repeated blood transfusions, which may be due to the presence of apoptotic

granulocytes and lymphocytes in blood stored for clinical transfusion **(106)**. In this context, it is intriguing that the infusion of donor apoptotic lymphocytes in a rat heart transplantation model induced allograft tolerance and was shown to be dependent on intact efferocytosis **(107)**. Despite this compelling evidence that the ingestion of apoptotic cells can inhibit antigen presentation and dendritic cell maturation, earlier studies indicate that antigen derived from ingested apoptotic cells could access the cytoplasm of the ingesting cell and be cross-presented on MHC class I molecules **(108)**. More recently, work by Nussenzweig and colleagues has established that $CD8^+$ $CD205^+$ DCs, which appear to be specialized for the uptake of dying cells, are much better than $CD8^-$ $33D1^+$ DCs for cross- presentation on MHC class I molecules **(109)**.

DCs acidify phagosomes at a slower rate than macrophages **(75)**, in keeping with data showing that DCs in comparison to macrophages generate low levels of lysosomal proteases and have a decreased ability to degrade internalized protein **(110)**. It is therefore not surprising that apoptotic cells contained in DC phagosomes can be observed in the afferent lymphatics of the gut **(111)** en route to lymph nodes. The slow degradation of ingested material by dendritic cells may allow an extended period of time to sample the microenvironment for danger signals, which in turn instruct the dendritic cells to initiate an immune response or assist in the maintenance of self-tolerance. Danger signals include not only signals received through pattern recognition receptors such as Toll-like receptors but also necrotic cells **(112)**. Necrotic but not apoptotic cell death has been shown to release heat-shock proteins, which in turn deliver a partial maturation signal to dendritic cells and activate their NF-κB pathway **(113)**. Furthermore, protein fragments chaperoned by heat-shock proteins and not intact proteins have been shown to be crucially important for the cross-presentation of antigens from cancer or infected cells for the priming of naïve $CD8^+$ T cells **(114)**. Therefore, the microenvironment in which apoptotic cell phagocytosis takes place appears to be critically important for the subsequent adaptive immune response. It is likely that apoptotic cells ingested by DCs in the absence of danger signals or concomitant necrotic cell death contribute to tolerance but otherwise might provoke autoimmune responses. It is important to keep in mind that the microenvironment is profoundly influenced by the molecules produced by the corpse-eating phagocytes and that the balance of appropriately disposed apoptotic cell versus primary necrotic or secondary necrotic cells (i.e., in clearance failure) may be critically important.

Failed Clearance and Autoimmunity

The prototypic autoimmune disease SLE is characterized by the development of specific antibodies to intracellular antigens that are clustered on the surface of apoptotic cells **(115)**. The notion that apoptotic cell debris represents the

autoantigen led to a series of studies that showed that immunization with apoptotic cells can drive the immune response and result in the production of autoantibodies **(116)**. This suggests that apoptotic cell clearance defects contribute to the development of autoimmunity. Indeed, patients with C1q deficiency and other defects of the complement pathway develop SLE **(117)** and C1q knockout mice develop spontaneous systemic autoimmunity with a marked excess of noningested apoptotic cells in the kidney **(118)**. Experiments using knockout mice of other components of the complement pathway including C3 and C4 also associated with susceptibility to SLE showed delayed clearance of apoptotic bodies by resident peritoneal macrophages **(119)**. Mice deficient in other receptors and bridge molecules implicated in the clearance of apoptotic cells such as MFG-E8 **(5)**, Mer **(120)**, and CD31 **(121)** also exhibit autoimmune disease. It is important to keep in mind, however, that genes from the nonautoimmune strains 129 and C57BL/6(B6) commonly used for generating knockout mice can induce a lupus-like disease and that a 129-derived interval on distal chromosome 1 is strongly linked to autoantibody production in the absence of any disrupted gene **(122)**. SP-D and MBL knockout mice show defective clearance of apoptotic cells; despite this, however, MBL knockout mice do not develop autoimmunity even when they are aged on a lupus-prone background for 18 months **(123)**, which shows that clearance failure does not inescapably lead to autoimmunity. Intriguingly, mice deficient in CD14 also show a clearance defect leading to the persistence of apoptotic cells in multiple organs and do not develop autoantibodies or autoimmune disease **(124)**. Taken together, these data suggest that the failure of apoptotic cell clearance in itself is not sufficient to initiate autoimmunity and raises questions regarding other mechanisms that instruct the immune response in the context of clearance failure. It is important to keep in mind that despite clearance failure, strong immunosuppressive signals such as the generation of TGF-β are induced by those cells that indeed get ingested or even just through binding to the phagocyte.

Exploitation of Apoptotic Cells by Tumors and Pathogens

In some ways, the most convincing evidence for the antiinflammatory consequences of apoptotic cell phagocytosis is the exploitation of these immune-inhibitory signals by pathogens and tumors. *Plasmodium falciparum*-infected erythrocytes inhibit the maturation of DCs by binding to CD36, a known recognition receptor for apoptotic cells. Infected DCs still secrete TNF-α but fail to activate T cells and secrete IL-10 **(125)**. This response can be mimicked by antibodies to CD36 or apoptotic cells and suggests that the pathogen and apoptotic cells engage the same pathway regulating DC function. It appears that *Plasmodium* almost inadvertently profits from using the same entry mechanism as apoptotic cells, whereas other pathogens

not only exploit recognition mechanism but, in addition, profit from the microenvironment created by apoptotic cell phagocytosis. Intense lymphocyte apoptosis occurs in Chagas disease, a debilitating cardiac illness caused by the protozoan *Trypanosoma cruzi*. In a mouse model of the disease interaction of apoptotic, but not necrotic, T lymphocytes with macrophages infected with *T. cruzi,* parasite growth is fueled in a manner dependent on prostaglandins, transforming growth factor-β and polyamine biosynthesis **(126)**. Furthermore, the vitronectin receptor is critical, in both apoptotic cell binding to phagocytes and the induction of prostaglandin E2/TGF-β release and ornithine decarboxylase activity in macrophages. These results suggest that continual lymphocyte apoptosis and phagocytosis of apoptotic cells by macrophages have a role in parasite persistence in the host. These intracellular parasites appear to use varied means to gain entry into their preferred location, the macrophage phagosome, including uptake via Fc receptors. However, interesting reports **(127**, **128)** suggest that they express PS on their surface, which may also contribute to uptake, and by inference would likely also alter the macrophage phenotype toward a less activated state, perhaps contributing to a more advantageous intracellular habitat. A related, but more complex, Trojan horse mechanism has been described **(129**, **130)** in which *Leishmania* are first taken up into neutrophils and then secondarily into macrophages when the neutrophils (still containing the living *Leishmania*) become apoptotic. The secondary uptake of the apoptotic neutrophils was, as usual, associated with the production of TGF-β with its potentially important antiinflammatory and antiimmunogenic consequences. A blunted immune response to rapidly growing tumors is frequently observed and thought to be at least in part mediated by the immune-inhibitory effects of apoptotic cell phagocytosis. Reiter et al. showed that the exposure of bone marrow-derived macrophages (BMDM) to apoptotic (but not necrotic) tumor cells inhibits their cytotoxity and nitric oxide production in response to IFN-γ and LPS **(131)**. Furthermore, unstimulated BMDM exposed to apoptotic tumor cells enhanced growth of life tumor cells by 40%. Therefore, treating cancers with chemotherapy or radiation that leads to massive tumor cell apoptosis is likely to inhibit macrophage-mediated antitumor responses. Much emphasis is currently being placed on utilizing some of these effects in enhancing immune responses to cancer on the one hand **(132)** or suppressing immune responses, for example, in transplantation on the other hand **(133)**.

These examples clearly illustrate the profound effects of apoptotic cell recognition on the outcome of the immune response to pathogens and tumors. It shows that pathogens and tumors use endogenous antiinflammatory pathways to aid their survival. The challenge for the future is to effectively and coordinately manipulate the clearance of dying cells to develop new therapies for inflammatory and autoimmune disease and prevent inappropriate immune inhibition in the context of pathogens and cancer.

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Chapter 12
Systems Biology Approaches to the Study of Apoptosis

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Abstract Today, we can avail of comprehensive information on the molecular mechanisms of apoptosis signaling that was gathered during decades of intense research. This chapter presents how mathematical approaches in the field of cellular signaling are used to integrate this complex and heterogeneous information into computational models with the aim to elucidate the functional properties of apoptotic signaling networks. Mathematical modeling allows one to describe properties of signaling systems that emanate from the interplay of the system's individual components and has a longstanding and successful history in the fields of physics, chemistry, and their applied engineering sciences. Systems analyses can serve to describe and identify signaling dynamics, molecular switches, thresholds, and feedback regulatory mechanisms and allow systems properties such as stability and robustness toward external perturbations to be identified. Crucially, systems analyses can also serve to generate novel qualitative and quantitative research hypotheses, which in turn allow for more focused experimental research approaches. This chapter provides a concise and critical overview on the current state of systems biology in the field of apoptotic signaling and the methodology employed.

Keywords apoptosis · systems biology · computational modeling · caspases · inhibitor-of-apoptosis proteins · death receptors · mitochondrial outer membrane permeabilization · apoptosome

Introduction

Cell death signaling, and here specifically apoptotic signaling, has been a major international research focus for several decades now and continues to be of central interest to hundreds of bioscience research groups worldwide. By now,

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the rapid speed of discovery of molecular components involved in the control of cell death processes has amassed a tremendous amount of information. Many of the key components of cell death signaling networks are described in great detail, as outlined in the previous chapters of this book. These components include genes and proteins, and their interactions within and across intracellular compartments, but also comprise metabolites and cellular bioenergetic processes. With the description of these smallest individual elements of apoptotic signaling, biomolecular research has reached its reductionist limit.

Even though we can avail of this detailed information on the individual elements, this alone is not sufficient to understand the functionality and responsiveness of a signaling network as a whole. Many features of signaling networks such as molecular switches, systems sensitivity, (bi)stability, or response robustness emerge as system properties from the interplay of its individual elements **(1–5)**. This interplay can be described using models of various levels of abstraction **(6)**. For example, block diagram models are commonly used and helpful to depict signaling pathways and networks, allowing the visualization of interactions and reactions [Figs. 12.1(A) and (B)]. With an increase in pathway

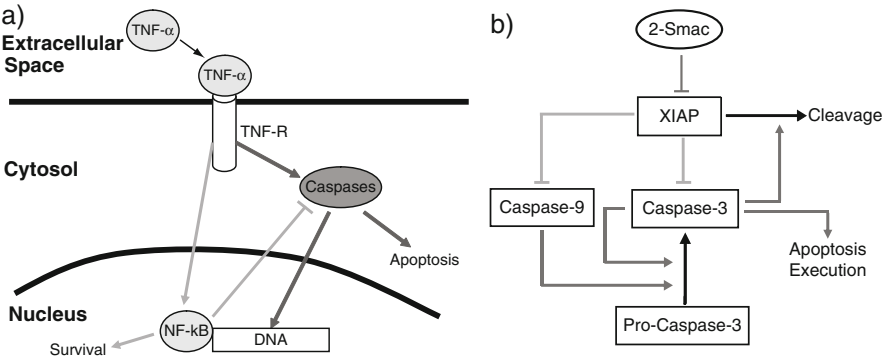

Fig. 12.1 Block Diagram Models. Block diagram models are widely used to visualize signaling networks both at low and high levels of abstraction. The relationships of the individual network components are typically depicted by *arrows* highlighting activation and inhibition mechanisms. In the following two examples, pro-survival signaling is depicted in light grey, cell death signaling is shown in dark grey arrows. **(A)** Abstract block diagram model for TNF-α-induced signaling. The general responses following TNF-α stimulation across three biological compartments are shown. TNF-α can bind to specific cell surface receptors that transmit the signal from the extracellular space into the cytosol. Both NF-κB-dependent pro-survival signaling as well as the activation of apoptotic caspases are induced. This model massively simplifies the underlying molecular complexity involved in these signaling pathways. Even though abstract, it is sufficient to display the two antagonizing routes of signaling. However, the static nature of this block diagram model cannot explain under which conditions cell survival or cell death signaling will prevail. **(B)** Block diagram model of the key components of the apoptosis execution network. At higher detail than the model in (A), this model displays the interaction of individual proteins at the molecular level, taking binding stoichiometry into account. While sufficient for displaying the key properties of the network, the model cannot describe which combinations of individual protein concentrations will result in apoptosis execution or caspase inhibition

understanding, over the years block diagram models of apoptotic signaling have become more and more detailed. Hypotheses for experimental studies, such as the potential consequences arising from inhibiting specific reactions or overexpressing or depleting individual network components, can be drawn from these static models. However, the generated predictions are of a qualitative nature and allow only limited or no insight into the kinetic behavior of the system.

Based on the interplay of network components as described by block diagrams, mathematical models are suitable to integrate additional knowledge about the processes. For example, mathematical models of intracellular signaling can describe the individual interactions and reactions by their respective association/dissociation constants and catalytic activities. Furthermore, the quantitative proportions of the individual system components can be defined, and for spatial models or models defining separate compartments, their location can also be taken into account. Such models allow the investigation of not only steady-state conditions but also temporal dynamics following network stimulation or perturbation in detail. In addition, a comprehensive description of a biological signaling network by experiments alone would require monitoring dozens of variables over time and space in parallel, which currently cannot be accomplished technically. Mathematical systems models thus allow the description of complex and/or rapid molecular dynamics where experimental approaches reach their limitations.

Systems biological research of apoptosis signaling currently is in a transition between the "proof-of-principle" phase during which the methodological tools required are introduced and established in the field and the phase of applying systems approaches to generate and answer new biological research hypotheses. Mathematical modeling studies of apoptotic signaling, either solely theoretical or in combination with experiments, have already contributed to the identification and functional explanation of signaling kinetics, switches, thresholds, and feedback regulatory mechanisms **(7–11)**. Other mathematically more complex approaches from the fields of control theory and engineering were introduced in a biological setting and were helpful in describing systems properties such as stability and robustness toward external perturbation **(4**, **8**, **10**, **12–14)** and allowed complex data sets to be reduced to smaller sets of parameters that were sufficient to predict cellular apoptotic responses **(15–17)**. Based on the published literature, in the following we provide an overview of the mathematical approaches and research accomplishments in the currently still developing, but rapidly expanding, field of systems biology of apoptosis.

Deterministic Modeling of Apoptotic Signaling

Deterministic models of apoptotic signaling are commonly based on the use of ordinary differential equations (ODE) and so far are probably the most successful mathematical approaches to describe the molecular dynamics during cell

death signaling. An ODE model describes the temporal evolution of states, which in a biological context are typically concentrations of, for instance, proteins, metabolites, or ions. ODE models assume a spatially homogeneous distribution of the network components within the modeled cell or cellular compartment.

An ODE model consists of a list of all reactions that can take place in the modeled network and the respective reaction rates and stoichiometries that describe the kinetic and quantitative relationship of reactants and products. As many key processes of apoptotic signaling are already known in great detail, rates for the modeled reactions often can be taken directly from published biochemical literature. These comprise k_{on} and k_{off} rates for binding/dissociation reactions or k_{cat} and K_m constants to describe enzymatic activities. Most reactions in signaling networks are modeled according to the law of mass action; i.e., the reaction rate is proportional to the reactant concentrations. When justifiable by the biological evidence, other rate models such as Michaelis-Menten kinetics or Hill kinetics can be used as well.

Due to the prominent use of ODE models in apoptosis research, we describe here a simplified example for ODE model generation based on mass action kinetics. We consider two biochemical reactions involving the proteins A, B, and C:

$$\begin{aligned} &\text{binding/dissociation} && R_1\text{: A} + \text{B} \leftrightarrow \text{AB}, \\ &\text{irreversible cleavage} && R_2\text{: C} + \text{A} \rightarrow \text{C} + \text{A}^I + \text{A}^{II}. \end{aligned} \tag{12.1}$$

Reaction R_1 is reversible and biologically reflects protein dimerization such as reversible inhibitor-target interactions. The irreversible reaction R_2 can be interpreted as an enzyme C cleaving protein A into two fragments A^{I} and A^{II} without being modified itself. Following the law of mass action, the reaction velocities v_1 and v_2 for reactions R_1 and R_2 are proportional to the product of the reactant concentrations. The reversible R_1 reaction is additionally balanced between forward and backward reactions:

$$\begin{aligned} v_1 &= {k_1}^{\text{on}} * [A] * [B] - {k_1}^{\text{off}} * [AB], \\ v_2 &= {k_2}^{\text{cleave}} * [A] * [C]. \end{aligned} \tag{12.2}$$

Based on these reaction velocities, the temporal change in concentration of each of the involved proteins and protein complexes can be described by an ordinary differential equation:

$$\begin{aligned} &\frac{d[A]}{dt} = -v_1 - v_2, \qquad \frac{d[A^I]}{dt} = \frac{d[A^{II}]}{dt} = v_2, \qquad \frac{d[B]}{dt} = -v_1, \\ &\frac{d[AB]}{dt} = v_1, \qquad \frac{d[C]}{dt} = 0 \Rightarrow [C] = \text{const}. \end{aligned} \tag{12.3}$$

This is the set of coupled ODEs that fully describe the reactions system of Eq. (12.1). A more comprehensive and detailed description of a universal mathematical formalism for the modeling of biochemical reactions can be found in the literature **(18)**. Thus, ODE models are ultimately a collection of relatively simple individual reactions or processes. Typically, the reaction rates and protein concentrations need to be backed up by the published literature or should at least be based on justifiable assumptions or estimations.

Fussenegger et al. introduced ODE modeling into apoptosis research by developing a temporal model of effector caspase activation that already integrated components of the extrinsic as well as the intrinsic pathways into a unified mathematical description with 19 state variables **(9)**. Even though many reaction rates were estimated due to a lack of further available data at the time, qualitatively the model compared reasonably well to previously published experimental kinetics of caspase activation in cell populations. It thus showed that the understanding of the signaling processes at that time was already sufficient to describe some key features of apoptotic responses. Since then, ODE models repeatedly were successfully employed in the modeling of apoptotic processes (Fig. 12.2 and Color Plate 2).

Initially, most efforts concentrated on systems analysis of death receptor-mediated apoptosis (see Chapter 5), the activation of caspase-8, and its direct link to and positive feedback with effector caspase-3 (so-called type I signaling) **(7**, **8**, **19–21)**, which is preferred by some cells over the mitochondrial pathway (type II signaling) **(22)**. In a theoretical study, Eissing et al. deciphered on a systems level that intracellular inhibitors for both caspases-8 and -3 are required to reproduce the delay times between apoptosis initiation and execution that can be observed experimentally **(8**, **22)**. Furthermore, the study mechanistically predicts that the signaling system can respond with rapid activation kinetics of caspase-3, resulting in efficient and irreversible cell death execution. Interestingly, the predictions resembled the experimental kinetics of caspase-3 activation that were observed during cell death execution via the mitochondrial pathway **(23)**, suggesting that even though different routes can lead to apoptosis execution, the response kinetics on the level of effector caspases may be largely identical.

Combining biochemical data from population analyses with mathematical modeling, Bentele et al. thoroughly analyzed the CD95 death receptor pathway in human B lymphoblastoid SKW 6.4 cells **(7)**, a cell line that is believed to predominantly signal via the type I pathway. Based on the identification of the critical system parameters, they developed a model that includes key elements from both the type I pathway as well as the mitochondrial pathway such as the formation of the death-inducing signaling complex (DISC) and the apoptosome. The final model covered 41 molecules and 32 reactions, with the individual model parameters being estimated from quantitative experimental data and literature. Significant insight was gained into the signaling dynamics during CD95 death receptor-mediated apoptosis by generating the temporal protein profiles in response to maximal and submaximal receptor stimulation. The

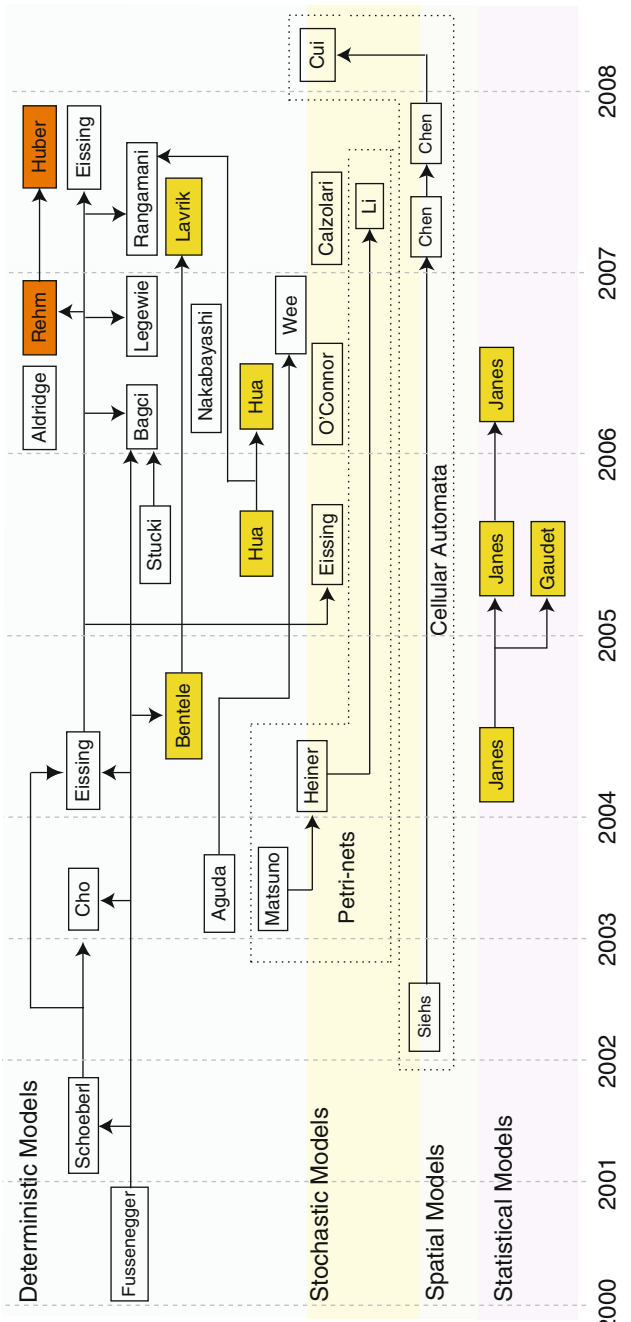

Fig. 12.2 History of apoptotic systems modeling. Published systems biology studies were classified into four methodological categories and ordered chronologically. *Arrows* indicate an influence or logical connection between different studies with respect to the adoption of biological or methodological information (*see* Color Plate 2)

model furthermore identified proteins and their concentration ranges controlling cellular responsiveness and resistance to death receptor stimulation. For example, a threshold concentration for c-Flip was calculated and experimentally confirmed to switch off cellular responsiveness to CD95 at submaximal doses, a finding that was experimentally followed up in further detail in a subsequent study **(7, 24)**.

A similar modeling approach for CD95-induced apoptosis with a stronger emphasis on the mitochondrial pathway was performed by Hua et al. **(19)**. Of note, many parameters of this model were estimated from experimental responses of cell populations of Jurkat T lymphocytes; the modeled kinetics therefore do not correspond well to the rapid signaling kinetics that would be expected during apoptosis execution at the single-cell level **(23, 25)**. Nevertheless, the model qualitatively could show that the dominance of either type I or type II signaling could be switched by the expression level of caspase-8 alone, which indicates that neither of the two signaling pathways requires its own specific network topology.

In addition to apoptosis initiation, death receptor stimulation in parallel can induce survival and proliferation, leading to a scenario where both responses directly compete [Fig. 12.1(A)] **(26)**. So far, initial ODE models were developed that took NF-κB-dependent survival and proliferation signaling as well as components of apoptotic pathways into account **(27, 28)**. Similarly, even though not exclusively related to death receptor-induced signaling, other studies theoretically analyzed the competition between cellular death and survival signaling with a stronger focus on transcriptional responses and kinase cascades **(29, 30)**. The answer to whether death or survival signaling prevails in such scenarios is clearly context-dependent and thus a systems problem. Further studies in this direction, however, will require a much closer link with parallel experimental data to validate the models and verify their predictive capacity.

In most cells, extrinsically-induced apoptosis requires the mitochondrial pathway for signal amplification and cell death execution. The death receptor pathway converges here with the intrinsic pathway at the level of mitochondria (see Chapter 6). Several theoretical as well as experimental studies were aimed at analyzing systems properties of apoptotic cell death following mitochondrial permeabilization **(10, 11, 31–33)**.

Mitochondrial permeabilization initiates the complex process of cytochrome c-induced apoptosome formation **(34)**. Modeling of this process is still in its infancy due to the complexity of the processes involved. Systems studies on the molecular mechanism of apoptosome formation and its efficiency address valid theoretical questions on regulatory mechanisms in the core reaction network of apoptosome formation. These include the potential cooperativity of the individual reactions as well as combinatorial problems in the generation of fully matured heptameric apoptosome complexes **(32, 33)**. However, so far apoptosome models lack sufficient integration and representation of the experimental knowledge available. Given that regulation of apoptosome formation was repeatedly implicated in developmental processes as well as in carcinogenesis

and cellular chemoresistance **(35, 36)**, more detailed systems studies could carry great potential to further analyze the complexity of the apoptosome formation process and the regulation of apoptosome activity.

The subsequent apoptosis execution network following apoptosome formation and Smac release was analyzed on a systems level by Rehm et al. **(11)**. The problem of describing the processes of apoptosome formation in detail was bypassed by employing an input function for apoptosome generation that was derived from experimental data. This study tightly coupled the systems analyses to experimental measurements of the real-time kinetics of apoptosis execution in individual HeLa cervical cancer cells. To this end, the input and output of the systems model were programmed to be directly comparable to experimentally observable signals of mitochondrial permeabilization/depolarization and intracellular effector caspase activity **(37)**. The model described the intracellular molecular signaling dynamics during apoptosis execution using a network of 53 reactions and identified that caspase inhibitor XIAP can inhibit apoptosis execution only at concentrations above a critical threshold value. Furthermore, the IAP-antagonist Smac was mathematically predicted to have little or no influence on the molecular dynamics during apoptosis execution in HeLa cells. Due to the direct comparability of the mathematical model and experimental measurements at the single-cell level, quantitative predictions could be validated by subsequent experiments. The model furthermore was sufficient to generate qualitative predictions for other cell types as well. The analytical tools used in this study were subsequently also published as a freely accessible web service **(38)**. Another detailed theoretical study analyzing the XIAP-caspase interplay during the apoptosis execution phase identified an unexpected positive-feedback mechanism in the signaling system that, together with positive feedbacks directly dependent on caspase activity, contributes to the irreversibility of apoptosis execution: The binding of XIAP to the relatively large pool of caspase-3 sequesters significant amounts of XIAP away from caspase-9, which in turn can activate additional caspase-3 **(10)**.

Stochastic Modeling of Apoptotic Signaling

A stochastic or random process is such that from a known starting point, the system can evolve to different outcomes. Brownian motion, the random movement of a particle in suspension, is an example of such a stochastic process **(39)**. One source of stochasticity is the general cell-to-cell variability that can be expected within a population of cells, called *extrinsic noise*. To reproduce a population's behavior, extrinsic noise can be modeled employing ODE-based cell ensemble models or models of population dynamics **(40–42)**.

A second source of stochasticity is cell-intrinsic noise. While for sufficiently large numbers of molecules, stochastic effects with respect to whether or not a reaction will take place usually average out, low reactant concentrations can

potentially result in stochastic variability of the resulting cellular response **(12, 43)**. For such intrinsic noise, stochastic models using either discrete or continuous quantities exist. While the temporal profiles of continuous quantities can be described by stochastic differential equations, modeling discrete quantities requires employing a chemical master equation or stochastic Petri nets **(44, 45)**. So far, however, Petri net models of cell death signaling have yet to develop beyond the proof-of-principle stage that established a methodological framework for this technique **(46–48)**. In contrast, using the chemical master equation allowed us to analyze whether the low numbers of active caspases present in a cell before entering the apoptosis execution phase can lead to stochastic cell death responses of type I cells **(12)**. Interestingly, this systems analysis identified that stochastic noise arising from low numbers of active caspases may be suppressed by continuous exchange with a larger pool of caspases bound to IAPs, which could act as a "molecular buffer." Therefore, the cellular asynchrony in apoptosis execution that can be observed on the population level **(49)** may largely result from extrinsic noise such as cell-to-cell variability.

Spatial Modeling of Apoptotic Signaling

Even though nonspatial apoptotic models are reasonable assumptions for most cases of apoptotic cell death signaling, spatial control of caspase-dependent signaling may be of significant importance during several physiological processes. For example, caspases were implicated in cellular differentiation and proliferation signaling, including localized and sublethal activation of effector caspases **(50)**. Even though the mathematical methodology to extend ODE models to spatial reaction/diffusion models based on partial differential equations (PDEs) is well established, PDE models have not yet been employed in apoptotic systems biology.

A different methodology for spatial modeling uses cellular automata (CA) **(51)**. CA models describe spatial processes by the movement of individual particles in lattice grids. Diffusion and transport processes can be implemented by stepwise movement of the individual reactants in the lattice in relation to their diffusion coefficients. Interactions such as enzymatic catalysis or binding/dissociation can only take place if the reactants meet each other in the grid. CA models can also be extended to include stochastic processes. As a first conceptual study, Siehs et al. applied a CA model to describe the homo- and heterodimerization of members of the Bcl-2 family in the outer mitochondrial membrane **(52)**. This approach was elegantly extended by Chen et al. to a significantly more comprehensive model that allowed an irreversible and switch-like induction of mitochondrial permeabilization to be remodeled by Bax activation **(13)**. Still, due to the lack of sufficient quantitative experimental data, CA models currently are rather

qualitative tools for the analysis of apoptotic signaling. However, in the light of the multitude of conflicting hypotheses on the molecular mechanisms of mitochondrial permeabilization, systems analyses by CA models already carry great potential for evaluating which of these hypotheses are more appropriate to reproduce experimentally observable data and to generate robust biological responses **(14, 53)**.

Bistability of Apoptotic Signaling Networks

Perturbations of cellular apoptosis susceptibility were repeatedly implicated as major contributors to degenerative and proliferative diseases. A key requirement for apoptotic signaling networks therefore is a robust insensitivity to mild proapoptotic stress to avoid excessive or unwanted apoptosis. In contrast, strong apoptotic stimuli must result in the efficient activation of effector caspases to ensure cell death execution.

The transition from a state of cell survival toward a state of efficient effector caspase activation should ideally be a fast and irreversible decision. Indeed, it was experimentally shown that apoptotic signaling comprises rapid and irreversible all-or-none responses such as mitochondrial outer membrane permeabilization and effector caspase activation **(23, 25)**. From a systems point of view, such a switch-like behavior can be achieved by bistability **(54)**, an emergent systems property that results from the interaction of the systems components but not from the individual components themselves **(5)**. A *bistable system* consists of two stable steady states that are robust to small perturbations. In apoptotic signaling, one steady state corresponds to cell survival, and the other corresponds to cell death.

The concept of systems bistability and its necessity for apoptotic cell death decisions were first highlighted by Eissing et al. when analyzing death receptor-mediated type I signaling **(8)**, and subsequently were implicated in various signaling processes during the initiation and execution of apoptotic cell death **(10, 13, 14, 21, 30, 32, 53)**. Importantly, the robustness of the bistable behavior was shown to be dependent on both the network structure as well as the parameter ranges **(4, 10, 32, 55)**. While these studies emphasize the importance of all-or-none and irreversible decision switches during apoptosis, they also suggest that these responses can become gradual and/or reversible if the signaling network is sufficiently altered in topology or quantitative composition. Correspondingly, it was experimentally shown that a sharp threshold concentration of the inhibitor-of-apoptosis protein XIAP can separate conditions of robust all-or-none apoptosis execution from conditions where effector caspase activation becomes sublethal **(11)**. Furthermore, mild caspase activation indeed has repeatedly been reported as a requirement for several physiological processes of cellular differentiation and proliferation **(50)**.

Statistical Models of Apoptotic Signaling

Apart from modeling the mechanistic detail of molecular interactions, generating abstract models based on large sets of experimental data has repeatedly served useful to elucidate cause-and-effect relationships among apoptotic stimuli, signal transduction processes, and their consequences for cellular fate **(15–17, 56)**. One of the biggest challenges here is to integrate heterogeneous data from different experimental approaches into one computational framework **(56)**. The complexity of the data can mathematically be tackled by statistical analyses. After data clustering, these simplify multidimensional data by identifying its principal components as well as predict cellular responses to system perturbations by applying statistical regression procedures **(57)**. Similar analyses can also be applied to large data sets generated directly by mathematical ODE models, combining the mechanistic detail of the signaling networks with statistical modeling procedures **(58)**.

Such multivariate statistical analyses were comprehensively employed by Janes et al. to analyze TNF-α-induced cell death and survival signaling in human colon carcinoma cells in the absence or presence of additional prosurvival factors such as insulin or epidermal growth factor **(15–17)**. Besides successfully tackling the complexity of the data, they surprisingly also found that following TNF-α-induced proapoptotic signaling, anti- and proapoptotic autocrine signaling loops are being activated in sequence and may significantly contribute to the cell fate decision. Indeed, targeted experimental studies supported these predictions and highlight the power of multivariate statistical analysis of experimental data generated by high-throughput methods **(15)**.

Current and Future Challenges in Systems Modeling

The requirement for quantitative experimental data increases with the rapid development of systems biological approaches, while historically such data were seldom required to answer classical research hypotheses in cell biology. Mathematical systems models, especially those designed to resemble specific cell types, require data such as binding/dissociation constants, enzyme activities, or intracellular protein concentrations. Binding/dissociation constants for many key proteins involved in apoptotic signaling are already described with high accuracy. In contrast, assigning specific enzyme activities in a mathematical model is more challenging, as, for example, the activities of one and the same caspase toward different substrates can differ significantly **(59–63)**. Furthermore, the lack of rigorously standardized protocols adds to variability in published data. It thus requires an excellent understanding of the biochemical methodology to appropriately interpret the published literature and choose biologically meaningful parameters. Finally, and probably most limiting for the modeling of specific cell types, very little quantitative data has been

published on intracellular protein concentrations, as historically such data were seldom required. However, platforms and databases for quantitative cellular data are now being developed **(38)**. In addition, repositories such as KEGG and BioBase provide additional pathway information as well as proteomic and kinetic data **(64, 65)**. The extended description, standardization, and normalization of heterogeneous experimental data are likely to significantly enhance upcoming systems studies in apoptosis signaling **(38, 56)**.

Similar trends can be seen on the side of computational model generation. Standardization is driven by the need for program code compatibility between different modeling softwares and has resulted in markup languages for systems biology, curated databases, and repositories for validated models **(66–69)**.

Ultimately, the success of systems approaches toward understanding apoptotic signaling will lie in a successful combination of both mathematical theory and experimental practice. As the fields of mathematics and biology share little or no scientific language, a widespread and successful generation of interdisciplinary teams with expertise in both fields continues to be a major challenge. Even though systems approaches toward intracellular signaling are still sparsely used, they probably will gain much wider acceptance in the future and will prove helpful in elucidating the properties not only of apoptotic but also of necrotic and autophagic signaling networks.

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Part II
Apoptosis in Model Organisms

Chapter 13
Programmed Cell Death in Plants: Apoptotic but Not Quite

Naohide Watanabe and Eric Lam

Abstract Plants have evolved distinct pathways and strategies from those of animals to cope with environmental stresses as well as microbial invaders. This is in part due to the sessile nature of plants as well as their particular mode of cellular organization and architecture. Whereas apoptosis in animals is a common mechanism for the clearance of unwanted cells by neighboring cells or circulating macrophages, the presence of a rigid cell wall in plants precludes this cellular cleansing strategy, and instead other mechanisms more akin to autophagic cell death would be required to remove undesirable cells. In spite of the apparent importance of programmed cell death (PCD) in plants, a molecular description of the key executioners and their control pathways remains elusive. Nevertheless, some of the common cytological changes that have been observed between plant PCD and animal apoptosis suggest that the underlying strategies for dismantling eukaryotic cells may have evolved before the divergence of these two kingdoms. Recent studies on the involvement of various proteases and the highly conserved Bax inhibitor-1 protein in control of plant PCD are consistent with this notion, and their further study should reveal novel insights on the cell death engine in plants.

Keywords Programmed cell death · Plants · Caspase-like protease · BI-1 · Reactive oxygen species · ER-stress · Metacaspase

Introduction

Apoptosis, a Greek work meaning "dropping off or shedding of leaves or petals from plants," was originally used in the seminal work of Kerr, Wyllie, and Currie to describe the so-called programmed cell necrosis observed when

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animal cells undergo cell death upon physical trauma **(1)**. The term is an apt moniker, as it implies an innate, ordered process whereby damaged or unwanted parts of the organism are removed. In the decades following this original work, the field of programmed cell death (PCD) has steadily grown in biomedical research, and through a combination of genetic, biochemical, and molecular approaches, apoptosis has became the most well-understood form of PCD in eukaryotes. The botanical origin of this moniker, however, is a bit ironic since the advances in our understanding of plant PCD control mechanisms have still remained largely phenomenological and the molecules that comprise the death engine of a plant cell still need to be identified. Since the mid-1990s, the study of PCD mechanisms in the plant field has largely focused on the cell death observed during plant-pathogen interaction and the formation of tracheid elements in the xylem **(2, 3)**. More recently, several other model systems, such as heat stress in *Arabidopsis* cell cultures **(4)**, the pollen death during self-incompatibility (SI) interaction in poppy **(5)**, the programmed degeneration of suspensor cells during embryogenesis of a pine tree *Norway spruce* **(6)**, and the programmed death of specific zones in leaves of the lace plant **(7)**, are showing promise as fertile grounds to make important contributions to our understanding of PCD control in plants. A common feature that has consistently been found in these different PCD models is the activation and apparent requirement of one or more caspase-like protease (CLP) activity, in contrast to the cell death during xylem formation, where CLP is apparently not involved. In addition, cell shrinkage, chromatin condensation, and DNA fragmentation before the compromise of nuclear integrity as revealed by TUNEL assays are commonly observed. These are all classic cellular morphologies described for apoptosis and distinct from necrotic cell death. In striking contrast to these apparent morphological and biochemical similarities to animal apoptosis, sequenced plant genomes revealed the absence of plant homologues to several key regulators in animal PCD. These include canonical caspases, BCL-2-related proteins, and p53, a key transcriptional regulator in animal cell death. Thus, PCD in plants must be controlled via a distinct set of regulators that can perform similar functions to these well-studied components of the apoptosis circuit. The elucidation of plant PCD control will thus benefit our ability to improve plant tolerance to biotic and abiotic stresses for agriculture and forestry, and it may also provide new strategies for manipulating cell death in animal systems as well.

The comparison between PCD mechanisms in plants and animals is also important from an evolutionary perspective. Blackstone and Green hypothesized that the generation of reactive oxygen species (ROS) from the mitochondrion may be a common link for PCD activation in different eukaryotes that is a consequence of the symbiotic relationship between the protomitochondrion and the progenitor of eukaryotes that had engulfed it **(8)**. Although the direct participation of cytochrome c in activating plant PCD has not been demonstrated, the mitochondrial permeability transition has been commonly observed **(9)**. Furthermore, the heterologous expression of mammalian Bax,

a pro-death regulator of the BCL-2 family that localizes to the mitochondrial membrane, can activate PCD in both yeast and plant systems **(10, 11)**. Together, these observations point to the mitochondrion as the organelle that may intimately link cell death evolution in eukaryotes through its ability to generate ROS as a signal to jump-start the death engine. Using the yeast system for rapid positive screens with cDNA libraries, genes that can suppress cell death upon Bax expression have been identified from both plant and mammalian systems **(12, 13)**. One particular gene, Bax inhibitor-1 (BI-1), was first identified from the Reed lab and found to be a highly conserved transmembrane protein that can function to suppress cell death across kingdoms and can repress multiple cell death pathways. These characteristics suggest the control level at which BI-1 and its homologues in yeast and plants operate should shed light on an ancient component of the cell death engine that may be targeted by the protomitochondrion. Plastids, a second organelle of endosymbiotic origin, have also been shown to play important roles in plant cell death signaling through its roles in ROS homeostasis and lipid metabolism. Whether BI-1 can also suppress cell death induced by plastid-derived signals would be an interesting question to test in the future. In another effort to identify common regulators of PCD from phylogenetically distant organisms, proteases that are structurally related to caspases have been identified through structure-based iterative searches of existing genome databases **(14)**. In yeast, protozoan, and plant genomes, where canonical caspases are not present, an ancient cysteine protease family called metacaspase with structural similarities to caspases is found. Interestingly, these proteases do not have CLP activity, although their roles in PCD have been shown in yeast and plant systems **(15–17)**. Together with the observation that one or more CLP may be downstream from metacaspases in cell death induction, the idea that caspases in metazoans may have arisen more recently to replace metacaspase functions is a possible scenario.

In this chapter, we describe the current state of our understanding in components that are likely to play an important role in the cell death machinery of plants. Since several recent review articles have already been written in this field **(18, 19)**. We focus mainly on pertinent discoveries from the past several years. Our intention in the context of this volume is to contrast the mechanisms and pathways that may serve as conserved switches in cell death control among eukaryotes.

Morphological Changes During Plant PCD

The sessile nature of plants necessitates a more flexible body plan that is responsive to changes in the environment. This is achieved in large part through the ability to maintain and create new meristems, which essentially are organized stem cell populations that can be regulated through the actions

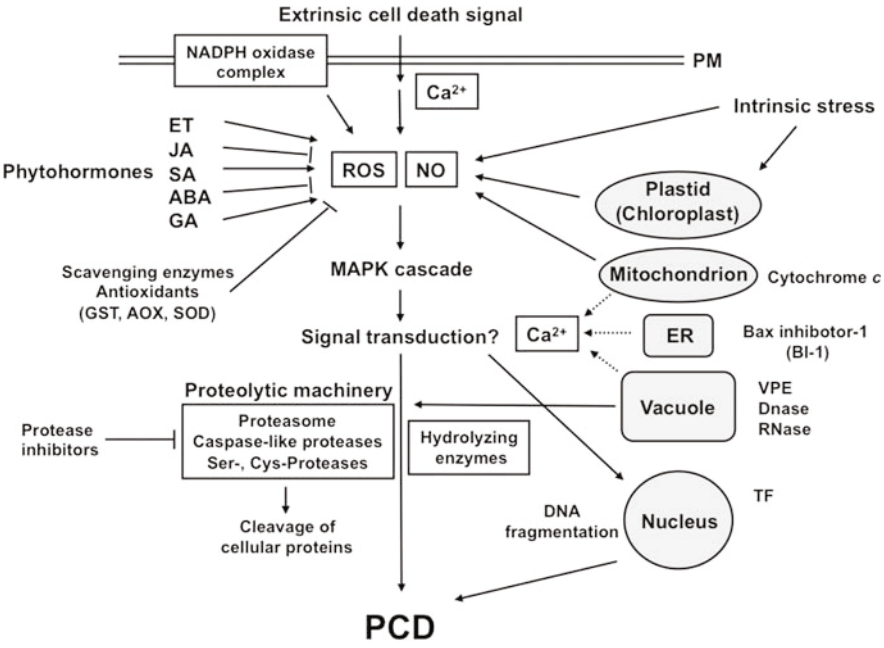

Fig. 13.1 A working model of programmed cell death in plant cells. The main mediators and proteins as well as the regulatory sites for selective processes involved are shown. See the text for detail. ABA, abscisic acid; AOX, mitochondrial alternative oxidase; ER, endoplasmic reticulum; ET, ethylene; Cys, cysteine; GA, gibberellic acid; GST, glutathione S-transferase; JA, jasmonic acid; MAPK, mitogen-activating protein kinase; NO, nitric oxide; PCD, programmed cell death; PM, plasma membrane; SA, salicylic acid; Ser, serine; SOD, superoxide dismutase; TF, transcription factor; VPE, vacuolar processing enzyme

of multiple phytohormones. The phytohormones such as ethylene, abscisic acid, cytokinin, auxin, and gibberellic acid have all been found to affect PCD in different plant cell types and developmental contexts **(3**, **18)** (see Fig. 13.1). Thus, as with growth factors and hormones in animal systems, phytohormones in plants can serve to regulate both cell proliferation and cell death in order to control the growth of the organism. PCD can also be used to alter plant architecture as a function of the season **(18)**, such as leaf senescence in autumn, or the natural perforations in mature leaves of the lace plant **(7)**. Although there are no circulatory systems that can transport cells and other molecules within plants, plant cells are "connected" to their neighbors through plasmodesmata channels, whereas specialized phloem and xylem cells carry out the bulk flow of water, nutrients, and other molecules within plants **(20)**. These conduits of vascular plants in fact are exploited by plant viruses for systemic infection of their hosts. The timely induction of the hypersensitive response cell death (HR-cell death), in which the intercellular connections are rapidly closed off due to apparent shrinkage of the plasma

membrane in the dying cell, has been shown to be an important component of virus resistance to limit systemic spread of the pathogen **(21)**.

Aside from the presence of a rigid cell wall that precludes cell migration and engulfment, plant cells also contain a big vacuole that can take up more than 80% of its total volume. These fundamental differences in cellular architecture to those of animal cells thus limit the extent that PCD in plants can be compared to apoptosis in metazoans. In a few cases, dying plant cells have been reported to fragment into smaller packets of cellular material reminiscent of apoptotic bodies **(22)**. In most cases, however, vacuole disruption and/or plasma membrane shrinkage were observed **(3**, **18**, **19)**. The cytological events during developmental cell death observed upon the transdifferentiation of xinnia mesophyll cells into tracheids *in vitro* are perhaps the most well characterized **(3)**. In this case, vacuole collapse is apparently the critical event that leads to the demise of the cell, and the timing of this event is controlled by a combination of the auxin/cytokinin ratio and the brassinosteroid production in the cell suspension. From these considerations, plant PCD most likely involves a "self-eating"—autophagic—process that would require its own components to carry out its own execution and removal. The recent interest in nonapoptotic cell death pathways, both autophagic and necrotic, in animals again points to the fact that these types of PCD pathways may be more ancient than apoptosis and could be more widely distributed cell death mechanisms in nature **(23)**.

The coexistence of multiple cell death pathways was shown previously in a study on interdigital cell death of the mouse **(24)** and is further apparent in the developmental cell death of the *Drosophila* salivary gland, where autophagic components may be involved in the activation of downstream caspases **(25)**. More recent studies with death receptor activation in mammalian cells also suggest that a nonapoptotic cell death with features of both autophagy and necrosis, called *necroptosis*, can occur when caspase activities are suppressed **(26)**. In plant PCD models, although the role of autophagy in HR-PCD has recently been examined by reverse genetic approaches such as virus-induced gene silencing and antisense RNA suppression of the AtATG6/Beclin 1 homologue in *Arabidopsis* **(27**, **28)**, it appears to function more in the process of limiting the systemic propagation of cell death signals rather than playing an essential role in the execution process. Further studies with mutants that have lesions in different autophagy genes will be necessary to confirm and extend these observations as well as to test the possibility that some of these genes may also exert their action on cell death control through regulation of vesicular transport or the maturation that may be needed for the intracellular transport of key death regulators **(29)**. As has been observed in the heat-stressed activated cell death model system with *Arabidopsis* cultured cells, careful observation of the dying cells' morphologies may be necessary to help define the contribution of different cellular pathways in the suicide process **(4)**.

Mediators of PCD Signals in Plants

Reactive Oxygen Species (ROS) as Effector and Mediator

ROS such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and nitric oxide (NO) have been recognized as signaling molecules to control many biological systems. The involvement of ROS in a variety of cell death systems is well characterized in plants, while the contribution of NO in plant PCD remains obscure. The inter- and intracellular generation of ROS resulting in oxidative burst is widely accepted to activate HR cell death **(18, 30)**. Signaling responses of ROS include the activation of mitogen-activated protein kinases and changes in gene expression leading to PCD. In *Arabidopsis,* a network of at least 152 genes controls ROS metabolism **(31)**. This network regulates the rates of ROS production and ROS scavenging in different cellular compartments such as chloroplasts and mitochondria and modulates the steady-state level of ROS for signaling as well as defense meanings. In leaf cells, an intricate balance exists between H_2O_2 and O_2^- production in the chloroplast and peroxisome during photosynthesis and the activities of the ROS-scavenging enzymes superoxide dismutase, ascorbate peroxidase, and catalase **(31, 32)** (also see Fig. 13.1). Many unfavorable environmental conditions such as cold, heat, UV radiation, and exposure to ozone, fungal toxin, and pathogens lead to oxidative stress due to increased ROS production and/or impaired ROS detoxification **(31, 33)**. Although the major intracellular sources of ROS in plant cells are plastids, mitochondria, and peroxisomes, the endogenous production of ROS from the endoplasmic reticulum (ER) and related intracellular compartments has been proposed to act as part of the intracellular communication system that regulates the stress response **(34)**. ROS are thought to be important for the regulation of several developmental processes, including root hair growth and elongation, apical dominance, leaf shape, tracheary element maturation, trichome development, aleurone cell death, and senescence **(32–34)**. Thus, ROS networks of production and action seem to be complex and can vary depending on different physiological conditions.

The role of ROS in plant PCD has been most extensively studied during the HR cell death in response to pathogens, when ROS are generated rapidly and transiently at the site of infection. This process, called *oxidative burst*, is mediated by plasma membrane-localized plant respiratory burst oxidase homologue (Rboh) proteins **(30)**, which are homologous to the mammalian 91-kDa glycoprotein subunit of the phagocyte oxidase ($gp91^{phox}$) or NADPH oxidase. This protein was initially described in mammalian neutrophils as a multicomponent complex mediating microbial killing. *Arabidopsis* plants have a multigene family of 10 *Rboh* genes (*AtrbohA–J*). Studies of mutants in members of this gene family have shown that *AtrbohD* and *AtrbohF* contribute to superoxide and hydrogen peroxide formation in the oxidative burst caused by bacterial and fungal pathogens **(35, 36)**. While specific ROS sensors in plants

remain elusive, ample data point out different components of the ROS signal network, including kinases, phosphatases, and redox-responsive transcription factors. For example, the zinc-finger proteins LSD1 and LOL1 are negative and positive regulators, respectively, of superoxide-induced cell death in *Arabidopsis* **(37, 38)**. They have been proposed to act as a molecular rheostat to sense and transmit the superoxide-derived signal. The phenotype of *lsd1* mutants is uncontrolled, spreading cell death (runaway cell death, rcd) that is initiated by superoxide. A triple mutant with *lsd1* and *atrbohD/F* showed uncontrolled cell death even under growth conditions that normally exhibit minimal *lsd1* cell death phenotype **(36)**. A recent study demonstrated that a bZIP transcription factor, AtbZIP10, is an important positive mediator of the uncontrolled cell death phenotype in *lsd1* mutant **(39)**. In wild-type *Arabidopsis* plants, LSD1 is required for the cytoplasmic retention of AtbZIP10 **(39)**. In *double mutants* of *atbzip10/lsd11*, the rcd phenotype of *lsd1* is suppressed, whereas the overexpression of AtbZIp10 in *lsd1* mutant background accelerates the *lsd1* phenotype. It thus appears that AtbZIP10 serves as a positive factor for HR cell death and basal defense pathways that are negatively controlled by LSD1 activity. However, AtbZip10 overexpression in a wild-type background does not significantly alter the progression of HR cell death and defense responses against fungal pathogens. It is thus proposed that LSD1 and AtbZIP10 act antagonistically in these processes, allowing the exact adjustment of the cell death response following the perception of stress signals that lead to cell death **(39)**.

The function of Rboh proteins was initially thought to be involved in ROS production during HR cell death, but more recent studies showed that ROS generated by Rboh activity may also be involved in mediating stomatal closure induced by the phytohormone abscisic acid **(40)**. Furthermore, Rboh activity was shown to be required for plant development since mutants in various *Atrboh* genes are affected in growth and development: Plant size and root length are reduced in knockout mutations in *AtrbohF* and in the *AtrbohD/F* double mutant, while root length is reduced and root hair elongation is impaired in *AtrbohC* mutants **(41, 42)**.

Role of Salicylic Acid

In contrast to animals, plants synthesize salicylic acid (SA) and activate SA-dependent physiological programs. SA accumulates dramatically in plant cells in response to challenges by a diverse range of phytopathogens and is essential to the establishment of both local and systemic-acquired resistance. The inhibition of SA accumulation in plants by expressing SA hydroxylase or through loss-of-function mutations in *NPR1,* an important signal integrator downstream from SA, revealed that SA is essential for cell death activation in some mutants but contributes to signal amplification or even to suppression of cell death in others **(43)**. This observation suggests that SA acts in a feedback loop

both upstream and downstream of cell death and that SA can function as a prodeath and a prosurvival signal, depending on its concentration and cellular context. Importantly, both SA and ROS signals are thought to act together in a positive-feedback loop to promote HR cell death, since exogenously applied H_2O_2 stimulates SA synthesis and ROS generation that depends on active SA synthesis **(44)**. SA accumulation can downregulate ROS scavenging systems that, in turn, can contribute to increased overall ROS levels following pathogen infection **(45)**. More recently, the interplay between SA accumulation and Rboh-dependent ROS generation in *Arabidopsis* plants was characterized genetically. In fact, ROS produced by AtrbohD and AtrbohF antagonizes the SA-dependent procell death pathway to restrict uncontrolled cell death beyond the site of HR **(36)**, suggesting that cross-talk between ROS- and SA-dependent cell death pathways seems to be very complex. Both ROS and SA signals might mediate distinct functions in different cellular and spatial contexts, and in relation to other regulatory signals. For example, SA and the phytohormone jasmonic acid (JA) were shown to either synergize or mutually antagonize their signaling functions at different concentrations **(46)**.

Involvement of Calcium and Its Related Signaling

Calcium (Ca^{2+}) is a well-known and universal intracellular messenger that can control a broad range of cellular processes in eukaryotes. As plants and animals have evolved, however, Ca^{2+} signaling systems have diverged and become more complex. In plants, Ca^{2+} signals are implicated to function in most aspects of growth and development as well as stress responses against abiotic stresses, mechanical wounding, and pathogen attacks **(47)** (see Fig. 13.1). Elevated calcium levels have been observed during developmental PCD such as trachaery element differentiation, aerenchyma formation, aleurone cell differentiation, and leaf senescence, in addition to HR cell death. Earlier studies showed that Ca^{2+} channel blocker lanthanum chloride ($LiCl_3$), which targets the plasma membrane-localized form, can block H_2O_2-induced cell death in soybean cells, bacteria-induced HR cell death in *Arabidopsis,* and camptothecin-induced cell death in tomato cells **(18)**. Consistent with a critical role for the transient elevation of Ca^{2+} levels in defense signaling, three *Arabidopsis* mutants that constitutively express defense responses and spontaneously form HR-like lesions were found to contain defects in Ca^{2+}-related proteins. On the one hand, the *cpn1*/*bon1* mutation disrupts a copine-encoding gene **(48)**. Copines are a newly identified class of Ca^{2+}-dependent phospholipid binding proteins that have been detected in diverse organisms ranging from ciliates to humans **(49)**. On the other hand, the *defense-no-death1* (*dnd1*) *Arabidopsis* mutant that has a null mutation in the *CNGC2*/*DND1* gene and displays no visible HR cell death links cyclic nucleotide monophosphate-dependent Ca^{2+} influx to NO generation and PCD of plants to pathogen infection **(50**, **51)**. Recently, another

Arabidopsis CNGC mutant, *cpr22* (*atcngc11/12*), exhibited spontaneous lesion formation resembling that of pathogen-induced HR cell death but also showed alterations in pathogen resistance and expression of some defense-related genes in the absence of pathogens **(52)**. Recent molecular and biochemical study revealed that the cell death phenotype of *cpr22* mutants has all the characteristic hallmarks of PCD and that the chimeric calcium channel, ATCNGC11/12, is at the point or upstream of the calcium signaling step necessary for development of the HR. The finding that cell death induction by ATCNGC11/12 is apparently mediated at least in part by activation of the vacuolar processing enzyme (VPE), which exhibits caspase-1-like activity and localizes to the vacuole **(53)**, correlated with the previous observation that an influx of Ca^{2+} can initiate cell death and leads to vacuole collapse in plant cells **(54)**.

In animal cells, the intracellular calcium level plays a critical role in death pathways triggered by apoptotic stimuli. In fact, the amount of calcium ion stored in the ER determines the sensitivity of the cells to apoptotic cell death **(55, 56)**. Translocation of calcium ions from the ER to the mitochondrion appears to be required to induce cell death by some apoptotic signals, while alteration of calcium levels in the ER could initiate apoptosis **(57, 58)**, suggesting the existence of cross-talk between the ER and mitochondrial cell death pathways. Present data also indicate that calcium signaling is an important mediator of various forms of plant PCD, but a well-defined calcium switch and its related cellular processes mediated by either the ER or mitochondria in plants have yet to be elucidated.

Modulation of PCD by Lipid-Related Signals

Sphingolipids consist of a diverse group of lipids that contain a relatively large hydrophobic moiety, known as ceramides, that includes a sphingoid or long-chain base amide linked to a fatty acid. Sphingolipids not only are ubiquitous membrane components in bacteria and eukaryotic cells but also act as second messengers to regulate stress response, cell proliferation, and apoptosis **(59)**. The balance between the bioactive sphingolipid ceramide and its phosphorylated derivative has been proposed to modulate the amount of PCD in eukaryotes. In plants, several studies indicated that fumonisin B1 (FB1) and *Alternaria alternate lycopersici* (ALL) toxin, two fungal toxins that specifically inhibit ceramide synthetase activity, cause apoptotic cell death in various plant species **(22, 60)**. By contrast, the exogenous administration of ceramides was shown to induce apoptotic cell death in *Arabidopsis* cells in a calcium-dependent manner **(61)**. More direct evidence was obtained from genetic studies as well. *Arabidopsis ACD11* encodes a sphingosine transfer protein, and a mutation in this gene causes a lesion mimic phenotype characteristic of PCD **(62)**. Similarly, *Arabidopsis ACD5* encodes a ceramide kinase that exhibits high specificity for ceramides but not other sphingolipids, and a loss of function of *ACD5* causes spontaneous

cell death phenotypes **(63)**. These findings strongly suggest that the maintenance of sphingolipid homeostasis is important for proper regulation of PCD in plants. However, the mechanism of sphingolipid-mediated PCD in plant cells remains unclear.

Mechanisms for PCD Activation: The Role of Proteases and Core Regulators in Plant PCD

The Role of Proteases

Many proteases involved in apoptotic processes have yet to be determined, but several are already well characterized. Caspases, a group of specialized cysteine proteases, traditionally held the predominant roles as key players of execution. However, recent evidence indicated that noncaspases, which include calpains, cathepsins, granzyme B, Omi/HtrA2, and the 26S proteasomes, have roles in mediating and promoting apoptosis **(64–68)**. Since plant PCD and apoptosis share many physiological and morphological features, it is reasonable to suspect that protease components of PCD may be conserved between the kingdoms. Several proteolytic systems, in fact, have been suggested in plant PCD and its associated processes. The particular combination of protease activities can be variable, depending upon both the origin of cell death stimuli and the cell type involved **(18**, **69**, **70)**. Recently, two proteases with caspase-like activities have been identified as belonging to two different protease families that are not closely related structurally to animal caspases. Various other protease families have also been implicated, which suggests that all these proteases, collectively called *death proteases*, can potentially form complex protease networks for plant PCD **(71)**.

Caspase-Like Proteases

Although there are no canonical caspase homologues in plants, indirect evidence for the involvement of caspase-like proteases (CLPs) to control PCD activation has been suggested from pharmacological studies by many laboratories using various cell death models **(5**, **70–75)**. This conclusion is largely based on the following observations: (1) Many plant extracts, obtained from cells undergoing PCD, contain endopeptidase activities cleaving synthetic caspase-specific substrates; (2) these CLP activities are not inhibited by general cysteine and serine proteases inhibitors; (3) synthetic caspase inhibitors can block plant PCD and the associated morphological and biochemical changes; (4) natural caspase substrates [e.g., bovine and plant poly(ADP-ribose)polymerase] can be cleaved by plant CLPs. However, most of the evidence to date is correlative and needs to be verified by conclusive molecular and genetic evidence. Cell death in most of the reported cases can also be blocked by some of

the general cysteine and serine protease inhibitors. This implies that plant cell death is regulated both by proteases with caspase-like properties in terms of substrate specificity as well as by other unidentified proteases. Bozhkov et al. **(76)** found that induction of caspase-6-like activity (VEIDase activity) correlates with embryonic cell death during somatic embryogenesis in *Norway spruce* cell culture. Specifically, autophagic cell death is observed in the suspensor, a structure that is eliminated in the final steps of embryogenesis in either somatic or zygotic embryos. Inhibition of the VEIDase activity suppressed the observed PCD, resulting in embryonic aberrations **(76)**. Aside from these pharmacological and biochemical studies, the use of baculovirus p35 protein, a broad-range caspase inhibitor that can effectively suppress apoptosis, in transgenic plants, nicely demonstrated that it can block cell death in several experimental systems: *Agrobacterium tumefaciens*-induced PCD in maize embryonic callus **(77)**, AAL toxin- (from *Alternaria alternata* f. sp. *lycopersici*) induced cell death in transgenic tomato plants **(78)** or nonhost and gene-for-gene HR cell death upon bacteria or virus challenge in transgenic tobacco plants **(21)**, and UVC-induced PCD in transgenic *Arabidopsis* plants **(79)**. These physiological inhibitor studies support an important role for CLPs during cell death in plants.

Vacuolar Processing Enzyme (VPE)

VPE, a plant cysteine protease that belongs to the legumain family and that is also found in animals (family C13), was identified as a potential plant counterpart to animal caspases that is essential for some pathogen-induced HR **(80–82)**. Although VPE does not share significant sequence similarity to animal caspases, it exhibits caspase-1-like activity (YVAD-hydrolyzing activity) and disrupts the vacuole during pathogenesis and development, consequently contributing to PCD **(80, 82)**. VPEs have an alternative usage of aspartate or asparagine at the substrate cleavage site, which is consistent with the structural modeling of legumains and caspases that belong to the same clan of protease family (CD) **(74, 83)**. Interestingly, recombinant VPEs do not cleave caspase-3 substrate (DEVD substrate), although their activities are sensitive to caspase-3 inhibitor (DEVD-fmk), indicating that VPEs are not responsible for caspase-3-like activity in plant tissue undergoing PCD **(82)**. Furthermore, it was suggested that p35 inhibits VPE's caspase-1-like activity *in vitro* **(82)**. In *Arabidopsis* plants mutated at the *VPEγ* locus, which expresses the major form of VPE in vegetative tissues, relatively small effects on pathogen-induced HR cell death and resistance were observed with mature leaves **(81)**, while a strong suppression of cell death induction by FB1 mycotoxin was reported for young seedlings of *vpeγ* **(82)**. These contrasting results suggest that the importance of VPE in PCD induction may vary depending on developmental stages and other factors. More detailed studies of VPE and its associated CLP activity would also be needed to define the role of this normally vacuole-localized protease to orchestrate cell death events in the cytosol and elsewhere in the cell **(73)**.

Metacaspases

Two new groups of caspase-related cysteine proteases, designated as paracaspase (found in metazoans and slime mode) and metacaspase (found in fungi, protozoa, and plants), were identified by iterative database searches **(14)**. In general, metacaspases and paracaspases possess the catalytic dyad of histidine and cysteine that comprises the active site of the caspases, but they otherwise display low overall sequence similarity (family C14). This suggests that metacaspases in plants, fungi, and protozoa may be at the evolutionary root of caspases. Although metacaspases (MCPs) have been considered to be strong candidates for caspase homologues in plant, yeast, and protozoa, the biochemical characterization of metacaspases from *Arabidopsis* and *Norway spruce*, budding yeast, and protozoa have demonstrated that they are arginine-/lysine-specific cysteine proteases and do not cleave any synthetic caspase substrates *in vitro* **(15**, **16**, **84–87)**. Similar to caspases, however, their activity also involves internal cleavage; point mutation at their active site cysteine residue, predicted from structural comparison with caspases, can abolish their protease activity. Thus, in spite of their difference in target site preference, MCPs seem to have structural similarities with caspases. Very recently, it was shown that human paracaspase MALT1 acts as an arginine-/lysine-specific cysteine protease; this activity is required for optimal transcription factor NF-kB activation and the production of interleukin-2 in T cells **(88**, **89)**. However, no correlation with cell death regulation was observed for paracaspases.

In the *Arabidopsis thaliana* genome, there are three type I (e.g., AtMCP1a–1c/AtMC1–3) and six type II MCPs (e.g., AtMCP2a–2e/AtMC4–9) **(15**, **90)**. It has been suggested that the constitutive overexpression or a disruption of some AtMCP members does not lead to an obvious phenotype (i.e., cell death phenotype or abnormalities in normal plant growth and development) under normal growth conditions **(17**, **91)**, our unpublished results]. Redundancy seems to exist between each subfamily member, or other factors may be necessary to control MCP activities *in vivo* **(92)**. Transcripts of several AtMCPs are rapidly upregulated in plant tissues in response to pathogen challenges, during a variety of abiotic stresses, and in senescing leaves and floral tissues **(74)**, our unpublished results]. Furthermore, our *in silico* analyses revealed that each AtMCP member shows different tissue- and growth stage-specific expression patterns, suggesting that some members of this family may be involved in the regulation of PCD and some may be involved in other processes (unpublished results). Also, each MCP activity may be controlled at multiple posttranslational levels. For example, the activity of AtMCA9/AtMCP2f could be controlled by a proteinous inhibitor AtSerpin1/AtSAS1, its autocatalytic processing depending on pH at weak acidic condition (pH 5–6), and *S*-nitrosylation **(15**, **91**, **93)**. Interestingly, some of the recombinant AtMCPs, with the exception of AtMCA9/AtMCP2f, showed a pH optimum at around neutral pH (7–8) and clear Ca^{2+}-dependency for their activation with autocatalytic processing *in vitro* **(15–17**, **84)**. These finding suggest the possibility that multiple factors may be used to control the physiological

activities of plant MCPs. Cellular functions for a specific metacaspase member may be inferred on the basis of distinct subcellular localizations and/or specific tissue and temporal expression patterns.

Two *Arabidopsis* MCPs (AtMCP1b and 2b) and a *Leishmania major* MCP (LmjMCA) were shown to functionally complement a null mutation of the *Saccharomyces cerevisiae* metacaspase *YCA1/MCA1*, which is the best-characterized metacaspase in terms of its biological function implicated in the death of aging yeast and cells exposed to different environmental stresses (see Chapter 14 in this volume). This indicates that MCPs belong to a conserved protease family that has similar biological activities in plants, protozoa, and yeast **(16**, **17**, **85)**. Furthermore, it was shown that yeast PCD can indeed be suppressed by the pancaspase inhibitor zVAD-fmk, which does not show any significant inhibitory effect on either recombinant YCA1 or AtMCPs. This observation suggested the presence of a cryptic CLP downstream of YCA1 that is required for PCD induction **(16)**. In addition, evidence of a positive-feedback mechanism for reactive oxygen species (ROS) as activator of MCP action during yeast PCD was observed **(16)**. Thus, although ROS triggers the activation of YCA1 or AtMCPs in yeast cells at the early stage of oxidative stress, further MCP-dependent ROS amplification seems likely to precede the onset of chromatin destruction. Interestingly, it was recently shown that one of the type II metacaspase genes in *Arabidopsis*, *AtMC8/AtMCP2e*, is highly upregulated temporarily by oxidative stress induced by UVC and H_2O_2 treatment and plays a role in activation of the subsequent cell death **(17)**. Further studies will therefore be needed to decipher the mechanisms and pathways of MCP-mediated cell death, which would be important for the control of plant development and stress response.

Saspases

Plant subtilisin-like proteases are serine endopeptidases that exist as large multigene families in plants **(94)**. Some subtilisin-like proteases have been associated with plant development **(95**, **96)** and ER stress response **(97**, **98)**. Recently, Coffeen and Wolpert purified and characterized two subtilisin-like proteases from the tissues undergoing victorin-induced PCD in oat (*Avena sativa)* seedlings **(99)**. The authors named these two enzymes *saspase* because of their caspase-like cleavage specificity (aspase) and active-site serine residue. Purified saspases showed maximal activity at neutral pH and cleaved many types of synthetic caspase substrates with different efficiencies: They showed high levels of peptidase activity toward a range of caspase substrates (VAD-, VKMD-, VNLD-, and VEHD-AFC) but not toward other caspase substrates (DEVD-, WEHD-, and VEID-AFC) and a range of other general protease substrates (e.g., subtilisin A, cathepsin B, casein, chymotrypsin, and aminopeptidase substrates). However, direct genetic evidence showing that these enzymes are responsible for victorin-induced PCD is still lacking **(99)**. It should be noted that saspases are not caspase-like proteases in a strict sense, as they appear to be serine proteases. However, this report strongly implies the existence of plant

proteases that exhibit substrate specificity toward caspase recognition sites, which can be abolished by synthetic caspase inhibitors.

Other Proteases

Several studies on particular noncaspase-like proteases and their role in HR cell death have recently been reported. Interestingly, many plant defense-associated proteases are secreted into the apoplast, including RCR3 **(100)**, CDR1 **(101)**, and cathepsin B **(102)**. Papain (family C1) is a small protease with broad substrate specificity and is believed to play an important role in plant growth and development as well as plant defense response **(71)**. Using their natural inhibitor cystatin and the cell-permeable pharmacological inhibitor E-64d, it was shown that E-64d can suppress H_2O_2- and pathogen-induced cell death in plants and that the overexpression of cystatin in plant cell suspensions blocks PCD and the induction of protease activity in response to H_2O_2 or avirulent bacteria **(103–105)**. The role of papain-like proteases is intriguing with the genetic identification of RCR3 and cathepsin B, secreted papain-like proteases of tomato **(100, 102)**. CDR1 is an aspartic pepsin-like protease (family A1) that, when overexpressed, causes constitutive disease resistance to *Pseudomonas* bacteria **(101)**. These studies indicate a key role in plant defense not only for cysteine proteases but also for endogenous protease inhibitors that may act to counter the action of these and other types of proteases.

Role of Core Regulators in Plant PCD

A large class of conserved core regulators related to the BCL-2 protein family plays a prominent role as prodeath or antideath proteins in animal apoptosis. In spite of the apparent lack of BAX and other BCL-2-related genes in plant genomes, several studies have revealed that the transgenic expression of mammalian anti- or prodeath proteins in plants can influence regulatory pathways of cell death activation or suppression **(18, 19)**. These observations thus argue for conserved cell death signaling mechanisms in eukaryotes and the conservation of a core switch in the PCD regulatory pathway. To date, however, only small numbers of endogenous plant genes that show significant sequence similarity to animal apoptotic genes have been characterized.

BAX Inhibitor-1

BAX inhibitor-1 (BI-1) was originally identified from a human cDNA library as a suppressor of Bax-induced lethality in yeast **(12)**. This gene encodes a small protein of 25–27 kDa with six or seven predicted transmembrane domains and is thought to be predominantly localized in the membrane of the endoplasmic reticulum (ER). At present, BI-1 is believed to be one of the few evolutionarily

conserved proteins that can act as a broad-spectrum cell death suppressor in mammals and plants **(90, 106)**. In mammalian cells, BI-1 was shown to interact with BCL-2 but not Bax or Bak, as shown by *in vivo* cross-linking and co-immunoprecipitation studies. Importantly, the overexpression of BI-1 can suppress apoptosis induced by Bax, etoposide, staurosporine, and growth factor depletion, but not Fas (CD95). Conversely, BI-1 antisense induced apoptosis in cancer cell lines, suggesting a new type of regulator of cell death pathways controlled by BCL-2 and Bax **(12)**. In plants, BI-1 is thought to be mainly localized on the ER membrane, as shown by GFP-fusion protein localization **(107)**. BI-1 is expressed in various plant tissues, and its expression level is enhanced during senescence and under various types of biotic and abiotic stresses **(90, 106)**. The overexpression of BI-1 from various plant species has also been shown to suppress BAX-, pathogen-, or abiotic stress-induced cell death in a variety of cells from yeast, plant, and mammalian origins **(90, 106)**. These observations support the idea that BI-1 could have conserved function in diverse organisms. Genetic analysis of *Arabidopsis BI-1* (*AtBI1*) demonstrated that *AtBI1* is dispensable for normal plant growth and development, but plays a protective role against both mycotoxin- and heat stress-induced PCD **(108)**. Recently, the function of mammalian BI-1 was shown to link to the protection of cells from ER stress-induced apoptosis **(109, 110)**. Similarly, a recent study demonstrated by reverse genetic approaches that the AtBI1 level is a critical determinant for plant survival under ER stress, demonstrating that BI-1 is a highly conserved core component that plays a pivotal role as a cell survival factor that is required to delay the onset of PCD upon ER stress response **(111)**. In animal systems, accumulating evidence indicates that both mitochondria-dependent and -independent cell death pathways likely mediate apoptosis in response to ER stress **(56, 57)**. The ER might serve as a site where apoptotic signals are generated through several mechanisms including BAK-/BAX-regulated Ca^{2+} release from the ER. It was recently reported that a plant calmodulin (AtCaM7) can interact with AtBI1 *in vitro* and *in vivo* and that the overexpression of AtBI1 can modulate cell death of tobacco cells induced by a calcium pump inhibitor that causes ER stress in animal cells **(112)**. This suggests the possible connection between AtBI1 and calcium homeostasis. By analogy with animal systems, controlling the calcium content of the ER triggered by stresses might be a key process to activate the downstream pathways that promote cell death in plant cells. Therefore, clarifying the regulation process in ER stress-induced cell death and its connection with other biotic and abiotic stress response pathways may thus provide us with new insights into common and distinct mechanisms leading to their tolerance in plants. In addition, the ability of an ER-localized BI-1 to suppress prodeath signals generated from the mitochondria (e.g., via Bax expression) suggests that ER stress may be a convergence point that continuously monitors the internal state of the cell and that the role of BI-1 as a "rheostat" is an ancient switch that may serve to determine the threshold at which to induce cell death **(113)**.

BON and BAP Families

The *BON* gene was identified from a genetic screen of *Arabidopsis* mutants that are defective in temperature homeostasis and have enhanced disease-resistance phenotype **(48)**. The *BON1* gene encodes one of the copines, a protein that is a calcium-dependent membrane-binding protein and is tightly associated with the plasma membrane and promotes aggregation of lipid vesicles *in vitro*. Importantly, BON1 associates with another protein, BAP1, which can suppress the *bon1* phenotype when it is overexpressed **(48)**. BAP1 is a membrane-associated protein containing a C2 domain with a calcium-dependent phospholipid-binding activity and acts as a negative regulator of defense response against pathogens **(114, 115)**. *BAP1* and related genes encode highly conserved proteins across eukaryotic kingdoms, suggesting the ubiquitous importance of their function in cellular processes. In the *Arabidopsis* genome, there are three *BON* genes (*BON1–3*) and two *BAP* genes (*BAP1* and *BAP2*), suggesting that some redundant and overlapping functions may exist. In fact, genetic analyses demonstrated that the loss of function of *BON1* combined with that of *BON2* and *BON3* leads to extensive cell death phenotypes resembling HR cell death **(115)**. Also, double-knockout mutants of *BAP1* and *BAP2* were shown to confer seedling lethality, whereas the overexpression of both *BAP* genes in wild-type plants suppresses PCD induced by bacterial pathogens, the proapoptotic protein Bax, or oxidative stress **(116)**. The responsiveness of the *BON* and *BAP* genes to diverse stimuli suggests that they may serve as signaling molecules or directly act to maintain calcium or lipid homeostasis during stress responses, since the imbalance of these parameters can likely trigger unwanted cell death activation. BON and BAP should act downstream of the production of H_2O_2, since the overexpression of these genes did not alter the onset of H_2O_2 production during Bax-induced cell death **(116)**. It is interesting to note that BI-1 is also responsive to a variety of environmental stresses and has a similar cytoprotective function with the BON and BAP protein families **(107, 108, 111)**. Thus, it would be interesting to study whether the BON and BAP proteins could be functionally connected with BI-1, which was shown to interact with calmodulin and maintain calcium homeostasis **(112)**.

BAG Protein Family

BCL-2-associated athanogene (BAG) family proteins were originally identified by their ability to associate with the antiapoptotic protein BCL-2, thereby synergistically enhancing cell survival **(117)**. BAG family proteins were also found to interact with heat-shock protein 70 family members (Hsc70/Hsp70) through their BAG domain and can modulate, either positively or negatively, the functions of these chaperone proteins. Therefore, BAG family proteins are characterized as co-chaperones. In animal cells, BAG proteins were shown to regulate diverse physiological processes, including apoptosis, tumorigenesis, neuronal differentiation, stress responses, and the cell cycle **(118, 119)**. Recent

findings demonstrated that the BAG domain is evolutionarily conserved, and BAG domain-containing proteins have been described and/or studied in a variety of organisms from yeasts, animals, and plants **(118**, **120**, **121)**. While information is available on the biological roles of animal BAG proteins, very little is known about the biological function of BAG-like protein in plants. In plants, the first BAG-like gene was functionally identified in a screen for calmodulin-binding protein from an *Arabidopsis* cDNA library. This BAG protein, called AtBAG6, was shown to induce a cell death phenotype of yeast cells consistent with the PCD phenotype, and the overexpression of AtBAG6 in *Arabidopsis* plants caused the dwarfism phenotype and formed disease-like necrotic lesions on the leaves **(122)**. The cell death-stimulating activity of AtBAG6 was shown to depend on its calmodulin-binding motif, suggesting that this protein might be controlled by calmodulin and possibly Ca^{2+} **(122)**. Phenotypic characterization of knockout mutants of AtBAG6 (*atbag6*) revealed that this mutant exhibited earlier flowering and shorter vegetative and reproductive phases, producing more branched roots and influorescence compared with wild-type plants **(121)**. In addition, *atbag6* mutants were shown to exhibit dramatically enhanced susceptibility to a necrotrophic fungal pathogen, *Botrytis cinerea,* which causes gray mold disease on numerous dicotyledonous plants. This indicates that AtBAG6 has a role in limiting pathogen colonization and spread in infected tissues. In the *Arabidopsis* genome, seven BAG-like genes have been identified **(121)**. Although a knockout mutant of *AtBAG4* (*atbag4*) exhibited very similar growth and developmental phenotype with *atbag6* mutants, the development of disease symptom by infection of *B. cinerea* in *atbag4* plants was comparable with that in wild-type plants **(121)**. Interestingly, it was shown that the overexpression of AtBAG4 in *Arabidopsis* and tobacco plants conferred resistance to a wide range of abiotic stresses such as cold, drought, oxidative stress, and high salinity without visible cell death-related phenotype, as seen in *AtBAG6*-expressing plants **(121)**. These observations strongly suggest that plant BAG family members are also multifunctional and remarkably similar to their animal counterparts, as they regulate PCD processes ranging from pathogen attack to abiotic stress and development. It should thus be the subject of considerable interest to study the roles of other BAG-like genes in plants to see if they all have a similar biological function or not, especially with respect to how these proteins may function under normal and various stress conditions.

Future Perspectives

Since the publication of the first edition of this volume, significant steps have been taken to identify several plant gene families that are good candidates for key cell death regulators. The highly conserved nature of these proteins, such as BI-1, BAG, and MCP, are consistent with the idea that they play essential roles

in eukaryotic cell homeostasis. Of critical importance at this juncture is to delineate more clearly the modes of action and regulation for these regulators. For example, a recent report indicated that proteolytic processing of a protozoan MCP is not necessary for its protease activity *in vitro* **(87)**, suggesting that these proteases may have a distinct mode of regulation from the canonical caspases that require cleavage for their activation. If this is confirmed as a general property of all MCPs, then the key issue that would need to be addressed will be the regulatory switch that allows the cell to control MCP proteolytic activity. Obviously, the *in vivo* substrate targets and inhibitors for these proteases that are relevant for PCD activation will be another important area of research.

For the BI-1 and BAG proteins, their ER localization and properties as co-chaperones respectively suggest that ER stress and general protein homeostasis may be a key regulatory switch that integrates diverse cell death signals emanating from metabolic and environmental cues. In this regard, an important step would be to decipher the biochemical activity of BI-1 proteins and how it may attenuate cell death. One possible model would be its function to regulate calcium mobilization from the ER **(109**, **110**, **123)**. The elucidation of the mechanism of BI-1 function would be a critical step in understanding this conserved switch of cell death in eukaryotes.

Lastly, as previously discussed **(18)**, the lack of definitive cell death mutants in plants suggests that multiple pathways of cell death may coexist in parallel downstream from various PCD signals. With the number of likely cell death mediators that have been identified (i.e., VPE, MCP, BI-1, BAP, and BAG), the combinatorial genetic manipulation of multiple switches should now be possible in a single plant line. This approach should allow us to tease out this possible functional redundancy and help to sort out the relative importance of different cell death pathways in different cellular context and development cell death models.

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Chapter 14
Tracing the Roots of Death: Apoptosis in *Saccharomyces cerevisiae*

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Abstract The modulation of cell death is a complex process. Its deregulation can lead to several types of diseases ranging from cancer to neurodegenerative disorders in humans. The finding that *Saccharomyces cerevisiae* can also undergo apoptosis has opened doors to investigate programmed cell death in a clear-cut model organism that unifies both technical advantages and a eukaryotic "cell room." So far, cell death in yeast has been described under multiple conditions, including exposure to different drugs, failure in several cellular processes, or heterologous expression of human proapoptotic genes. Yeast apoptosis has also been shown to occur in physiological scenarios such as aging or failed mating, thus suggesting a teleological explanation for the death of a unicellular organism. Finally, several yeast orthologues of crucial metazoan apoptotic regulators have been identified and characterized, including a caspase and the apoptosis-inducing factor. Uncovering apoptosis in yeast as well as in other fungi and unicellular parasites is of great medical interest. Moreover, it is helping to decipher the molecular mechanisms of cell death in higher organisms.

Keywords yeast apoptosis · aging · programmed cell death · stress · unicellular organism

Introduction

The budding yeast *Saccharomyces cerevisiae* was the first eukaryotic organism with a completely sequenced genome **(1)**. Its characteristics regarding technical advantages for biological studies have made yeast a preferred research tool. These include not being pathogenic, rapid growth, inexpensive accessibility,

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and, most importantly, easy DNA modification accompanied with simple mutant isolation. The disruption of a specific gene or replacement by a modified DNA sequence can be easily achieved through homologous recombination, which has allowed a variety of systematic genome-wide studies in yeast. Furthermore, yeast has proven valuable for the detection of general protein-protein interactions via the two-hybrid screening system.

Thus, the easy handling and technical benefits of yeast resemble those of bacteria but coupled to the functional advantage that yeast is a eukaryote. Therefore, it is particularly suitable as a model organism for diverse questions in general cell biology of higher eukaryotes. In fact, numerous studies have systematically analyzed the function of mammalian proteins in yeast. An example for that is given by the cdc48 protein **(2)**, which led to the first description of apoptosis in yeast **(3, 4)**. Since then, several yeast orthologues of crucial mammalian apoptotic proteins have been found, conserved proteasomal, mitochondrial, and epigenetically regulated cell death pathways have been outlined, and physiological death scenarios such as chronological and replicative aging have been described. Furthermore, assays for apoptotic and/or necrotic cell death such as viability, DNA fragmentation, exposition of phosphatidylserine, cell integrity, or ROS accumulation are routinely used in the field of yeast programmed cell death (PCD) **(5)**.

This chapter primarily aims at giving an overview of the different triggers that can lead to apoptosis in yeast (the routes to death) and the conserved molecular players that are known so far to be involved in this process (the roots of death). Moreover, it attempts to clarify the physiological sense as well as the medical implications of yeast apoptosis (the roofs of death) and briefly summarizes the current knowledge about other unicellular organisms undergoing PCD (the rooms for death).

The Routes to Death—Triggering Yeast Apoptosis

For mankind, there is just one way to enter this world but a thousand ways to leave it. Similarly, the routes leading to death in yeast are multiple and include exogenous and endogenous triggers. Diverse drugs, heterologous expression of proapoptotic human genes, defects in cellular processes, aging, or mating stress all lead to an apoptotic phenotype in yeast.

Exogenous Induction of Yeast Programmed Cell Death

Hydrogen Peroxide and Acetic Acid

The first evidence establishing reactive oxygen species (ROS) as key regulators of yeast apoptosis (see ahead) came from experiments exposing *Saccharomyces cerevisiae* to low doses of H_2O_2. While high doses of H_2O_2 lead to a necrotic

phenotype, low doses induce apoptosis, similar to oxidative stress achieved by glutathione depletion **(4)**. The cell actively participates in this death process, as the inhibition of translation by cycloheximide prevents the development of apoptotic markers in response to H_2O_2. Several molecular players seem to be involved, including the yeast caspase *YCA1* **(6)** and the apoptosis-inducing factor *AIF1* **(7)**, whose respective knockouts cause enhanced resistance to H_2O_2. Interestingly, also a deletion in *RHO5*, a small GTPase with a Ras-like effector region, leads to reduced cell death upon H_2O_2 induction. Rho5p also appears to interact with the thioredoxin reductase Trr1p, a key component of the cytoplasmic thioredoxin antioxidant system **(8)**.

In the meanwhile, H_2O_2 has become the most commonly used drug to induce yeast apoptosis together with another compound: acetic acid **(9, 10)**. Treatment with acetic acid leads to cytochrome c release associated with enhanced mitochondrial membrane potential and the loss of COX activity **(11)**. Consistently, acetic acid treatment has been shown not to induce apoptosis in cells that lack mitochondrial DNA and hence are respiratory-deficient (rho0 cells). Additionally, cell death is at least partially prevented when cytochrome c is disrupted **(11)**. A series of other studies has connected cytochrome c release with yeast apoptosis, including heterologous expression of human Bax **(12, 13)** or human α-synuclein **(14)** as well as deletion of the histone chaperone *ASF1*/*CIA1* **(15)** or exposure to hyperosmotic stress **(16)**. Nevertheless, it remains elusive if the pathway downstream of cytochrome c release is connected to the formation of an apoptosome-like structure and subsequent caspase activation as known from the mammalian apoptotic system. So far, neither homologues of the mammalian apoptosome-associated factor (Apaf) nor functionally related proteins have been found in yeast.

Recent studies have further elucidated details in acetic acid-induced PCD. Catalase activity is reduced upon treatment, speculated to be due at least in part to catalase degradation by a proteolytic system **(17)**. Indeed, a temporary activation of the proteasome seems to be needed during acetic acid-induced PCD **(18)**. Intriguingly, such a cross-talk between the antioxidant and the proteolytic systems is known to occur in mammalian cell death and plays a role in the apoptosis/necrosis transition **(19)**. Moreover, the absence of the yeast orthologues (AACs) of adenine nucleotide translocator (ANT), a protein involved in the proapoptotic mitochondrial outer membrane permeabilization (MOMP) through the so-called permeability transition pore, was shown to prevent cytochrome c release and to protect cells exposed to acetic acid but not to H_2O_2. However, the absence of AAC proteins did not completely prevent cell death **(20)**, pointing toward the existence of different but partly redundant apoptotic pathways induced by H_2O_2 and acetic acid, respectively.

Other External Triggers: Drugs, Toxins, Metals, and Radiation

A series of other external triggers has been reported to induce an apoptotic phenotype in yeast, including heat stress **(21)**, UV irradiation **(22)**, hypochlorous

acid (HOCl) **(23)**, or ethanol **(24)**. Also, when exposed to salt (NaCl), yeast cells undergo apoptosis that can be rescued by deletion of yeast caspase *YCA1* **(25)** or expression of human Bcl-2, similarly as it has been found in mammalian cells **(26)**. Additionally, SFK1 has been identified as a suppressor molecule of FK506 (an inhibitor of the mammalian apoptosis regulator calcineurin) and seems to play a significant role in salt-mediated yeast apoptosis: It improves growth at high NaCl concentrations in the presence of FK506, but induces death via the mitochondrial pathway under conditions of low salt levels **(27)**.

Compounds beneficial or needed at subtoxic levels can induce apoptosis at higher concentrations. Besides high concentrations of glucose or sorbitol (hyperosmotic stress) **(16)**, copper and manganese also lead to apoptosis at moderately toxic levels apparently via two different mitochondrially regulated pathways **(28)**. An excess of iron also leads to yeast death, probably in a programmed fashion **(29–31)**. Interestingly, *ISC1*, a neutral sphingomyelinase that can be activated by cardiolipin and is involved in the biosynthesis of ceramide, may play a central role in this process. Besides resulting in shortened chronological life span, the deletion of *ISC1* derives in an *YCA1*-dependent higher H_2O_2 sensitivity, which can be suppressed by iron chelation. Strikingly, *ISC1* deletion associates with an upregulation of the iron regulon that increases iron levels, known to catalyze (Fenton reaction) the production of the highly reactive hydroxyl radicals **(31)**. Another metal involved in yeast apoptosis is calcium, whose cytosolic concentration is raised following amiodarone- or α-factor-induced PCD. This eventually leads to mitochondrial fragmentation in a process dependent on the yeast suicide proteins Ysp1p **(32)**. A further link between yeast apoptosis and Ca^{2+} is given by the calcineurin/calmodulin system, which is proposed to inhibit programmed cell death and has been linked to ER stress regulation **(33)**.

In nature, the functional potential of yeast undergoing PCD has been recognized and hijacked by other organisms or competing strains. The plant toxins ricin **(34)** and osmotin **(35)**, for instance, trigger apoptosis as a form of plant defense against pathogenic fungi; in the case of osmotin via activation of the Ras pathway of apoptosis **(35, 36)**. Upstream of this pathway, the receptor-like polypeptide Pho36p, a homologue of the mammalian receptor for the hormone adiponectin, mediates osmotin apoptosis induction **(37)**. Furthermore, exposure to toxins produced by coexisting virus-carrying killer strains also leads to PCD in yeast **(38–40)**. Here, Yca1p is needed and misused (from the viewpoint of the attacked strain), as deletion of the metacaspase leads to reduced toxin sensitivity **(39)**. Also amphibians have evolved mechanisms to kill yeast, probably in order to counteract the invasion of fungal pathogens: An amphibian-derived peptide (dermaseptin family) was recently shown to trigger Yca1p-independent, but Aif1p-dependent, yeast apoptosis **(41)**.

Diverse medically relevant drugs (including antitumor drugs) have been shown to induce yeast apoptosis in *S. cerevisiae* **(42)**. These include aspirin **(43, 44)**, the antitumor synthetic lipid edelfosine **(45)**, the antimitotic microtubule-stabilizing agent paclitaxel **(46)**, and the antibiotic bleomycin **(47)**, also used in

cancer therapy. For the highly toxic but also antileukemic agent arsenic, the proapoptotic action has been shown to depend on the mitochondrial translocase *TIM18* **(48)**. Furthermore, low doses of valproic acid, a short-chained fatty acid widely used in neuropsychiatric disorders and with antitumor activity, induces yeast apoptosis *YCA1*-dependently **(49, 50)**. Interestingly, cell death triggered by valproic acid, which is accompanied by neutral lipid accumulation, can be prevented upon deletion of the histone deacetylase (HDAC) *SIR2* **(51)**. Of note, valproic acid has been identified as an inhibitor of HDACs in mammalian cells **(52)**. Moreover, treatment of yeast cells with amiodarone, an antiarrhythmic drug, leads to a Ca^{2+} boost **(53)** and eventually to mitochondrially mediated cell death (32). Recently, a DNA microarray analysis of the yeast's transcriptional response to amiodarone showed an overlap to calcium stress responses as well as a downregulation of genes involved in all stages of cell cycle control. Interestingly, nutrient-responsive genes were also affected by amiodarone, though independently of Ca^{2+} **(54)**. Other antitumor drugs (e.g., 5-fluorouracil or coumarin) have been shown to be cytotoxic for yeast but have not been directly linked to apoptosis, partly due to a lack of investigation into the induced cell death markers **(42)**. Finally, a series of antifungal drugs has been described to induce yeast apoptosis, among them the metal cation chelator Ciclopirox olamine **(55)** or the antifungal antibiotic Pradimicin **(56)**.

A more physiological possibility of exogenous apoptosis induction is used by yeast itself when a suitable mating partner is absent or after unsuccessful mating, probably as a mechanism to eliminate unfertile cells. Exposure of haploid cells to low doses of its correspondent mating pheromone results in the activation of the MAP kinase signaling cascade (including the key kinase Ste20p), the subsequent elevation of intracellular Ca^{2+}, and an increase in mitochondrial activity, eventually leading to cytochrome c release and apoptosis **(32, 57)**. In contrast, Zhang et al. proposed a rather necrotic type of death upon pheromone exposure **(58)**. Apart from mating also sporulation following meiosis of diploid cells is coupled to apoptosis **(59)**.

Heterologous Expression of Apoptosis Modulators

Heterologous expression of the human key apoptotic inducer Bax in yeast leads to cell death, cytochrome c release, ROS accumulation, and apoptotic features such as DNA fragmentation or phosphatidylserine externalization **(4, 13, 60, 61)**. Moreover, Bax-induced cell death in yeast has been shown to depend on mitochondrial lipid oxidation **(62)**. Cardiolipin, even though not essential for Bax insertion into mitochondria **(63)**, may play an important role in the synergistic effect of Bax and the proapoptotic human truncated Bid (tBid) on yeast mitochondria **(64)**. Conversely, the cell death inhibitor BI-1 (Bax inhibitor 1) from plants and human cells is able to suppress apoptosis following Bax expression or hydrogen peroxide treatment in yeast **(65)**. In addition, heterologous expression of Bcl-2 or Bcl-xL prevents Bax-induced lethality **(12, 66–68)**, enhancing resistance to H_2O_2 **(69)** and acetic acid **(70)**. Bcl-2 expression was further

shown to reduce the mutation frequency and extend the chronological life span in yeast **(71)**. The data obtained upon heterologous Bax expression in yeast **(72)** clearly argue for a specific action of Bax in cell death activation rather than an uncontrolled perforation of the cell or the mitochondria through pores formed by Bax. A possible requirement of the yeast permeability transition pore complex (PTPC) or mitochondrial respiration still remains a matter of debate **(73)**. However, Ott et al. recently solved one of the pending questions in the field of mitochondrial outer membrane permeabilization: Does Bax act on a channel or pore in order to trigger cytochrome c release? The answer is yes. Using heterologously expressed Bax in yeast, Ott et al. were able to show a strict requirement for the TOM complex **(74)**.

Recently, yeast cells in the exponential phase were shown to be hypersensitive to H_2O_2 treatment in a Yca1p-dependent fashion upon heterologous expression of one of the main toxic triggers of the neurodegenerative Parkinson's disease (PD), α-synuclein (α-syn), or of two mutants associated with the early onset of PD (A30P, A53P) **(14)**. Flower et al. further showed that overexpression of *YPP1* is able to suppress the lethality of A30P but not of wild-type α-syn or A53T, suggesting a specific endocytic pathway for A30P vacuolar degradation **(75)**. Moreover, a conserved link between ER-Golgi traffic and α-syn toxicity involving the Rab GTPase Ypt1p has been established based on the yeast model **(76–79)**. During chronological aging, heterologous expression of α-syn leads to a decrease in life span that is accompanied by increased levels of both apoptotic and necrotic markers **(80)**. Accelerated death is thereby neither dependent on the apoptotic key players Aif1p, Yca1p, or Nma111p nor on autophagy. Intriguingly, the chronological life span of cells lacking mitochondrial DNA (rho0) was not affected by wt-α-syn or A53T, pointing out a strict requirement of functional mitochondria for α-syn toxicity **(80)**.

Endogenous Induction of Yeast Programmed Cell Death

Mutation-Induced Deregulation of Cellular Homeostasis

Defects in several cellular processes including N-glycosylation, chromatid cohesion, and ubiquitination have been shown to cause yeast apoptosis **(81–83)**. DNA damage and replication failure do also trigger cell death in yeast **(84–86)**. An important source of DNA damages, including gross chromosomal rearrangements (GCR), is thereby oxygen metabolism and ROS **(87–89)**. Only recently could tRNA methyltransferase 9 (*TRM9*) be identified as a tRNA modifier that can promote the increase in levels of key DNA damage-response proteins **(90)**. DNA replication stress takes a central position in explaining genome instability, which is fundamental during aging in all eukaryotes. In yeast, both aging models (chronological and replicative aging; see ahead) are affected by replication stress **(91)**. Interestingly, a recent study shows that lack of the RecQ helicase *SGS1*, whose human orthologues are implicated in premature-age diseases

(Werner and Bloom syndromes), leads to an increase in GCR frequency during chronological aging. Intriguingly, deletion of the protein kinase *SCH9* but not calorie restriction (see ahead) prevents the age-dependent defects of the *SGS1* disruptant **(92)**. Furthermore, the absence of factors connected to mRNA decapping leading to mRNA stability triggers the generation of oxygen radicals and apoptotic cell death **(93)**. Such a factor is *LSM4*, whose C-terminal part was recently shown to be responsible for its association to processing bodies (P-bodies). P-bodies play an important role in mRNA decapping and increase in number upon H_2O_2 treatment **(94)**.

Aging

Replicative Aging

Mother cell-specific or yeast replicative aging serves as a model for the aging of proliferating cells, especially asymmetrically dividing stem cells. The replicative life span represents the number of divisions an individual mother cell undergoes before dying **(95)**, which in yeast averages from 25 to 35. Each cell undergoing cell division does it in an asymmetrical manner, resulting in a mother and a daughter cell. The newborn daughter cell acquires the undamaged cellular material and is therefore reset to a young cell status. This process includes the asymmetrical distribution of mitochondria, in whose normal inheritance *PEX6*, a peroxisome biogenesis gene, has been shown to play a role **(96)**. In contrast to the daughter, the mother cell, characterized by a remaining bud scar, retains the damaged contents of the progenitor cell and is therefore one division step older. This eventually leads to death of the replicatively aged cell and is accompanied by typical apoptotic markers such as ROS accumulation, phosphatidylserine externalization, and DNA fragmentation **(97)**. Replicative aging is also characterized by the formation of extrachromosomal circles of rDNA (ERCs), which accumulate exponentially and eventually lead to the death of the mother cell **(98)**. Fob1p, a nucleolar protein required for DNA replication fork blocking, promotes ERC formation. Deletion of *FOB1*, accordingly increases the replicative life span **(99, 100)**. An important molecular player involved in replicative aging is Sir2p, an NAD-dependent histone and protein deacetylase homologous to the aging-regulating mammalian sirtuins. Upon overexpression, Sir2p has been shown to increase the replicative life span **(101, 102)**. It has also been involved in the asymmetrical distribution of oxidatively damaged proteins associated with Hsp104p during cell division **(103)**. Intriguingly, an additional HDAC, *HST2*, was recently shown to mediate *SIR2*-independent life-span extension via calorie restriction **(104)**. HDACs and thus epigenetic regulation of cell death play an important role during aging. In replicatively aged cells, an acetylating transcriptional complex (termed SLIK) activates the expression of stress-response genes leading to inhibition of death and longevity. Other transcriptional complexes that include HDACs like *RPD3* and *HDA1*, on the contrary, suppress the same sets of genes, achieving the opposite outcome **(105)**. Furthermore, mitochondria play an

essential role: Their increasing dysfunction during replicative aging leads to the activation of the retrograde response. This intracellular signaling pathway, of which the retrograde regulatory protein Rtg2p is a key mediator, activates genes that counterbalance the mitochondrial dysfunction via the SLIK complex **(105)**. Moreover, the mitochondrial-dependent energy availability is central, as enhanced respiration leads to replicative life-span extension but ATP depletion to premature death **(106, 107)**.

Chronological Aging

Yeast chronological aging serves as a model for aging of human postmitotic cells. The chronological life span represents the time a population remains viable in the postdiauxic and stationary phase **(108)**. Nutrient availability is restricted within a chronologically aged population; to promote survival of the culture, unfit cells must therefore die in order not to "waste" and by their death to release nutrients for the benefit of the fitter cells. Thereby, chronologically aged yeast cells die showing markers of apoptosis **(7, 109)**. Deletion of the mitochondrially located proapoptotic nuclease EndoG (see ahead) also prolongs life span, but only if respiration is enhanced **(110)**. It has been shown that chronological life-span extension requires transcription factors like Msn2p and Msn4p and activation of stress-response genes like the superoxid dismutases (SOD) **(111)**. Indeed, deletion of *SOD1* or *SOD2* leads to early death **(112)**, and several mitochondrial mutants are unable to enter the quiescent G0 state, which is crucial for the survival of stationary-phase cultures **(113)**. This places mitochondrial function including respiration at a crucial position during chronological aging **(73)**.

Chronological Versus Replicative Aging: Differences and Crossroads

Together with their respective peculiarities, replicative and chronological aging have clear differences and partly opposing mechanisms. For example, Msn2p and Msn4p, necessary for stress resistance during chronological aging, reduce the replicative life span, suggested to happen partly by increasing Sod2p expression **(114)**. Indeed, Sod2p overexpression increases the chronological life span but decreases the replicative life span **(115)**. Furthermore, while during replicative aging high Sir2p levels are associated with longevity, during chronological aging it is reduced Sir2 activity that promotes life span under nutrient-poor conditions **(116, 117)**.

However, both aging models seem to be connected to some extent, as the replicative life span of chronologically aged mother cells decreases with age **(118)**. Also, both chronological and replicative longevity are associated with high alcohol dehydrogenase (Adh1p) levels **(119)**. A further crossroad is given by the cell's nutritional situation. Accordingly, aging involves the regulation of three nutrient-dependent kinases: *TOR1/2*, *SCH9* (Akt/PKB), and PKA. The TOR complex 1 (TORC1) includes either Tor1p or Tor2p and is sensitive to rapamycin. The deletion of *TOR1* or *TOR2* as well as rapamycin treatment

leads to a starvation-like phenotype that correlates with longevity **(120**, **121)**. The deletion of *SCH9*, an orthologue of the proaging Akt/PKB proteins in worms, flies, and mice **(122)**, equally results in a life-span extension **(123)**, which can also be achieved by directly or indirectly abrogating the activity of the cAMP-dependent protein kinase A mediated primarily by its subunit Tpk3p **(107)**. Actually, PKA activation is placed downstream of a signaling pathway involving the Ras protein Ras2p. Hyperactivation of Ras2p, therefore, results in enhanced apoptosis, leading to a shortened replicative as well as chronological life span **(124**, **125)**. This signaling activation can be achieved by mutations decreasing actin dynamics/promoting actin aggregation and requires mitochondria for its execution **(36)**. Intriguingly, a protective function for the FMN oxidoreductase Oye2p regarding oxidative damage has been suggested, and two cysteine residues in actin have been proposed as sensors for oxidative stress **(126–128)**. Cells lacking *OYE2* show premature death during chronological aging and exhibit markers of apoptosis, a phenotype that can be reversed by mutation of the actin regulatory cysteines **(126)**.

Consistent with the observations that the aging process is accelerated by the activity of nutrient-dependent kinases and upregulation of their pathways, nutrient-poor conditions achieved by reducing the glucose content in the medium lead to life-span extension, comparable to the longevity-associated effect of calorie restriction (CR) in mammals **(129**, **130)**. A recent study has revealed that the serine/threonine kinase Rim15p and the stress resistance transcription factors Msn2p, Msn4p, and Gis1p are required for chronological life-span extension caused by CR or by deficiencies in *RAS2*, *TOR1,* and *SCH9*. The authors also show that CR can further promote longevity of the extremely long-lived mutants lacking Tor, Sch9, and Ras signaling and that this is only in part Rim15p-dependent, thus suggesting that further Rim15-independent mechanisms are required for maximum life-span extension **(131)**.

Autophagy and Cell Death: Companions or Competitors?

A process intimately related to scarce nutrition is autophagy, a cellular response of "self-recycling" that promotes survival and plays a significant role in different human diseases **(132)**. In contrast to the selective protein degradation in the ubiquitin-proteasome pathway, autophagy results in the formation of autophagosomes, vacuolar bulk degradation, and subsequent amino acid and lipid release into the cytosol **(133**, **134)**. Upstream of autophagic execution, sensing of nutrient availability is given by the TORC1 complex and also cooperatively by PKA and Sch9p **(135**, **136)**. A central role in coordinating autophagic function is adopted by Atg6p, the yeast orthologue of mammalian beclin1 **(137)**. However, autophagy may not be restricted to its prosurvival activity: Under certain circumstances, it also constitutes an alternative cell death pathway. Apoptosis and autophagy, therefore, represent two distinct responses to stress that in mammals act in a cooperative or competitive manner depending on the cellular context **(138–140)**. In yeast, the mitochondrial protein Uth1p

might be associated with autophagic cell death. While its deletion provides resistance to rapamycin, it is also involved in diverse stress responses and aging and is required for death triggered by the heterologous expression of human Bax **(141–143)**. However, the deletion of *UTH1*, while preventing mitochondrial lipid oxidation and ROS production, does not inhibit Bax insertion into the mitochondrial outer membrane or cytochrome c release **(143)**. Interestingly, Uth1p is specifically involved in the selective autophagic degradation of mitochondria **(144, 145)**, and mitochondrial disappearance has been suggested to be crucial for the involvement of autophagy in PCD **(146)**.

The Roots of Death—Mapping the Molecular Machinery

Being able to interact with the PCD machinery requires the identification of molecular players connected to the regulation of the death process. In recent years, a rising number of proteins have been shown to be involved in yeast PCD under specific conditions (Fig. 14.1 and Color Plate 3). It has therefore become apparent that the PCD core machinery in yeast is conserved and may represent the molecular roots of death. This is not only interesting from an evolutionary point of view but also confirms yeast as a valuable model for PCD research.

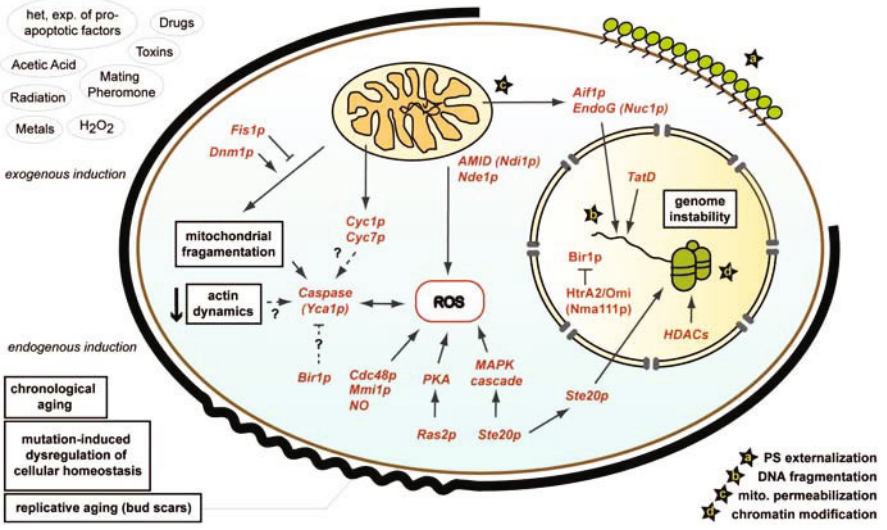

Fig. 14.1 The molecular machinery of yeast apoptosis. Exogenous and endogenous induction of yeast apoptosis leads to the activation of the basic molecular machinery of cell death, which is configured by conserved apoptotic key players such as the yeast caspase Yca1p, the yeast homologue of mammalian HtrA2/OMI (Nma111p), or the apoptosis-inducing factor Aif1p. Furthermore, it involves complex processes like histone modification, mitochondrial fragmentation, cytochrome c release, and cytoskeletal perturbations (*see* Color Plate 3)

Small Molecules as Mediators

ROS were the first mediators to be characterized as essential during yeast apoptosis **(4)** and have been confirmed as the central regulators ever since **(147)**. Emerging evidence points toward other small molecules that share this involvement as mediators in yeast apoptosis. For instance, nitric oxide (NO) was recently shown to be produced by an arginine-dependent mechanism in H_2O_2-induced apoptotic cells and to S-nitrosate glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which has been implicated in mammalian apoptosis as well **(148)**. The blockage of NO synthesis decreased both GAPDH S-nitrosation as well as intracellular ROS accumulation, thereby leading to increased survival. During the physiological stress scenario of chronological aging, NO scavenged by oxyhemoglobin led to a delayed onset of cell death and to decreased levels of superoxide anion **(149)**. Interestingly, a recent two-dimensional gel electrophoresis study to identify the changes in the proteome upon H_2O_2 treatment supports the involvement of NO-target GAPDH in cell death by revealing GAPDH alterations in apoptotic cells **(150)**.

Moreover, ammonia was also shown to mediate a cell death signal, in this case during the development of multicellular yeast colonies. Here, an ammonia signal triggers the localization of cell death to the colony's center, allowing the rest of the population to exploit the released nutrients. In colonies lacking the Sok2p transcription factor, the resulting inability to produce ammonia leads to decreased life span of the colony and death throughout the whole population **(151)**.

Proteasome and Proteases

The first molecular evidence for yeast apoptosis arose from experiments carried out in a *CDC48* mutant (*cdc48*^{S565G}) which was shown to exhibit an apoptotic phenotype **(3)**. This death has recently been linked to mitochondria: *cdc48*^{S565G} mutants show proteomic alterations in mitochondria, release of cytochrome c to the cytosol, and increased ROS production **(152)**. Interestingly, the identification of *CDC48* as a PCD player in yeast led to the characterization of the mammalian orthologue p97/VCP **(153)** and the related *mac-1* in *C. elegans* **(154)** as antiapoptotic factors. VCP is crucial for endoplasmic reticulum-associated protein degradation (ERAD) **(155)** and plays a role in polyglutamine neurodegeneration **(156)**.

The identification of the yeast metacaspase *YCA1* and its characterization as an orthologue of mammalian caspases **(6, 157)** have allowed the testing of dependency on caspase activity in diverse cell death scenarios. In conditions of oxygen stress, for example, the disruption of *YCA1* leads to decreased cell death and reduced formation of apoptotic markers **(6)**. Furthermore, *YCA1* dependency has been shown for numerous cell death scenarios, among them valproic acid-induced cell death **(50)** as well as apoptosis following defects in

different cellular processes, including the loss of ubiquitination control **(81)**, altered mRNA stability **(93)**, and defective initiation of DNA replication **(85)**.

During chronological aging, deletion of *YCA1* initially results in better survival but eventually leads to an accumulation of predamaged cells, as the apoptotic cleaning function is partly abrogated in the culture. In fact, in a direct competition, the *YCA1* knockout is outlived by the is wild-type control when aged **(109)**. The importance of Yca1p-mediated apoptosis in removing damaged cells is furthermore emphasized by studies showing that *YCA1* disruption increases the level of oxidized proteins **(158)**.

Interestingly, regulated cell death during long-term development of yeast multicellular colonies is independent of *YCA1* **(151)**. This example shows the importance to underline that the involvement of Yca1p is not necessary for all apoptotic scenarios. In fact, a rough estimation of the published yeast apoptosis scenarios counts approximately 40% as caspase-dependent, meaning they can be rescued at least in part by the deletion of *YCA1*. For instance, defective N-glycosylation in cells lacking Ost2p, the yeast homologue of the mammalian defender of apoptosis-1 (DAD1) protein, leads to apoptosis that is not altered by additional deletion of *YCA1* **(82)**. Moreover, apoptotic death mediated by Nuc1p, the yeast homologue of endonuclease G, or the yeast apoptosis-inducing factor Aif1p, is *YCA1*-independent **(7**, **41**, **110)**.

Even though the presence of other *YCA1*-independent caspase-like activities has been proposed **(109)** and may exist, these findings suggest that, also in yeast, PCD is not synonymous to caspase activity. In mammals, the existence of alternative, caspase-independent PCD modalities that may be at least as important as the classical caspase-determined pathways has become evident. This idea was confirmed with the discovery of the caspase-independent PCD executioner AIF [apoptosis-inducing factor; **(159**, **160)**] and is now broadly recognized.

Another protease involved in yeast apoptosis is Nma111p (nuclear mediator of apoptosis), the yeast homologue of the proapoptotic mammalian HtrA2/Omi. Deletion of *NMA111* reduces apoptotic markers, while overexpression of Nma111p increases cell death upon elevated temperature or H_2O_2 treatment **(161)**. Unlike its mitochondrial localized human counterpart, Nma111p resides in the nucleus and the proapoptotic function depends on its serine protease activity **(161)**. It was recently shown that Bir1p, the only known inhibitor-of-apoptosis (IAP) protein in yeast, which is localized to the cytoplasm and the nucleus, is a substrate for Nma111p. When challenged with oxidative stress, *BIR1* disruptants show enhanced apoptosis. Consistently, the overexpression of Bir1p reduces cell death, an effect that can be antagonized by the simultaneous overexpression of Nma111p. Overexpression of Bir1p also affects chronological aging by delaying cell death, whereas in this scenario *BIR1* disruption has no significant effect **(162)**. Along with its function in apoptosis, Bir1p, like its closest metazoan homologues deterin (flies) and survivin (mammals), also plays a role in chromosome segregation and cytokinesis **(163**, **164)**.

Within the nucleus, further factors have been described to play a role in PCD regulation besides Nma111p. One of them is involved in the essential step of DNA fragmentation/degradation. By sequence comparison with *C. elegans* apoptotic nucleases, Qiu et al. identified eight potential apoptotic nucleases, one of them being the ds DNA endo-/exo-nuclease *Tat-D*. Under mild H_2O_2 stress conditions, the *Tat-D* null mutant shows better survival compared to the wild-type strain, while overexpression of the nuclease leads to higher death rates and an apoptotic phenotype **(86)**.

Another important nuclear process is epigenetic cell death control. As described for mammals **(165)**, histone H2B phosphorylation is also necessary for cell death induction upon oxidative stress in yeast **(166)**. A further study showed the unidirectional cross-talk between lysine 11 (K11) deacetylation by the HDAC Hos3p and subsequent serine 10 (S10) phosphorylation by the Ste20p kinase in the histone H2B tail (H2Bt). A nondeacetylable H2Bt lysine mutant was resistant to H_2O_2-induced death, while a mutant mimicking a permanent deacetylated state strongly elicited cell death **(167)**. Remarkably, two further HDACs (Rpd3p and Hda1p) that caused cell death independently of H2Bt deacetylation **(167)** have been shown to counteract the longevity-promoting effect of the H2B-acetylating enzyme Gcn5p during replicative aging **(105)**. Moreover, the kinase Ste20p is required for pheromone-induced apoptosis in yeast **(57)**. Therefore, H2B epigenetics may be cross-linked to replicative life-span control and/or the MAPK cascade in a manner yet to be defined **(168)**.

Mitochondrial Factors: Toxic When Released

As mentioned earlier, mammalian apoptosis does not necessarily need caspase activity to occur, and a key protein in one of these caspase-independent ways of death is the apoptosis-inducing factor (AIF). With the identification of an AIF homologue in yeast **(7)**, it has become clear that the roots of cell death may harbor at least in part different PCD types beyond caspase-dependent cell death pathways. Like its mammalian counterpart, yeast Aif1p translocates from the mitochondria to the nucleus when apoptosis is induced (by H_2O_2, acetate, or aging), leading to chromatin condensation and DNA degradation. Furthermore, as in mammals, cyclophilin A is required for Aif1p-induced cell death **(7, 169, 170)**. Disruption of *AIF1* inverts these death effects, and the deletion mutant shows better survival during H_2O_2 and acetate treatment as well as during aging **(7)**. Besides its lethal function, mammalian AIF, which is an NADH oxidase, also contributes to the cell's vitality via its redox function and is central for optimal oxidative phosphorylation and for an effective antioxidant defense **(171)**. Consistently, yeast *AIF1*, which exhibits a high degree of similarity to mammalian AIF in its oxidoreductase domain, has vital functions for respiration **(172)**.

Also, for the AIF homologous mitochondrion-associated inducer of cell death (AMID), a mitochondria-associated protein implicated in caspase-independent apoptosis **(173)**, a yeast homologue (*NDI1*) has been described and linked to cell death **(174)**. Disrupting this NADH dehydrogenase responsible for intramitochondrial NADH decreases ROS production and elongates chronological life span, although with a loss of survival fitness. To a lower extent, this effect on aging is also displayed by the knockout of *NDE1*, which catalyzes the oxidation of NADH at the cytosolic side of the inner mitochondrial membrane **(174)**.

Recently, a yeast orthologue of mammalian endonuclease G (EndoG), *NUC1*, was identified and its apoptotic function characterized **(110)**. Mammalian EndoG is mitochondrially located and translocates to the nucleus upon apoptosis induction **(175**, **176)**. Büttner et al. showed that the same is true for overexpressed Nuc1p and demonstrated that the apoptotic phenotype is drastically enhanced when the mitochondrial localization sequence is partially lacking. The disruption of *NUC1* inhibits apoptotic cell death when mitochondrial respiration is increased but enhances necrotic death when oxidative phosphorylation is repressed, discriminating between vital and lethal functions of *NUC1*. Finally, *NUC1*-mediated death is *YCA1*- and *AIF1*-independent but requires yeast homologues of the mammalian permeability transition pore, the karyopherin Kap123p, and histone H2B **(110)**. In a further study, a vital role of EndoG in the maintenance of polyploid cells was shown for yeast and a human tetraploid cell line **(177)**.

Mitochondria obviously play a central role in yeast cell death execution **(73)**; in humans, they represent an interesting target for medical purposes **(178)**. Along with their part in ROS generation, mitochondria also guard several factors that, upon cellular stress, turn into proapoptotic agents (*AIF1*, yeast AMID, yeast EndoG). A further mitochondrial protein, cytochrome c, is release when apoptosis is triggered via heterologous Bax or acetic acid induction **(11**, **68)**, even though the downstream pathway still remains unclear (see earlier). All these mitochondrial proteins bear both lethal and vital roles, similar to many apoptotic-relevant proteins in mammals **(179**, **180)**. Coupling these two activities allows a functional switch upon a change in localization, thus allowing death to occur in an effective and redundant manner while at the same time disrupting the normal cellular processes in which these factors are involved.

Moreover, in humans as in yeast, mitochondrial shape transitions are critical for cell death. Mitochondrial fragmentation (fission) in humans is regulated by the dynamin-related protein 1 (Drp1) and has been shown to play a causal role in PCD **(181**, **182)**. In addition, a recent study identified a new Drp1 inhibitor (mitochondrial division inhibitor-1, mdivi-1) that is able to block mitochondria division and Bax-driven mitochondrial outer membrane permeabilization during apoptosis (183). Fannjiang et al. showed that the yeast homologue of Drp1 (Dnm1p) stimulates mitochondrial fragmentation and degradation followed by apoptosis upon treatment with diverse death stimuli **(10)**. Two Dnm1p interactors with a fission function in healthy cells also regulate cell death: While Mdv1p/Net2p consistently promotes cell death, Fis1p unexpectedly inhibits mitochondrial

fission. The deletion of *FIS1* therefore leads to an increase in cell death, which can be annulled by an additional deletion of *YCA1* **(10)**. Furthermore, the mitochondrial fission pathway of death has been involved in different scenarios such as PCD induced by amiodarone or mating factor, where mitochondrial fragmentation is dependent on Ysp1p **(32)**, M1 virus-encoded K1 toxin **(38)**, or ethanol **(24)**. Intriguingly, this pathway also seems to play a role in aging, as the deletion of *DNM1*, resulting in reduced mitochondrial fission, extends life span **(184)**.

A factor that relocates to mitochondria upon oxidative stress, *MMI1*, was recently shown to bear apoptotic functions **(185)**, similar to its human orthologue TCTP (translationally controlled tumor protein), which has been implicated in important cellular processes including apoptosis **(186)**. The deletion of *MMI1* leads to benomyl sensitivity, pointing toward a stabilizing effect on microtubules, and increases resistance to H_2O_2 as well as life span during replicative aging **(185)**.

The Roofs of Death—Physiological Sense and Medical Implications

Exploring yeast apoptosis raises at least two interesting questions. First, why should a unicellular organism commit suicide? And second, why should a multicellular human being study it? In other words, what is the physiological roof under which yeast apoptosis occurs, and what are the implications for the evolutionary roof of death, the human cell death system?

Physiological Sense

In yeast, apoptosis fulfills the same purpose known for multicellular organisms: It eliminates superfluous cells. However, understanding this physiological sense in yeast requires a conceptual change: It is important not to interpret yeast populations just as a group of unicellular organisms that have no communication activity with each other, but rather to view them as a multicellular community of interacting individuals in which the death of a single cell is beneficial for the whole population. In fact, this multicellular-like behavior of single-cell organisms is not restricted to eukaryotes: The ability of bacteria to communicate with each other via quorum-sensing signal molecules has become an important topic in current microbiology **(187)**. Intriguingly, bacteria also show forms of PCD, including the chromosomal toxin–antitoxin systems in *Escherichia coli* and the killing response of *Bacillus subtilis* during sporulation **(188)**. Interestingly, a recent study shows that the production of ROS is a common process of cell death triggered by bactericidal antibiotics **(189)**. Though very basic and not to be defined as apoptotic, bacterial PCD underlines the functional importance and evolutional advantage of cell death in populations of unicellular organisms.

In yeast, several physiological scenarios exist where the altruistic death of single cells promotes survival of the population as a whole (Fig. 14.2 and Color Plate 4) **(190)**. As already discussed, aged yeast cells die exhibiting markers of apoptosis

during chronological and replicative aging **(97**, **109**, **123)**. Therefore, in the natural situation of starvation, old and damaged cells undergo a suicide program rather than consume vanishing resources in the probably vain attempt to become young and repaired **(113)**. Also, their remaining cell mass represents additional nutrients for the rest of the population. Furthermore, this altruistic death may increase genetic variability by both enhancing the probability of somatic mutations through ROS generation and eliminating ancient genetic variants in the population. The development of multicellular yeast colonies is equally linked to apoptosis. Here the central region exhibits higher death rates in order to feed the colony margin **(151)**. In general, fitter cells profit from the death of damaged ones, which increases the survival possibilities of the population and, consequently, the spreading of the clone.

As mentioned earlier, the exposure of haploid cells to mating-type pheromones induces cell death when the corresponding mating partner is missing and mating fails **(57**, **58)**. Therefore, the population can be cleaned from infertile or otherwise damaged haploid cells, which is central for the expansion of the clone, as the mating process ensures meiotic recombination and increases genetic diversity, providing an adaptive advantage.

Aside from its advantages, having an autodestructive program also bears the risk that it might be misused from outside (Fig. 14.2 and Color Plate 4). Exposure

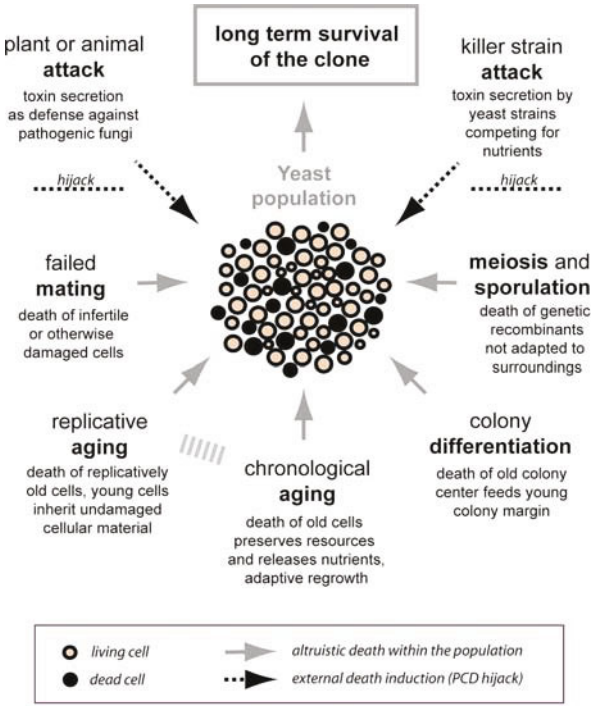

Fig. 14.2 Physiological scenarios and yeast apoptosis. A wild-type yeast population promotes its own long-term survival and spreading of the clone by eliminating unfertile, damaged, or genetically unadapted individuals. Death in the population may also be triggered by toxins from nonclonal enemy strains or higher eukaryotes that hijack the PCD machinery of yeast (*see* Color Plate 4)

to virus-encoded killer toxins produced and secreted by killer strains induces apoptosis in *S. cerevisiae* **(38–40, 191)**. Under conditions in which different colonizing yeast strains might be fighting for nutrients, killer strains may provoke cell death in competing yeast strains. In fact, it is significant that toxin-resistant rather than susceptible yeast strains are usually found in fruits and that one quarter of them are killers **(192)**.

Medical Implications

Yeast serves as a good model for medical and medicinal research **(193)** and with the finding of yeast apoptosis, this role as a model system has gained a new and crucial area. Understanding PCD is one of the biggest challenges in modern medicine, as it is a central process for the most devastating diseases of our time, including cancer as well as neurodegenerative, autoimmune, and cardiovascular diseases. Moreover, apoptosis is essential during processes like organismal development or tissue homeostasis. For these reasons, any further advance in the field of yeast apoptosis that provides new clues in understanding cell death in humans is meaningful. This may include insights in the relationship of different types of PCD (like apoptosis, necrosis, autophagy), the upstream signaling and execution pathways, or poorly understood regulation mechanisms (like epigenetics).

Importantly, apoptotic players shown to be conserved in yeast are of major clinical relevance; among these are caspases, involved in many physiological and pathological states, and AIF, whose absence can cause neurodegeneration, skeleton muscle atrophy, and dilated cardiomyopathy **(171)**. Another example is endonuclease G, whose translocation to the nucleus has been associated with the progression of several degenerative disorders, such as cerebral ischemia **(194)** and muscle atrophy **(195)**. Also, the inhibitor-of-apoptosis (IAP) protein family, to which the yeast Bir1p belongs, is emerging as a promising cancer therapy target, as several lines of evidence point to a crucial role in oncogenesis **(196)**. Moreover, emerging evidence suggests that mitochondrial dynamics (fusion and fission) are important in development, aging, and disease **(197)**.

Yeast apoptosis has also proven valuable for the investigation of neurodegenerative diseases such as Parkinson's disease **(198)**. Furthermore, it serves as a model for the aging of postmitotic cells (chronological aging) as well as stem cells (replicative aging), and it has shed light on the possibility of fighting fungal or protozoan pathogens at the level of PCD (see ahead). Finally, yeast might help to explore the pathophysiological implications of other types of PCD than apoptosis **(139)**.

Rooms for Death—Apoptosis in Other Unicellular Organisms

S. cerevisiae is not the only unicellular organism exhibiting markers of apoptosis. Indeed, programmed cell death has been described for model organisms like the yeast *Schizosaccharomyces pombe* or the slime mold *Dictyostelium discoideum* as well as for pathogenic fungi and protozoan parasites, showing that there is enough phylogenetic room to die for everyone.

Fungi

Schizosaccharomyces pombe

Another yeast proven to be an important model organism for basic research, especially in the field of cell cycle regulation **(199)**, is the fission yeast *S. pombe*. *S. cerevisiae* and *S. pombe* split early in evolution, probably at least one billion years ago **(200)**, not long after the actual diversification into fungi and animals. Consequently, they display striking differences in some fundamental characteristics **(201)**. Nevertheless, though not as well characterized for *S. pombe*, the existence of a cell death program appears to be a common feature for both model organisms **(202, 203)**. Indeed, the proapoptotic proteins Bak and Bax can induce cell death in *S. pombe* **(202, 204)**, and cells defective in triacylglycerol synthesis show a loss of viability accompanied by apoptotic markers such as nuclear DNA fragmentation, exposure of phosphatidylserine, and ROS accumulation **(205)**. Inappropriate mitosis and replication stress are also associated with ROS production and cell death in fission yeast **(206)**. In addition, Roux et al. showed that fission yeast undergoes chronological aging regulated via two nutrient-dependent kinases known to play a role during the aging of budding yeast: the cAMP-activated *PKA1* and *SCK2*, a homologue of *S. cerevisiae SCH9*. Interestingly, a double deletion of *PKA1* and *SCK2* showed an additive effect regarding life-span extension, suggesting that both kinases act in independent age-related pathways **(207)**. Recently, it was shown that ectopic expression of the mitochondrial endonuclease Pnu1p leads to cell death and DNA fragmentation in fission yeast, suggesting it to be the orthologue/homologue of the mammalian/budding yeast EndoG **(208)**. In fact, many homologues/orthologues of key apoptotic genes are present in fission yeast and may be characterized regarding their cell death function in the near future.

Candida albicans and Other Pathogenic Fungi

Candida albicans is one of the major pathogenic fungi, being especially threatening for severely immunocompromised patients as well as for patients undergoing chemotherapy or organ transplantation where infection is associated with a high incidence of mortality. *C. albicans* exhibits cell death with apoptotic and secondary necrotic features upon exposure to diverse stimuli including acetic

acid, H_2O_2, and the antifungal drug amphotericin B **(209)**. Interestingly, mutations that block Ras-cAMP-PKA signaling abrogate or delay, while mutations that stimulate signaling increase the apoptotic response induced by weak acid exposure **(210)**. The possible cell death function of a metacaspase-encoding gene remains to be elucidated **(157)**. Further fungi causing life-threatening diseases such as *Aspergillus fumigatus* and *Aspergillus nidulans* have been described to undergo PCD **(211, 212)**. In *A. nidulans*, sphingosine-induced apoptosis was shown to be dependent on mitochondrial function but independent of caspase activity and ROS formation **(211)**, while during sporulation, caspase-like activity could be detected **(213)**. For a series of other pathogenic fungi including *Magnaporthe grisea* and *Cryptococcus neoformas,* PCD has also been reported **(214)**.

Protozoan Parasites

Apoptotic markers have also been observed in a series of protists including nonparasitic organisms, such as the slime mold *Dictyostelium discoideum* **(215, 216)**, and different parasites like the malaria-causing *Plasmodium spp*. **(217)**. Of further clinical relevance is the finding of apoptotic cell death in different developmental stages of the kinetoplastid parasites *Leishmania* **(218)** and *Trypanosoma* **(219)**, which cause diverse forms of leishmaniasis and trypanosomiasis, respectively. However, the understanding at the molecular level of cell death execution in protists is still limited. Examples of molecular death players include Pdd1 in *Dictyostelium* **(220)**, AIF in *T. thermophila* **(215)**, Sir2 in *Leishmania* **(221)**, and endonuclease G *in Trypanosoma* **(222)**. Interesting candidates arise from the identification of metacaspases in unicellular organisms and plants **(157)**. Even though a lot of data show caspase-like activity during cell death in protists, there is still no clear picture to which extent and in which fashion this proteolytic activity is required for the death process, and this needs further investigation **(223)**.

Conclusions

The concept of programmed cell death being a privilege of classical multicellular organisms is certainly outdated. There exists no stringent border line dividing uni- and multicellular systems but rather a continuum with extreme forms of "uni- or multicellularity" that in most cases seems to exhibit functionally indispensable programs for evolutionary profit including cell death. During the last few years, evidence has accumulated that points toward a conservation of the basal apoptotic machinery and their key regulators between mammals and yeast. Certainly, the yeast model is not an exact copy of the complete mammalian system: For instance, components of the extrinsic apoptotic

regulatory process have not been identified so far. Nevertheless, the field of yeast apoptosis, though very young, is rapidly growing and may extend similarities to mammals to even a higher degree than assumed to exist to date. The fact remains that many conserved triggers and pivotal players have already been identified and insights have been gained for application in the human system, as exemplified by CDC48, first discovered to play an apoptotic role in yeast and later in human cells.

A main goal of further work must be to clarify the pathways and mechanisms underlying yeast apoptosis. Yeast as a simple and technically advantageous eukaryotic model organism may thereby uncover and help to understand ''universal'' activators, inhibitors, and mechanisms of programmed cell death, including the connection to cell cycle control, mitochondrial implications, epigenetics, and different forms of PCD (e.g., apoptosis, autophagy, necrosis). Current and future studies on yeast PCD could therefore be of great relevance for indirect (cancer, lipid deregulation, or protein fibril accumulation diseases) or direct (antifungal drugs) clinical applications. Nature has designed and evolved a complex network of deadly functions, whose roots might well reach the highest branches of death.

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Chapter 15
Programmed Cell Death in *C. elegans*

Monica Darland-Ransom, Yi-Chun Wu, and Ding Xue

Abstract Studies in the nematode *Caenorhabditis elegans* have established that programmed cell death is a genetically determined part of development and is controlled by a specific set of genes. These genes have been ordered into a pathway through genetic analyses. This cell death pathway is evolutionarily conserved and provides a basis for understanding programmed cell death in more complex organisms, including humans. This chapter discusses the activation, cell killing, and engulfment processes in the model organism *C. elegans*.

Keywords Cell death · Apoptosis · *C. elegans* · *ced* gene · *ces* gene · *egl* gene · *cps* gene · *crn* gene

Studies in the nematode *Caenorhabditis elegans* have established that programmed cell death is a normal, genetically determined part of development and is controlled by a number of specific genes. Genetic analyses have ordered these genes into a pathway. This cell death pathway is evolutionarily conserved and provides a basis for understanding programmed cell death in more complex organisms, including humans.

C. elegans as a Model Organism for Studies of Programmed Cell Death

C. elegans is a small (adult animals are approximately 1 mm in length), free-living worm with a short generation time (3 days at 20 °C) (Fig. 15.1). It can feed on the bacteria *Escherichia coli* and is cultivated on Petri dishes in the laboratory **(1)**. Because *C. elegans* is small and transparent, its internal structures can

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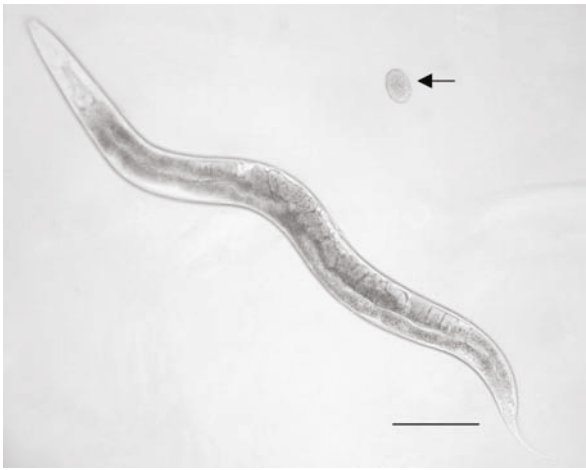

Fig. 15.1 A *C. elegans* adult hermaphrodite and an embryo viewed using bright-field microscopy. An *arrow* indicates the embryo. Dorsal is up and anterior is to the left for the adult. The bar represents 0.1 mm

be visualized with the light microscope. Furthermore, with high-magnification differential interference contrast (DIC) optics or Nomarski optics, cell divisions and cell deaths can be observed and followed in living animals **(2–4)**. For these reasons, *C. elegans* has been an excellent model organism for experimental analyses and has proven to be exceptionally well suited for the study of programmed cell death.

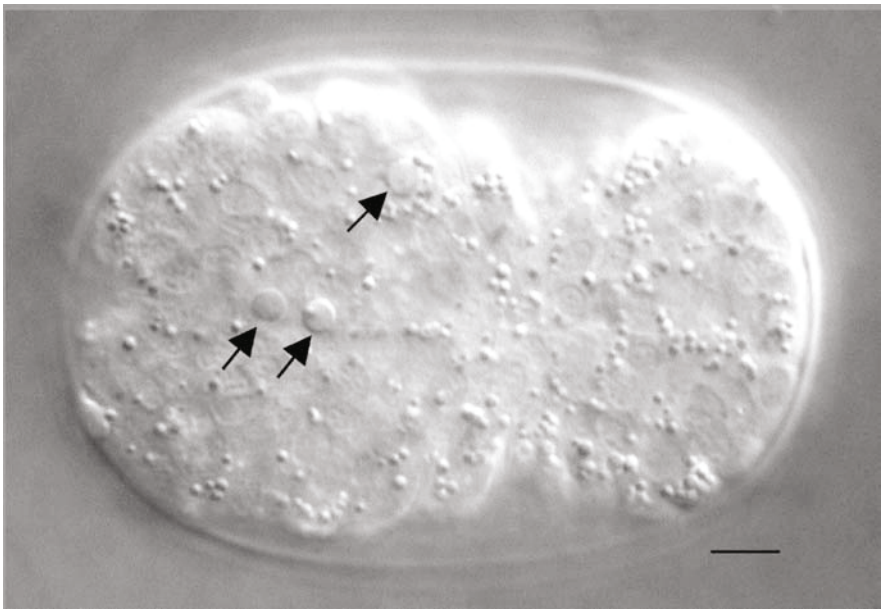

Fig. 15.2 A Nomarski photomicrograph of an embryo with apoptotic cells. Three cells indicated by *arrows* underwent programmed cell death in a bean-/comma-stage embryo and exhibited the refractile raised-button-like appearance indicated by *arrows*. The bar represents 5 μm

During the development of the *C. elegans* adult hermaphrodite from the fertilized egg, 1,090 somatic nuclei are generated by essentially invariant patterns of cell divisions. Of these 1,090 somatic cells, 131 undergo programmed cell death **(2–4)**. When observed with Nomarski optics, the dying cell adopts a refractile and raised button-like appearance (Fig. 15.2). When viewed using an electron microscope, the cells that undergo programmed cell death in *C. elegans* display characteristic features of apoptosis observed in mammals, including cell shrinkage, chromatin aggregation, and phagocytosis of cell corpses **(5)**. The entire process of cell death from the birth to the disappearance of the cell by phagocytosis occurs within approximately one hour **(2, 6)**.

The Genetic Pathway for Programmed Cell Death

Genetic approaches have been taken to identify genes that are involved in mediating different aspects of *C. elegans* programmed cell death. Genetic analyses have placed these genes in four sequential and distinct steps: first, the decision of whether individual cells should live or die; second, the activation of the cell-killing machinery in cells that are committed to die; third, the execution of the cell-killing process, which includes the fragmentation of chromosomal DNA and phagocytosis of the dying cell by its neighboring cell; and fourth, the degradation of the engulfed dead cell in phagocytes **(7)** (Fig. 15.3). Genes acting in the first step affect only specific cells. By contrast, genes that function in the second through fourth steps appear to affect all cell deaths.

In the following sections, we first review our current understanding of genes that function as global regulators of cell death activation and then discuss how these regulators may be controlled by cell-type-specific genes to control the life vs. death fates of specific cells. Lastly, we focus on genes that function in the cell death execution process and genes that are involved in degrading engulfed apoptotic cells.

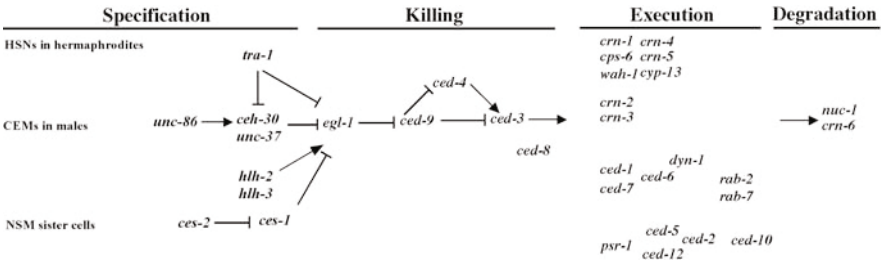

Fig. 15.3 Genetic pathway of programmed cell death in *C. elegans*. Four sequential steps of programmed cell death are indicated. In the cell death specification step, genes involved in regulating the death fates of three specific cell types (HSN neurons, sister cells of NSM neurons, and CEM neurons) are shown. Parallel pathways mediate the engulfment of cell corpses (*ced-1, -6, -7* and *psr-1, ced-2, -5, -10, -12,* respectively) and apoptotic DNA degradation (*cps-6, wah-1, crn-1, -4, -5, cyp-13* and *crn-2, -3,* respectively)

Activation of Cell Death

The Genetic Pathway for Cell Killing

Three death-promoting genes, *egl-1*, *ced-3,* and *ced-4,* are required for most, if not all, programmed cell death in *C. elegans*. Strong loss-of-function (lf) mutations in any of these genes lead to the survival of essentially all cells that normally undergo programmed cell death during the development of wild-type animals **(8, 9)**. Genetic mosaic analyses were carried out to determine whether these death-promoting genes act in cells that are doomed to die or instead function in adjacent cells to promote the death of dying cells as "murderers." In these experiments, mosaic animals that contain both genotypically wild-type cells and genotypically mutant *ced-3* (or *ced-4*) cells were generated **(10)**. It was found that in these mosaic animals, cells that are genotypically wild-type are capable of undergoing programmed cell death and cells that are genotypically mutant for the *ced-3* (or the *ced-4*) gene fail to die. These findings indicate that both *ced-3* and *ced-4* genes function within dying cells to cause cell death and provide the first genetic evidence that cells undergoing programmed cell death die by a suicide mechanism.

In contrast to these death-promoting genes, the activity of the *ced-9* gene protects a majority of cells from undergoing programmed cell death during *C. elegans* development **(11)**. Loss-of-function mutations in *ced-9* cause embryonic lethality, as a consequence of massive ectopic deaths of cells that normally live **(11)**.

To understand how *egl-1*, *ced-3*, *ced-4,* and *ced-9* coordinate to regulate programmed cell death, two approaches have been taken to order their functions in a pathway leading to cell death. First, genetic epistasis analyses have been performed to define the relationships of the killer genes *egl-1*, *ced-3,* and *ced-4* with respect to the protector gene *ced-9*, taking advantage of the fact that the phenotype of mutants defective in any of these killer genes is opposite to the *ced-9(lf)* phenotype. Specifically, double-mutant combinations such as *ced-9*; *ced-3*, *ced-4 ced-9,* and *ced-9*; *egl-1* were generated, and the phenotypes of double mutants were compared with those of the respective single mutants. For example, *ced-9*; *ced-3* and *ced-4 ced-9* double mutants are viable and have extra surviving cells, as observed in *ced-3* and *ced-4* single mutants **(11)**. This result indicates that the ectopic cell deaths and lethality caused by loss-of-function mutations in *ced-9* are suppressed by loss-of-function mutations in *ced-3* or *ced-4* and are dependent on the activities of *ced-3* and *ced-4*. Therefore, *ced-9* likely acts upstream of *ced-3* and *ced-4* to negatively regulate the activities of these two death-promoting genes. By contrast, the *ced-9*; *egl-1* double mutant is lethal and exhibits a large number of ectopic cell deaths, as observed in the *ced-9(lf)* mutant, indicating that the loss of *egl-1* does not suppress ectopic deaths caused by the loss of *ced-9* **(9)**. Therefore, *egl-1* likely acts upstream to negatively regulate *ced-9*.

Transcriptional overexpression experiments have been used to help order the functions of the three death-promoting genes *egl-1*, *ced-3,* and *ced-4*. The overexpression of *egl-1*, *ced-3,* or *ced-4* in *C. elegans* can induce the deaths of cells in which one of these genes is ectopically expressed **(12)**. The resulting cell-killing effects vary in different genetic backgrounds. For example, cell killing caused by the overexpression of *egl-1* is greatly reduced in the *ced-3(lf)* or *ced-4(lf)* mutant **(9)**, suggesting that *ced-3* and *ced-4* act genetically downstream of *egl-1*. Similarly, cell killing caused by the overexpression of *ced-4* is strongly inhibited in *ced-3(lf)* mutants **(12)**. By contrast, cell killing mediated by the overexpression of *ced-3* does not seem to be affected by the absence of the *ced-4* activity **(12)**. These observations indicate that *ced-3* likely acts genetically downstream of *ced-4* (Fig. 15.3).

Molecular Identities of egl-1, ced-3, ced-4, and ced-9

CED-9 is similar to the product of human proto-oncogene *bcl-2* **(13)**, which plays a similar role in preventing apoptosis in mammals **(14–18)**. *ced-9* and *bcl-2* are two members of a large gene family that play important roles in regulating apoptosis in diverse organisms **(19**, **20)**. All members of this gene family contain at least one Bcl-2 homology region (BH domain), and some, such as CED-9 and Bcl-2, have up to four BH domains **(19)**. The *C. elegans* killer gene *egl-1* encodes a relatively small protein of 91 amino acids with a BH3 motif that has been found in all death-promoting Bcl-2/CED-9 family members **(9)**.

CED-3 belongs to a family of cysteine proteases called *caspases* (cysteine aspartate-specific protease), which cleave their substrates exclusively after an aspartate amino acid **(21)**. CED-3, like many other caspases, is first synthesized as a 56-kDa zymogen, which can be proteolytically activated to generate an active cysteine protease complex that contains a large subunit and a small subunit **(21**, **22)**. Assays of the protease activities of several mutant CED-3 proteins showed that the extent of reduction of CED-3 *in vitro* protease activity correlates directly with the extent of reduction of *ced-3 in vivo* cell-killing activity **(22)**. These findings indicate that the CED-3 protease activity is important for *ced-3* to cause programmed cell death in *C. elegans*. The *ced-4* gene encodes a protein similar to mammalian Apaf-1 (apoptotic protease activating factor-1), which is an activator of caspases during apoptosis in mammals **(23–26)**.

Molecular Model for the Killing Process

Molecular studies of *C. elegans* killer and protector genes not only reveal the molecular identities of these genes but also facilitate the biochemical and cell biological analyses of these genes. The biochemical studies have provided

important insights into how EGL-1, CED-3, CED-4, and CED-9 proteins function in a protein interaction cascade, leading to the activation of programmed cell death.

CED-4 has been shown to physically interact with CED-9 *in vitro* **(27)**. Endogenous CED-4 and CED-9 proteins co-localize at mitochondria in *C. elegans* embryos, as detected using antibodies against CED-4 and CED-9 proteins **(28)**. In embryos in which cells had been induced to die by the overexpression of *egl-1*, CED-4 assumed a perinuclear instead of mitochondrial localization pattern **(28)**. This translocalization of CED-4 is triggered by the interaction between EGL-1 and CED-9, as biochemical assays show that EGL-1 can bind CED-9 **(9**, **29)**, and this binding induces disassociation of CED-4 from the CED-9/CED-4 complex **(29)**(Fig. 15.4). The *ced-9* gain-of-function mutation *n1950*, which causes the substitution of glycine 169 by glutamate, impairs the binding of EGL-1 to CED-9 but does not affect the association of CED-9 with CED-4. As a result, this mutation inhibits the EGL-1-induced translocation of CED-4 and results in the inhibition of programmed cell death **(30)**.

In addition to interacting with CED-9, CED-4 has been shown to interact with CED-3 **(31**, **32)**. The binding of CED-3 and CED-9 to CED-4 may not be mutually exclusive **(31**, **32)**. This observation leads to the hypothesis that in living cells, CED-3, CED-4, and CED-9 may co-exist as a ternary protein complex in which CED-3 remains an inactive proenzyme **(32)**. However, the CED-3 subcellular localization pattern has not yet been determined. It is possible that CED-3 does not associate with the CED-4/CED-9 complex and exists as an inactive monomer elsewhere in the cell. In either case, the EGL-1-induced dissociation of CED-4 from CED-9 may allow the formation of a CED-3/CED-4 complex that results in the activation of the CED-3 zymogen (Fig. 15.4). Activated CED-3 proteases may cause cell death by cleaving key substrates and hence lead to systematic cell disassembly and phagocytosis of the dying cell by its neighboring cells.

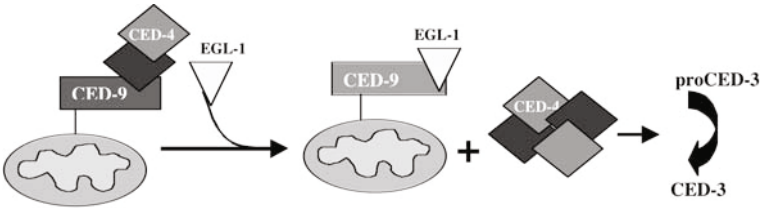

Fig. 15.4 The molecular model for the activation of programmed cell death. In living cells, CED-4 and CED-9 form a two-to-one complex, which is tethered to mitochondria through CED-9. CED-3 may associate with CED-4 at the mitochondria or exist elsewhere without binding to CED-4. However, in either case, CED-3 remains an inactive proenzyme in living cells. In the cells that are doomed to die, the death initiator protein EGL-1 binds to CED-9 and induces a conformational change of CED-9, which triggers the release of CED-4 (or the CED-4/CED-3 complex) from CED-9. Released CED-4 dimers undergo further oligomerization, which leads to CED-3 autoproteolytic activation

In addition to CED-4, CED-3 can interact with CED-9. Moreover, CED-9 is an excellent substrate of the CED-3 protease *in vitro*. Mutations that destroy both CED-3 cleavage sites in CED-9 markedly reduce the death protective activity of CED-9 *in vivo*, suggesting that CED-3 cleavage sites are important for the CED-9 death-protective function **(33)**. Cleavage of CED-9 by CED-3 generates a carboxyl-terminal product that resembles Bcl-2 in sequence and is sufficient to mediate interaction with CED-4 **(33)**. These results suggest that CED-9 may inhibit cell death in *C. elegans* by two distinct mechanisms. First, CED-9 may directly inhibit the CED-3 protease activity through its CED-3 cleavage sites, probably acting as a competitive inhibitor. Second, CED-9 may indirectly inhibit the activation of CED-3 by forming a complex with CED-4 through its carboxyl-terminal Bcl-2 homology regions.

Structural Analysis of CED-4, CED-9, and EGL-1

Structural analysis of the crucial components of the cell death machinery in *C. elegans* has provided important insights into the functions of these molecules and the mechanisms by which these molecules regulate cell death activation. The three-dimensional structures of the CED-9/EGL-1 complex and the CED-4/CED-9 complex have been determined **(34, 35)**. The structure of the EGL-1/CED-9 complex reveals that EGL-1 adopts an extended α-helical conformation and induces substantial structural rearrangements in CED-9 upon binding. Importantly, substitutions of residues in EGL-1 that localize at the interface of the EGL-1/CED-9 complex disrupt EGL-1 binding to CED-9, the release of CED-4 from the CED-4/CED-9 complex, and the cell-killing activity of EGL-1 *in vivo* **(34)**, confirming that the binding of EGL-1 to CED-9 is critical for cell death activation. Interestingly, through mutagenesis study, a surface patch on CED-9, different from that required for binding to EGL-1, is identified to be responsible for binding to CED-4, suggesting that EGL-1 and CED-4 bind to different regions of CED-9. The 2.6 Å crystal structure of the CED-4/CED-9 complex reveals an unusual two-to-one CED-4/CED-9 ratio in that one molecule of CED-9 binds to an asymmetric dimer of CED-4 but shares an interface with only one of the two CED-4 molecules **(35)**. EGL-1 binding induces significant conformational changes in CED-9 that result in the dissociation of CED-4 dimer from CED-9. The released CED-4 dimer further forms an oligomer, which facilitates the autoactivation of CED-3, possibly by induced dimerization or oligomerization of the CED-3 zymogen (Fig. 15.4). Importantly, the activation of the CED-3 zymogen can be recapitulated *in vitro* using homogeneous proteins of CED-4, CED-9, and EGL-1, suggesting that these three components identified by genetic analysis are sufficient to mediate CED-3 activation, although the presence of additional positive or negative regulators cannot be ruled out. Mutations that prevent the formation of the CED-4 dimers or oligomers abolish the cell-killing activity of CED-4 *in vivo* and the ability of

CED-4 to induce CED-3 autoactivation *in vitro*, indicating that the CED-4 oligomerization process is critical for CED-3 activation. Together, these structural studies provide significant mechanistic insights into the regulation of cell death activation in *C. elegans*.

Specification of the Life vs. Death Fates

Genetic studies in *C. elegans* suggest that the life vs. death decisions of individual cells are controlled by cell-type-specific regulatory genes **(36)**. These regulatory genes appear to control cell deaths by regulating the expression or activities of key components in the central cell-killing pathway. Control of the life vs. death fates of three specific cell types, HSNs, NSM sister cells, and CEMs, is discussed in this section.

HSN Neurons

The hermaphrodite-specific neurons (HSNs) of *C. elegans* that control the egg-laying process in hermaphrodites are generated embryonically in both hermaphrodites and males but undergo programmed cell death specifically in males, where they are not needed **(3)**. In these sexually dimorphic HSNs, the cell killer gene *egl-1* is under the direct transcriptional control of the *C. elegans* sex determination pathway. TRA-1A, a zinc-finger protein, is the terminal global regulator of somatic sexual cell fate and binds to the *egl-1* gene *in vitro* **(37)**. Specific mutations in a *cis*-regulatory element of the *egl-1* gene that disrupt the binding of the TRA-1A to the *egl-1* gene result in the transcriptional activation of *egl-1* in HSNs and subsequent deaths of HSNs not only in males but also in hermaphrodites **(37)**. Therefore, *tra-1* acts to prevent the cell death fate of HSNs in hermaphrodites by transcriptionally repressing the expression of the *egl-1* gene (Fig. 15.3).

Sister Cells of NSM Neurons

Two genes, *ces-1* and *ces-2* (cell death specification), are important in determining the life vs. death fates of the sister cells of two NSM (neurosecretory motor) neurons in *C. elegans* pharynx **(36)**. Either a loss-of-function mutation in *ces-2* or gain-of-function mutations in *ces-1* can prevent these two cells from adapting their normal apoptotic cell fates. Interestingly, the NSM sister cells undergo programmed cell death normally in *ces-1(lf)* mutants and *ces-1(lf)*; *ces-2(lf)* double mutants, suggesting that the activity of *ces-1* normally is suppressed to allow NSM sister cells to die and that *ces-2* likely acts upstream of *ces-1* to suppress its activity.

The *ces-1* gene encodes a Snail family zinc-finger protein **(38)**. CES-2, a putative bZIP (basic leucine-zipper) transcription factor **(39)**, can bind to the *ces-1* gene *in vitro* and may thus directly repress *ces-1* transcription **(38)**. These findings suggest that a transcriptional regulatory cascade may control the deaths of NSM sister cells in *C. elegans*.

The relationship of the *ces* genes with the death-promoting genes has been inferred from the phenotype of *ces-1*; *egl-1* double mutants **(9)**. In *ces-1(lf); egl-1(lf)* mutants, NSM sister cells survive as they do in the *egl-1(lf)* mutants. This observation indicates that *egl-1* likely acts downstream of *ces-1* to cause programmed death and *ces-1* may negatively regulate the activity of *egl-1* in these cells. Indeed, CES-1 binds to several Snail-binding sites of the *egl-1* gene *in vitro*, suggesting that CES-1 may directly inhibit *egl-1* expression **(40)**. Interestingly, these Snail-binding sites also contain an E-box motif, the DNA-binding site for bHLH (basic helix-loop-helix) DNA-binding proteins. Two *C. elegans* bHLH proteins, HLH-2 and HLH-3, appear to be important for the death of NSM sister cells *in vivo* and can form an HLH-2/HLH-3 heterodimer that binds to the Snail-binding sites/E-boxes in the *egl-1* gene *in vitro* **(40)**. CES-1 may inhibit the death of NSM sister cells by competing with HLH-2/HLH-3 for binding to the Snail-binding sites/E-boxes in the *egl-1* gene (Fig. 15.3).

CEM Cell Fate Specification

Cephalic male sensory neurons (CEMs), like HSNs, are born in both males and hermaphrodites but undergo cell death in hermaphrodite embryos. Genetic screens have been carried out to identify genes that regulate sex-specific CEM cell death **(41, 42)**. Several loss-of-function and gain-of-function mutations in one gene, *ceh-30,* lead to opposite sex-specific CEM cell death defects. Loss-of-function mutations in *ceh-30* cause ectopic death of CEMs in males, whereas gain-of-function mutations in *ceh-30* result in the improper survival of CEMs in hermaphrodites **(41, 42)**. Genetic analysis indicates that *ceh-30* acts downstream of the *C. elegans* sex determination pathway but upstream of, or parallel to, the *C. elegans* programmed cell death pathway to protect CEMs from undergoing apoptosis in males **(41)**. *ceh-30* encodes a BarH homeodomain protein. Interestingly, one of the *ceh-30*(*lf*) mutations and all *ceh-30*(*gf*) mutations alter two adjacent *cis*-elements in the second intron of *ceh-30*. Biochemical analysis indicates that one of the *cis*-elements contains a binding site for the POU homeodomain protein UNC-86, which plays a role in specifying the CEM cell fates, and the other contains a binding site for the terminal sex determination factor TRA-1A, which determines the sexual identity of CEMs **(41, 42)**. The amino-terminus of the CEH-30 protein also contains a conserved eh1/FIL domain that is important for the recruitment of the general transcriptional repressor UNC-37/Groucho **(41)**. Molecular genetic analysis indicates that

ceh-30 defines a critical checkpoint that integrates the sex determination signal, TRA-1, and the cell fate determination and survival signal, UNC-86, to control the sex-specific activation of the cell death program in CEMs through the general transcription repressor, UNC-37 (Fig. 15.3).

Engulfment of Cell Corpses

Two Partially Redundant Signaling Pathways Promote Cell Corpse Engulfment

Once a cell undergoes programmed death, the cell corpse is rapidly engulfed by one of its neighboring cells **(2, 6)**. Genetic analyses have identified several key genes, *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, *ced-10,* and *ced-12,* which function in the cell corpse engulfment process in *C. elegans* **(6, 43–46)**. Mutations in any of these genes block the engulfment of many dying cells and lead to the persistence of cell corpses. Genetic analysis of double-mutant combinations suggests that these genes fall into two classes: *ced-1*, *ced-6*, *ced-7* in one and *ced-2*, *ced-5*, *ced-10*, *ced-12* in the other. Single mutants or double mutants within the same class show relatively weak engulfment defects, while double mutants between two classes show much stronger engulfment defects. This finding suggests that dying cells likely present at least two different engulfment-inducing signals that can be recognized by distinct molecules from engulfing cells, and both signaling events are required for efficient and complete phagocytosis.

The engulfment process consists of three sequential steps: the recognition of a cell corpse by an engulfing cell, the transduction of the engulfing signal to the cellular machinery in the engulfing cell, and the phagocytosis of the cell corpse by the engulfing cell. The genes *ced-1*, *ced-6,* and *ced-7*, which define one engulfment pathway, appear to encode components of a signaling pathway involved in cell corpse recognition. CED-1 is similar to the human scavenger receptor SREC and may function as a corpse-recognizing phagocytic receptor since CED-1 protein was found to cluster around dying cells **(47)**. CED-7 is similar to ABC (ATP-binding cassette) transporters **(47, 48)** and may play a role in promoting or mediating cell corpse recognition by CED-1, as CED-1 receptors fail to cluster around dying cells in mutants defective in the *ced-7* gene **(47, 48)**. The CED-6 protein contains a PTB (phosphotyrosine-binding) domain **(49)** and may act as a signaling adaptor downstream of CED-1 and CED-7 (Fig. 15.5 and Color Plate 5).

The *ced-2*, *ced-5*, *ced-10,* and *ced-12* genes, which define the other engulfment pathway, also control the migration of specific somatic cells, the gonadal distal tip cells (DTC) of *C. elegans*. These four genes encode conserved components of the Rac GTPase signaling pathway involved in regulating the actin cytoskeleton rearrangement essential for cell corpse phagocytosis and cell migration. CED-2 is a CrkII-like adaptor, consisting of one SH2 and

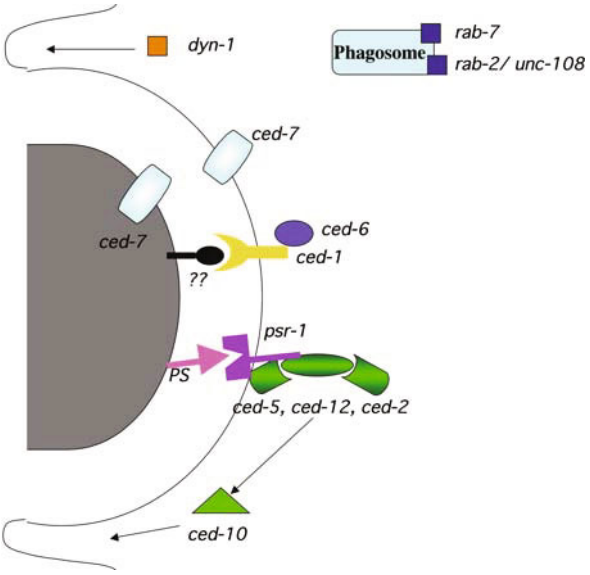

Fig. 15.5 The molecular model for the cell corpse engulfment process. Two partially redundant pathways mediate the engulfment process. CED-1 and CED-7 act on the surface of engulfing cells to mediate cell corpse recognition and to transduce the engulfing signal through CED-6 to the cellular machinery of the engulfing cells for engulfment. CED-7 also acts in dying cells. DYN-1 acts in the CED-1 pathway to promote the delivery of intracellular vesicles to the phagocytic cups and the maturation of phagosomes. RAB-2 and RAB-7 mediate lysosome fusion with phagosomes and are important for the degradation of internalized apoptotic cells. The CED-2/CED-5/CED-12 ternary complex mediates the signaling events from externalized phosphatidylserine (PS)/PSR-1 and other unidentified engulfing signal(s) and receptor(s) to activate CED-10 during phagocytosis (*see* Color Plate 5)

two SH3 domains **(50)**. CED-5 is similar to human DOCK180, which physically interacts with human CrkII **(51)**. CED-10 is a *C. elegans* homologue of mammalian Rac GTPase **(50)**, which controls cytoskeletal dynamics and cell shape change [for a review, see **(52)**]. CED-12 contains a potential PH (pleckstrin-homology) domain and an SH3-binding motif **(44–46)**. Transcriptional overexpression studies suggest that *ced-2, ced-5,* and *ced-12* function at the same step upstream of *ced-10* during the engulfment process. Biochemical analysis indicates that CED-2, CED-5, and CED-12 form a ternary complex *in vitro,* as do their human homologues **(44–46)**. Based on these findings, it has been postulated that the engulfing signal induces the formation and translocation of the CED-2, CED-5, and CED-12 ternary complex to the plasma membrane of engulfing cells and the subsequent activation of CED-10 GTPase, leading to the extension of membrane processes around cell corpses (Fig. 15.5). Recently, CED-10 was proposed to act downstream of *ced-1, ced-6,* and *ced-7* as well **(53)**.

The phagocyte receptors that act upstream of *ced-2, ced-5,* and *ced-12* are poorly understood. One of them is likely the PSR-1 protein, a *C. elegans* homologue of the human phosphatidylserine receptor **(54, 55)**. Both human PSR and worm PSR-1 bind preferentially to phosphatidylserine (PS) or cells with exposed PS, which has been suggested to be one of the engulfment-inducing signals **(56)**. The loss of *psr-1* impairs cell corpse engulfment in *C. elegans* and results in an increase in corpse persistence **(55)**. Genetic and biochemical analyses indicate that *psr-1* functions in the *ced-2, ced-5, ced-10*, and *ced-12* pathway, possibly through the direct interaction of the intracellular domain of PSR-1 with CED-5 and CED-12 to transduce the engulfment signal and to activate the phagocytic event **(55)**. As expected with a receptor for cell corpse engulfment, *psr-1* is required to act in the engulfing cell **(55)**. However, the cell corpse engulfment defect of the *psr-1(lf)* mutant is significantly weaker than that of the *ced-5(lf)* or *ced-12(lf)* mutant, suggesting that other surface receptors may act in parallel to *psr-1* to promote cell corpse engulfment through *ced-5* and *ced-12*.

Externalized PS as an "Eat-Me" Signal and the PS Externalization Process

The loss of phospholipid asymmetry on the plasma membrane has long been an early marker of programmed cell death. While it is known that phosphatidylserine (PS) is exposed on the outer surface of dying cells **(56, 57)**, how this process is activated is unclear. Three classes of proteins have been implicated in establishing and maintaining phospholipid asymmetry, including phospholipid scramblases, ABC transporters, and aminophospholipid translocases [for a review, see **(58, 59)**]. Phospholipid scramblases are ATP-independent, bidirectional lipid transporters proposed to equilibrate the lipid bilayer upon activation. ATP binding cassette (ABC) transporters, also called floppases, are a class of proteins that flip phospholipids and other molecules from the inner to the outer leaflet of the bilayer in an ATP-dependent manner. Aminophospholipid translocases, also known as flippases, are a class of Mg^{2+}-dependent type IV ATPases that flip PS and, to a lesser extent, PE from the outer leaflet of the lipid bilayer to the inner leaflet in an ATP-dependent manner (Fig. 15.6 and Color Plate 6).

C. elegans has eight phospholipid scramblase homologues (named *scrm* genes for scramblases), six aminophospholipid translocase homologues (named *tat* genes), and more than 50 ABC transporter homologues. Genetic analyses of some of these genes have revealed the involvement of all three protein families either in maintaining the phospholipid asymmetry in normal cells or in activating the PS externalization process during apoptosis. *C. elegans* SCRM-1 is partially responsible for PS exposure in apoptotic cells, and the loss of the *scrm-1* activity impairs cell corpse engulfment **(60)**. SCRM-1 is activated by the binding of a proapoptotic factor, the worm apoptosis-inducing-factor homologue (WAH-1), which is released from mitochondria during apoptosis **(60, 61)**. Therefore,

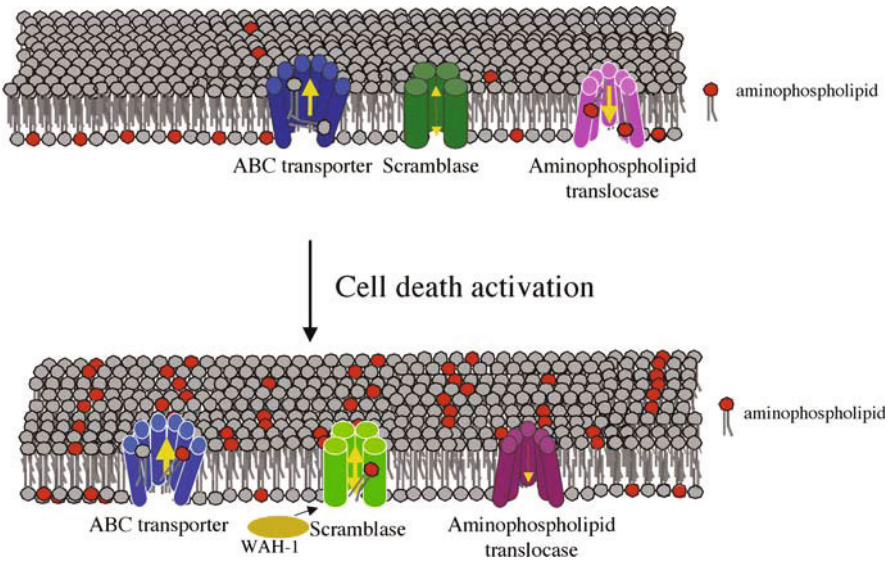

Fig. 15.6 Molecular model of PS externalization during apoptosis. Phospholipid asymmetry is maintained through the action of three classes of proteins: scramblases, ABC transporters, and aminophospholipid translocases. In a living cell, scramblases are not activated and show little to no activity, ABC transporters are only used to maintain lipid balance between the two bilayers, and the aminophospholipid translocases transport any externalized PS and PE to the inner leaflet. During apoptosis, scramblases are activated, for example, by WAH-1 released from mitochondria, randomly scrambling phospholipids on the membrane, ABC transporters may be activated to transport specific lipids to the outer leaflet, and the aminophospholipid translocase is inactivated, leading to PS externalization on the outer leaflet (*see* Color Plate 6)

WAH-1 and SCRM-1 present a mitochondria-to-cell-surface signaling pathway that activates the PS externalization process. Another scramblase, SCRM-3, has been implicated in PS exposure in apoptotic germ cells **(62)**. CED-7, an ABC transporter, has also been implicated in PS exposure in somatic apoptotic cells **(62)**. TAT-1, an aminophospholipid translocase, is required for the maintenance of PS asymmetry on the plasma membrane **(63)**, as loss of the *tat-1* activity causes ectopic PS exposure on the surface of normal cells. Interestingly, improper PS exposure on the surface of living cells in *tat-1(lf)* animals can result in the indiscriminate removal of living cells through a mechanism dependent on PSR-1 and CED-1, suggesting that externalized PS can serve as an engulfment signal for both living cells and apoptotic cells **(63)**.

Internalization and Degradation of Apoptotic Cells

While the recognition of apoptotic cells by neighboring engulfing cells and the signaling pathways that initiate the phagocytic response are well understood,

little is known about how apoptotic cells are internalized and degraded in engulfing cells. Recent studies have shown that a *C. elegans* large dynamin GTPase, DYN-1, plays an important role in engulfing cells to mediate the internalization and degradation of dying cells **(64)**. DYN-1 is proposed to promote delivery of intracellular vesicles to the phagocytic cups to support pseudopod extension and the internalization process and to phagosomes to support their maturation and eventual degradation of apoptotic cells **(64)**. Several Rab GTPases, UNC-108/RAB-2 and RAB-7, are involved in promoting the maturation of phagosomes and the degradation of apoptotic cells **(65–67)**. For example, it has been suggested that the CED-1 signaling pathway activates phosphatidylinositol 3-phosphate [PI**(3)**P] enrichment on the phagosomal membrane, which then recruits RAB-7 and leads to the recruitment and fusion of lysosomes to phagosomes and the formation of phagolysosomes critical for the degradation of dying cells **(67)**. Another Rab GTPase, RAB-2/UNC-108, is also involved in phagosome maturation by mediating the efficient recruitment and fusion of lysosomes to phagosomes and the acidification of the phagosomal lumen **(65**, **66)**.

Degradation of Chromosomal DNA in Dying Cells

The degradation of chromosomal DNA has been thought to be a crucial step and a hallmark of apoptosis. Apoptotic DNA degradation in *C. elegans* has been studied with the aid of DNA-staining techniques. For example, DAPI or Feulgen dye has been used to visualize DNA for *in situ* staining, and the TUNEL technique, which was initially developed to specifically label apoptotic cells **(68)**, has been applied to detect the DNA intermediates with 3'-hydroxyl ends during the chromosome degradation process.

When stained with TUNEL, only a small subset of cells that undergo programmed cell death in wild-type *C. elegans* embryos are TUNEL-positive **(69)**, suggesting that DNA degradation is a rapid process and that TUNEL only labels apoptotic cells during a transient intermediate stage. Interestingly, mutant embryos defective in *nuc-1,* which encodes a mammalian DNase II homologue, have many more TUNEL-reactive nuclei than do wild-type embryos **(69)**. This finding indicates that mutations in *nuc-1* allow the generation of TUNEL-reactive DNA breaks but block the subsequent conversion of these TUNEL-reactive DNA ends to TUNEL-nonreactive ones. Like *nuc-1* mutations, the mutation in the *cps-6* gene (CED-3 protease suppressor), which encodes a homologue of mammalian mitochondrial endonuclease G, also increases the number of TUNEL-reactive nuclei **(70)**. Interestingly, *cps-6*; *nuc-1* double mutants have more TUNEL-reactive nuclei than *cps-6* or *nuc-1* single mutants, suggesting that *cps-6* and *nuc-1* likely function in a partially redundant fashion to destroy TUNEL-reactive DNA ends. However, *cps-6* and *nuc-1* appear to play different roles during apoptosis. A loss-of-function mutation in the *cps-6* gene not only delays the appearance of embryonic cell corpses during development but also can

block the deaths of some cells if the activity of other cell death components is compromised **(70)**, suggesting that *cps-6* is important for the normal progression and execution of apoptosis. In contrast, mutations in *nuc-1* do not appear to affect either the execution of cell death or the engulfment of cell corpses. Furthermore, *nuc-1* mutants are also defective in the degradation of DNA from ingested bacteria in the intestinal lumens. These observations indicate that *cps-6* is a more specific cell death nuclease and likely functions at an earlier stage in the apoptotic DNA degradation process than *nuc-1* does. CPS-6 is the first mitochondrial protein shown to be important for apoptosis in invertebrates, underscoring the conserved role of mitochondria in regulating apoptosis.

Several candidate-based RNAi screens have been carried out in *C. elegans* to identify additional genes involved in mediating apoptotic DNA degradation and have led to the identification of eight new genes **(61, 71)**. These include *wah-1* (*wah*, worm AIF homologue), *crn-1* to *crn-6* (*crn*, cell death-related nucleases), and *cyp-13* (*cyp*, cyclophilins). The loss or reduction of activity in any of these genes results in the accumulation of TUNEL-positive cells in *C. elegans* embryos, suggesting that these genes are important for resolving TUNEL-reactive DNA breaks generated during apoptosis **(61, 71)**. In addition, a reduction in activity in most of these genes (with the exception of *crn-6*) causes a delayed appearance of embryonic cell corpses during development and reduced cell deaths in sensitized genetic backgrounds. Genetic and phenotypic analyses indicate that these genes act in multiple pathways and at different stages to promote DNA degradation and apoptosis, with *cps-6, wah-1, crn-1, crn-4, crn-5,* and *cpy-13* acting in one pathway and *crn-2* and *crn-3* in the other **(61, 71)**.

Like the human AIF protein, *C. elegans* WAH-1 localizes to mitochondria **(61)**. Importantly, ectopic *egl-1* expression induces WAH-1 translocation from mitochondria to nuclei in a CED-3-dependent manner, suggesting that the role of mitochondria in regulating apoptosis is conserved. The WAH-1 protein can physically associate with CPS-6 and enhance the endonuclease activity of CPS-6 **(61)**. Furthermore, CPS-6, CRN-1, CRN-4, CRN-5, and CYP-13, which are either endonucleases or exonucleases, appear to interact and cooperate with one another, possibly in a large DNA degradation complex named a degradeosome **(71)**, to promote stepwise DNA fragmentation, starting from generating DNA nicks, gaps, to double-stranded DNA breaks **(72)**. Like *nuc-1*, *crn-6* encodes a type II acidic DNase and does not seem to affect either the activation or the progression of cell death or the engulfment of cell corpses **(71)**. NUC-1 and CRN-6 may act at later stages of apoptotic DNA degradation, possibly in the phagolysosomal compartments of the engulfing cells to promote degradation of engulfed apoptotic cells.

Summary

Genetic studies in *C. elegans* have identified more than 30 genes that function in different aspects of programmed cell death. These genes have defined a programmed cell death pathway that is evolutionarily conserved. In *C. elegans,* the

life vs. death fates of cells appear to be controlled at the level of transcription. Once the decision to die has been made, a protein interaction cascade involving EGL-1, CED-3, CED-4, and CED-9 is responsible for the activation of the cell death machinery, which initiates various cell disassembly processes such as the apoptotic DNA degradation process involving CPS-6, WAH-1, CRN nucleases, and NUC-1. The dying cell presents at least two distinct engulfing signals to their neighboring cells for phagocytosis. These two signals are recognized and transduced by two partially redundant signaling pathways: CED-1, CED-6, and CED-7 in one and PSR-1, CED-2, CED-5, CED-10, and CED-12 in the other. Finally, the dying cell is completely degraded in the engulfing cell.

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Chapter 16
Cell Death in *Drosophila*

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Abstract The fruit fly, *Drosophila melanogaster*, has been instrumental in identifying pathways regulating development and signal transduction. *Drosophila* has been a relatively late entrant into the world of apoptosis, but with its powerful genetic tools, studies in the fruit fly are destined to contribute essential insights into cell death. *Drosophila* has homologues to mammalian apoptotic genes, and their function can be studied at the cellular level as well as in the developing organism. As in other organisms, cell death in the fruit fly removes damaged and unneeded cells. Beyond this, however, experiments in the fruit fly have provided unique insights into how cell death removes cells that initiate the incorrect developmental program. Furthermore, *Drosophila* studies have begun to elucidate how cell death is spatially regulated for precise pattern formation. In this chapter, we introduce the molecular players in *Drosophila* cell death, discuss current knowledge about cell death during development, introduce the idea that dying cells induce neighboring cells to proliferate, and finish with nonapoptotic roles for cell death proteins.

Keywords *Drosophila* · Apoptosis · Programmed cell death · Development

Introduction

The fruit fly, *Drosophila melanogaster*, has been instrumental in identifying pathways regulating development and signal transduction. *Drosophila* has been a relatively late entrant into the world of apoptosis, but with its powerful genetic tools, studies in the fruit fly are destined to contribute essential insights into cell death. *Drosophila* has homologues to mammalian apoptotic genes (Table 16.1), and their function can be studied at the cellular level as well as

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Table 16.1 Conserved intrinsic death machinery

	Mammalian	*Drosophila*	*C. elegans*
Caspases			
Initiator caspases	i.e., caspases-2, -8, -9, -10	Dronc, Strica, Dredd	CED-3
Effector caspases	i.e., caspases-3, -6, -7	Drice, Dcp-1, Decay, Damm	
Death activator	SMAC/Diablo, Omi/HtrA2	Rpr, Grim, Hid, Skl	
Inhibitor-of-apoptosis protein	cIAP1, cIAP2, XIAP, NIAP, Survivin	DIAP1, DIAP2	
Apoptosis-activating factor	Apaf-1	Dark/Hac-1/dApaf-1	CED-4
Bcl-2 family			
Multidomain proapoptotic	i.e., Bax, Bak, Bok	Debcl/dBorg-1/ Drob-1 /dBok	
Multidomain antiapoptotic	i.e., Bcl-2, Bcl-xL, Bcl-w	Buffy/dBorg-2	CED-9
BH3-only proapoptotic	i.e., Bad, Bid, Noxa, Bim		EGL-1

in the developing organism. As in other organisms, cell death in the fruit fly removes damaged and unneeded cells. Beyond this, however, experiments in the fruit fly have provided unique insights into how cell death removes cells that initiate the incorrect developmental program. Furthermore, *Drosophila* studies have begun to elucidate how cell death is spatially regulated for precise pattern formation. In this chapter, we introduce the molecular players in *Drosophila* cell death, discuss current knowledge about cell death during development, introduce the idea that dying cells induce neighboring cells to proliferate, and finish with nonapoptotic roles for cell death proteins.

Cell Death Pathways

Programmed cell death (PCD) refers to a genetically controlled form of cellular death in which surplus, obsolete, or damaged cells are removed in an orderly fashion. There are two main types of PCD: apoptosis, which is the focus of this chapter, and autophagy, which we cover briefly. Apoptosis was first recognized as a discrete process by the characteristic morphological changes it causes in cells—cytoplasmic and nuclear shrinkage, chromatin condensation and fragmentation, and membrane blebbing **(1)**. Only relatively recently have the molecular pathways behind these changes been elucidated.

The Apoptotic Machinery

Here we briefly describe the components of apoptosis in *Drosophila* and contrast them with their homologues in mammals [see **(2–4)** for reviews].

The workhorses of apoptosis are a category of aspartyl proteases called caspases. They are expressed ubiquitously in virtually all cells in an inactive zymogen form that must be cleaved to become active. Caspases cleave a wide range of cellular proteins, a process that has been called "protein surgery" **(5)**, with the ultimate result of causing the morphological changes described above. Caspases can be divided into two classes: long-prodomain initiator caspases, that cleave other caspases, and short-prodomain effector caspases, that cleave cellular substrates. *Drosophila* has members of each class.

The *Drosophila* initiator caspases are Dronc, Dredd (discussed in the upcoming section on extrinsic signals), and Strica. Dronc contains a CARD domain and is the core initiator caspase in flies **(6)**. Mutation of *dronc* ablates almost all embryonic apoptosis **(7)**. Strica contains a serine-/threonine-rich domain that has not been seen in any other caspase and whose function is unknown **(8)**. Strica has recently been shown to have a redundant function with Dronc in the death of nurse cells during oogenesis **(9)**.

There are four fly effector caspases. Drice is the core effector caspase in *Drosophila*, is activated by Dronc **(10)**, and is important for most embryonic cell death **(11, 12)**. Closely related to Drice are Decay, about which little is known, and Dcp1 **(13)**, that is necessary for proper developmental apoptosis in oogenesis **(14)**. Dcp1 also plays a redundant role to Drice in normal Dronc-induced apoptosis **(9, 15)**. The most recently cloned *Drosophila* caspase is Damm, that is homologous to mammalian caspase-6 and to the *Drosophila* initiator caspase Strica, but does not interact with the caspase inhibitor DIAP1 and may not require cleavage for activity **(16)**. Nothing is yet known about Damm's role in developmental apoptosis.

Caspase activation is regulated at the level of zymogen processing. The initiator caspases interact with death-promoting complexes via protein-interaction domains. The apoptosome, consisting of apoptotic protease-activating factor 1 (Apaf-1) and cytochrome *c* (cyt *c*), is responsible for cleaving and activating procaspase-9. Similarly, in flies Dark (*Drosophila* Apaf-1-related killer) is required for Dronc processing **(17, 18)**. However, the *Drosophila* apoptosome assembles in the absence of cyt *c* **(19)**, and there is considerable evidence that cyt *c* does not play a role in *Drosophila* cell death **(17, 18, 20–22)**. Another mechanism to regulate caspases is through binding to inhibitor-of-apoptosis (IAP) proteins. IAP proteins inhibit activated caspases, and most IAP proteins contain a RING (really interesting new gene) domain that encodes an E3-ubiquitin ligase **(23)**. During mammalian apoptosis, the SMAC/Diablo and Omi/HtrA2 proteins are released from the mitochondria to bind and antagonize IAP proteins. RING-mediated ubiquitination and degradation of IAPs liberates active caspases. The fruit fly functional orthologues to SMAC/Diablo and Omi/HtrA2 are Sickle (Skl), Reaper (Rpr), Hid, and Grim, often referred to as the RHG proteins. *rpr, hid,* and *grim* all reside within the *H99* deletion that was isolated in 1994 as the first genetic lesion that blocked all developmental death in the *Drosophila* embryo **(24)**. All of the *RHG* genes are regulated transcriptionally. Several are regulated through ecdysone-responsive

transcription factors **(25–27)** and *rpr*, *skl,* and *hid* transcription respond to ionizing radiation in a p53-dependent manner **(28, 29)**. The Hid protein is unique in that it is regulated both transcriptionally and posttranslationally (as described later). All four RHG proteins bind to DIAP1 (*Drosophila* IAP1) and induce DIAP1 autoubiquitination and degradation **(30)**. Several recent reviews address caspase and DIAP1 regulation **(3, 31–33)**.

The most upstream of the apoptosis regulators are the Bcl-2 family proteins. In mammals, a large family of Bcl-2 proteins integrates apoptotic signals to make the ultimate decision of whether a cell will live or die. The defining domain of the Bcl-2 family is the Bcl-2 homology (BH) domain. There are three types of Bcl-2 proteins: (1) proapoptotic and (2) antiapoptotic proteins that have four BH domains (multidomain) and (3) proapoptotic proteins that have only the BH3 domain. In response to apoptotic signals, BH3-only proteins bind to the proapoptotic multidomain proteins Bax and Bak, releasing inhibition of their function by antiapoptotic proteins such as Bcl-2 and Bcl-x_L. Bax and Bak cause mitochondrial outer membrane permeabilization (MOMP) and the release of proapoptotic factors such as SMAC/Diablo, Omi/HtrA2, and cyt c. *Drosophila* has two Bcl-2 proteins, Buffy/dBorg2 and Debcl/dBorg1/Drob1/dBok, both of which are multidomain proteins **(34–39)**, and no BH3-only proteins. Both Buffy and Debcl, when ectopically expressed, cause **(35, 39–43)** and inhibit **(35, 36, 43, 44)** cell death under different circumstances. Investigation into *buffy* and *debcl* mutants demonstrated that Buffy inhibits and Debcl promotes cell death triggered by irradiation of the embryo **(44)**. Analysis of the double *buffy debcl* mutant indicated that Debcl promotes apoptosis by inhibiting the antiapoptotic Buffy protein **(44)**.

Clearly, the apoptotic machinery is well conserved between *Drosophila* and mammals. Caspases are essential to the process and are regulated similarly in both organisms. However, although the mechanisms are the same, their relative importance differs between organisms. In mammals, caspase activation is tightly regulated, whereas in flies, a low level of caspase activation is constitutive, making the continuous production of DIAP1 necessary to ensure cell survival **(45, 46)**. This difference is reflected in the uncertainty of a mitochondrial requirement in *Drosophila* apoptosis. Intriguingly, neither Buffy nor Debcl is required for normal development, suggesting that PCD in the fly is regulated differently than in stress-induced death **(44)**. Furthermore, caspases, DIAP1, Dark, and Hid are all required for development, indicating that the difference between developmental PCD and stress-induced apoptosis is primarily at the level of Bcl-2 proteins. This information, together with the lack of involvement of cyt *c* in *Drosophila* apoptosis, suggests that mitochondria may not be as important in fruit fly apoptosis as in mammalian apoptosis. Indeed, Rpr, Hid, and Grim are not sequestered in the mitochondria to be released upon Bax/Bak activation. However, Rpr, Hid, and Grim are all localized to the mitochondria **(47–49)** and, importantly, Rpr and Grim require mitochondrial localization for killing **(47, 48)**. Several recent reports show that *Drosophila* Omi may be selectively released from mitochondria upon an apoptotic stimulus **(50–52)**. Mitochondrial fission, regulated in part by Drp1, has recently been

appreciated to play a role in mammalian apoptosis **(53)**. Similarly, in *Drosophila*, mitochondrial changes occur in dying cells and the inhibition of Drp1 reduces mitochondrial disruption and apoptosis **(21)**. Furthermore, Bcl-2 proteins may play a role in mitochondrial dynamics **(53)**, although such a role for the *Drosophila* Bcl-2 proteins has not been investigated. Thus, there is evidence that mitochondria in *Drosophila* play a role in stress-induced apoptosis and that Debcl, Buffy, Rpr, Grim, and Drp1 may all be involved in this.

Extrinsic Signals for Caspase Activation

Whereas the molecules and mechanisms involved in triggering, executing, and regulating apoptotic cell death within a cell are becoming well understood, only now are we beginning to understand the connections between this process and cell signaling, either extracellular or intracellular. Most of our understanding of cell death signaling has come from studies in the immune system or in tissue culture cells. For example, in the immune system, regulation through the TNF receptor and Fas mediate death signaling through the activation of initiator DED-containing caspases such as caspase-8 that, in turn, cleave and activate downstream effector caspases [reviewed in **(54)**].

Although flies do not have an adaptive immune system, they have a homologous pathway that includes a death receptor, Wengen, and its ligand, Eiger **(55)**. Their role in *Drosophila* development is still unclear. The *Drosophila* innate immune system employs the Toll and IMD pathways to guard against microbial infection, and both pathways result in the production of antimicrobial peptides rather than apoptosis. Dredd, the *Drosophila* homologue of caspase-8 **(56)**, is a downstream component of the IMD pathway. In this pathway, Gram-negative bacteria cause the activation of Dredd, which then mediates the activation of the transcription factor Relish, presumably via cleavage. Relish then translocates to the nucleus and induces transcription of the antimicrobial peptides specific for Gram-negative bacteria **(57)**.

Cell signaling also regulates cell death in organs and tissues. We are only beginning to look at developing epithelia in an effort to understand its spatial regulation (further discussed in the following sections); model organisms are essential for this. An important example of invoking cell death in response to external stimuli involves signaling through the Ras signal transduction pathway. Following stimuli from growth factors and the extracellular matrix, activated Ras signaling can regulate apoptosis, either positively or negatively, by controlling the activity of multiple effectors. In flies, the activation of Ras signaling can block cell death, at least in part by downregulating Hid expression and by suppressing Hid activity through phosphorylation **(58–60)**; interestingly, it can also lead to apoptotic cell death in surrounding cells that have not received ectopic Ras activation **(61)**, suggesting that a death-promoting factor is released to neighbors.

Tools of the Trade: How We Observe and Block Apoptosis in *Drosophila*

An introduction to apoptosis research in *Drosophila* would not be complete without a discussion of the techniques used to study it. Here we list the most commonly used methods of detecting apoptotic cells and of blocking the process of apoptosis.

Observing Apoptosis

Although the morphological hallmarks of apoptosis have been known for many years, it is often not feasible to use morphology as a screening tool. Cell morphology is difficult to see within the context of a tissue, and most morphological changes occur at the end stages of the process of apoptosis, leaving early apoptotic cells invisible. Therefore, the most common tools used to assay apoptosis are molecular ones. There are four main categories of molecular apoptosis assays:

- TUNEL (terminal uridine deoxynucleotidyl transferase dUTP nick end labeling): This assay works by labeling the ends of cleaved genomic DNA with a fluorescently labeled uridine.
- Cleaved caspase antibodies: As their name suggests, these antibodies detect the cleaved (active) forms of caspases, but not the inactive zymogens. Robust antibodies are commercially available that detect human cleaved caspase-3 (CM1, Idun Pharmaceuticals; or CC3, Cell Signaling Technology) and cleaved caspase-7 (CC7, Cell Signaling Technology). The CC3 antibody has been used extensively in *Drosophila*. By Western analysis, the antibody recognizes Drice, but *drice*-null embryos still show punctate staining. Thus, it is not clear what apoptotic epitope CC3 recognizes **(11, 62)**. The CC7 staining pattern suggests that it recognizes a different antigen than does the CC3 antibody (CBB, unpublished results). Caspase activation is a hallmark of apoptosis, but not all cells with activated caspases are doomed to die (see the upcoming section entitled "Demolishing or Remodeling?").
- Annexin V: Annexin is a protein that binds phospholipids normally found on the inner leaflet of the plasma membrane. An early indication of apoptosis is the translocation of phosphotidylserine (PS) to the outer leaflet. This can be recognized by fluorescently labeled Annexin V.
- Vital dyes: Several dyes mark cells using an exclusionary mechanism, including Acridine orange (AO), Propidium iodide (PI), and Trypan blue. Acridine orange fluoresces when intercalated in DNA. In a healthy cell, AO is sequestered in the lysosomes. When a cell dies, the lysosomal membrane potential is lost and the AO moves into the nucleus, where it fluoresces **(63)**. PI and Trypan blue are both excluded from healthy cells by their intact plasma membranes but accumulate in cells with disrupted membranes. These dyes

are not ideal for studying apoptosis, since they do not differentiate between different types of cell death. Nevertheless, AO is commonly used to visualize apoptosis in *Drosophila* tissues.

A good source for more detailed protocols for each of these methods is White et al. **(64)**.

Blocking Apoptosis

In order to study a process such as apoptosis, one must often impede it or prevent it altogether. This can be done genetically in *Drosophila* by deleting proapoptotic genes, as with the *H99* deficiency mutant, that ablates all embryonic death **(65)**. However, it is often more advantageous to block apoptosis at a later developmental stage. The ectopic expression of the Baculovirus inhibitor-of-apoptosis protein p35 or DIAP1 is often used to inhibit activated caspases in a tissue- and temporal-specific manner. Notably, p35 does not inhibit the *Drosophila* initiator caspase Dronc **(10, 66)**.

Early Use of Cell Death in Oogenesis and Embryogenesis

Cell Death in Drosophila *Oogenesis*

Drosophila oogenesis is divided into 14 stages as the germarium develops into a mature oocyte. During early stages (S1-S8) of oogenesis, the *Drosophila* egg chamber encloses a cluster of 16 interconnected germ-line cells surrounded by a monolayer of roughly 650 somatic follicle cells. One of these 16 germ-line cells will become the oocyte, while the other 15 will develop into nurse cells. The nurse cells support the oocyte by transporting proteins and RNAs into the oocyte (termed "dumping") through cytoplasmic bridges. Prior to this process, defective egg chambers are removed by apoptosis to ensure that only normal egg chambers are permitted to proceed through development. After dumping their contents, the remaining depleted nurse cells undergo apoptosis during stages 12 and 13 (Fig. 16.1) [reviewed in **(67)**]. The overlying follicle cells phagocytose the fragmented and condensed nuclei of the nurse cells. PCD during oogenesis does not require *rpr, hid, grim, skl, Cyt-c,* and *debcl* **(68, 69)**, but does require the caspases *strica*, *dronc*, *drice,* and *dcp-1* in redundant roles **(9)**. Thus, PCD during oogenesis utilizes a novel pathway leading to caspase activation.

Cell-to-cell signaling plays an important role in regulating nurse cell death. The BMP2/4 orthologue Dpp is expressed in the follicle cells, while its receptors are expressed in both the nurse cells and follicle cells. Several experiments suggest that Dpp signaling is central to regulating cell death during oogenesis. Reducing the activity of either Dpp or its receptor Saxophone in egg chambers produces defective anterior eggshell structures. In addition, germline cells

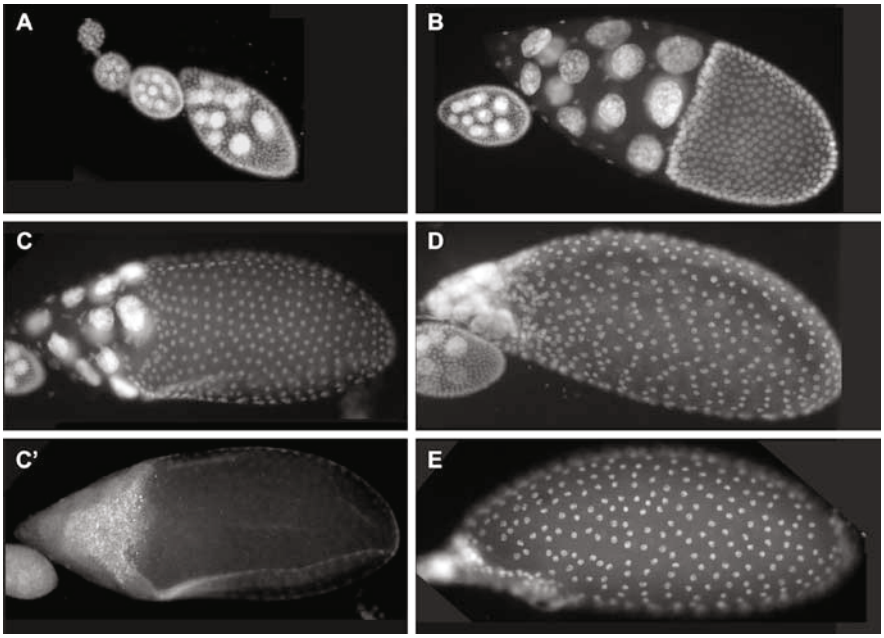

Fig. 16.1 Stages of *Drosophila* oogenesis and events of PCD. Parts (**A-E**) are labeled with DAPI to identify nurse cell nuclei (large nuclei) and follicle cell nuclei (smaller nuclei). (A) Portion of an ovariole showing the early stages of oogenesis to stage 8. (B) Stage 10 egg chamber next to smaller stage 6 egg chamber. (C) Stage 11 egg chamber. (C') Same egg chamber as in (C), labeled with antibody to cleaved caspase-3 (CC3) to indicate that activated caspases are detected at the stage of nurse cell dumping. (D) Stage 12 egg chamber; note that the nurse cell nuclei are fragmented and shrunken. (E) Mature egg chamber. All images were taken at the same magnification

mutant for *saxophone* display defects in actin bundle formation and failure of the nurse cells to complete cytoplasm transfer **(70)**, suggesting that Dpp signaling orchestrates programmed cell death in the germline.

Cell Death in the Embryonic Epidermis

Cell death is utilized throughout embryonic development, and the embryo has provided a number of important insights into the role of programmed cell death during normal epithelial patterning. An excellent example is the establishment of segments. Cell division and cell movement create 14 segments that represent the fundamental segmental units of the embryo. Each segment expresses *hedgehog* and *engrailed* at its posterior margin, while *wingless* is expressed within its anterior. Wingless- and hedgehog-expressing cells signal each other to stabilize segment boundaries [for a review, see **(71)**]. In late-stage wild-type embryos,

about 10 diverse epidermal cell types are generated within each segment; each cell type contributes to a different part of the cuticular covering to mark positional information.

Time-lapse microscopic studies using Acridine orange (AO)-injected embryos found that approximately 40–45 dying cells were detected in the ectoderm of each segment during stages 12–14 **(72)**. Although the number of dying cells varies from embryo to embryo, the pattern of cell death within each embryo is conserved. Approximately three-fourths of the dying cells were located in or immediately adjacent to the *engrailed* posterior stripe. Some dying cells form clusters at specific locations along the dorsal-ventral axis, and the expression pattern of *rpr* correlates closely with this distribution (Fig. 16.2). Dead cells are eventually engulfed by their neighbors **(73)**.

The segment polarity genes *wingless* and *hedgehog* and their signal pathway components play an essential role in patterning the embryonic epidermis. Loss of *wingless* signaling leads to abnormally small embryos with a number of patterning defects that are likely due to inappropriate cell death **(74–76)**. Using time-lapse imaging studies, Pazdera et al. **(72)** showed that the removal of *wingless* signaling resulted in a fivefold increase in the number of dying cells within the anterior of each segment; this death occurred in cells approximately six rows away from the *wingless*-secreting cells. Conversely, activating the *wingless* pathway by reducing *naked* function led to a sixfold increase in apoptosis in the posterior region **(72)**. The best evidence to date suggests that *wingless* and *hedgehog* do not directly activate the apoptotic pathway; instead, signaling through the *Drosophila* EGF receptor (dEgfr) is responsible. Cells in the *wingless* anterior strip secrete the dEgfr ligand, Spitz, to stimulate the

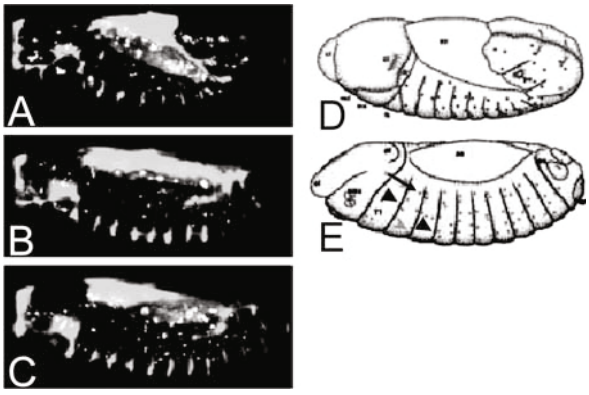

Fig. 16.2 Cell death pattern in the embryonic epidermis in live embryos. Apoptotic cells detected by Acridine orange (*white spots*) are shown relative to each segment border (*light gray*) in an embryo at stages 12, 13, and 14 (**A–C**). A map of cell death in the abdominal epidermis is illustrated during stage 11–12 (**D**) and stage 13–14 (**E**). The *arrow* and arrowheads indicate three clusters of dying cells along the dorsal-ventral axis. Adapted with permission from **(72)**

survival of cells in the *engrailed* posterior stripe. But a limited amount of Spitz is secreted and cells that do not receive Spitz die **(77)**.

A regional increase in cell death appears to be a common phenomenon of mutations that affect axis formation, suggesting that embryos monitor the precision with which segments are made. Surprisingly, correcting axis defects appears to use only cell death and not, for example, the cell cycle. In an elegant set of experiments, Namba et al. demonstrated that an overall increase in the gene dosage of the anterior determinant *bicoid* leads initially to an enlarged head region; however, the embryo compensates by increasing cell death in its anterior regions and decreasing it in the posterior **(78)**. A decrease in *bicoid* led to the complementary response. Cell cycle levels were unaffected. These experiments point to the central role of selective cell death to clean up the normal errors that occur during development. Cell death acts as a buffer against errors and provides precision to the emerging embryo. As we shall see later in this chapter, other tissues such as the wing and eye utilize death in much the same way.

Death in the CNS

During mid-embryogenesis, about 10 glial cells in the embryonic midline (midline glial cells) are generated. As development proceeds, the majority of these midline glial cells die, leaving typically three glial cells per segment to support axonal connections. Cell death of midline glial cells is caspase-dependent and requires *rpr, hid,* and *grim* **(79, 80)**. Analysis of mutations that remove different combinations of *rpr*, *hid,* and *grim* demonstrated that these three death activator genes normally act synergistically to promote cell death at the midline **(80)**.

Surface signaling through the dEgfr is important for the survival of midline glial cells **(79, 81, 82)**. The secretion of Spitz from neurons promotes the activation of the downstream effector MAPK, that in turn phosphorylates and inactivates Hid **(58–60)**. By calibrating this signaling, presumably the correct number of cells can be retained. How this calibration is achieved, however, is not understood.

wingless signaling is required for development of the embryonic brain **(83)**. As discussed earlier for the embryonic epidermis, the loss of *wingless* signaling results in the initial generation of the developing brain followed by massive apoptotic cell death. Again, the removal of incorrectly specified cells is a recurring theme in development; how mispositioned cells are recognized and removed represents an enduring mystery in the cell death field.

Embryonic Head Development

The development of the embryonic head is an example of a tissue that relies upon spatially regulated apoptosis for gross patterning. The emerging embryonic head

undergoes axial induction during a process of "head involution" in which the head is "swallowed" inward at the mouth regions. Normal head morphogenetic movements utilize widespread cell death during retraction of the clypeolabrum during early head involution, formation of the dorsal ridge, fusion and involution of dorsal structures of the pharynx and mouth cavity, and segregation of progenitor cells of the brain **(84)**. Although the early stage of head development such as formation of the dorsal ridge and the pharynx proceeds almost normally without apoptotic death, PCD is required for later stages of head development. *H99* mutant embryos fail to demonstrate normal morphogenesis associated with head involution, including retraction of the clypeolabrum, formation of dorsal pouch, and fusion of gnathal lobes, demonstrating a requirement for *rpr, hid,* and *grim* **(84)**. This suggests that shaping and reducing the size of the head are all important aspects of normal involution.

Cell Death Prior to and During Metamorphosis: The Mechanism of Cell Death Depends on the Larval Tissue Type

Metamorphosis is the fascinating process in which the larva is transformed into an adult fly and is regulated stage-specifically by changes in titer of the steroid hormone ecdysone. Following the roughly 24 hours of embryonic development, the first instar larva hatches. The larva eats continuously to promote growth and molts after one day into the second instar larva and a day later into the third instar larva. Third instar larvae continue to eat and grow for another two days. During this time, the imaginal discs that will give rise to adult structures proliferate and grow and very little cell death is observed. A sharp rise in ecdysone at the end of larval development triggers puparium formation and the onset of prepupal development. A second major ecdysone pulse 10 hours later initiates the prepupal-pupal transition. Pupal development then proceeds until ecdysone titers return to basal levels and the adult fly emerges, or "ecloses" **(85)**.

In the larva, there are essentially two types of tissues: endoreduplicative tissues, which become obsolete during metamorphosis, and progenitor cells, which will proliferate and grow into the adult body. The progenitor cells of the imaginal discs are located in the larva as dormant sac-like structures and have no real function for the larva **(86)**. Endoreduplicative tissues, on the other hand, such as salivary glands, midgut, and fat body, are made up of large polyploid storage cells with multiple functions that are vital for larval development **(87, 88)**. It is believed that the histolysis of the endoreduplicative organs fuels the proliferation and differentiation of imaginal discs into adult structures during metamorphosis, a time when the animal is not feeding.

A coordinated effort between two types of programmed cell death, apoptosis and autophagic cell death, occurs during metamorphosis **(89, 90)**. The process of autophagy, also called macroautophagy, involves the nonselective sequestration of cytoplasmic material into a double-membrane vesicle. This vesicle,

the autophagosome, fuses with a lysosome to form an autolysosome where the cellular contents are broken down **(91, 92)**. Autophagy is usually regarded as a cell survival mechanism, but it can lead to death of the cell. During metamorphosis there are instances where apoptosis is favored, usually for sculpting purposes, as seen during the development of the embryo and eye. However, for the degradation of major tissues such as salivary glands and the midgut, autophagic cell death takes center stage with the help of classical apoptotic factors to initiate the process. We will further explore the mechanisms of cell death during metamorphosis based on tissue type.

Autophagic Cell Death in Midgut and Salivary Gland Histolysis

Autophagic cell death plays a dominant role in histolysis of the midgut and salivary glands. Of the many transcriptional targets regulated by ecdysone signaling, a small set of transcription factor-encoding genes are initially transcribed to amplify the hormonal signal and further regulate secondary targets. A few of these early targets have been well-studied during histolysis of these tissues and include *Broad Complex (BR-C), E74A,* and *E93* **(93)**. These early transcription factors are responsible for regulating both death-activating and autophagy genes during metamorphosis **(25, 94–97)**.

In the instance of the midgut, the first titer of ecdysone at the end of larval development initiates cell death of the larval midgut. Dramatic changes of the midgut then follow between two to six hours after puparium formation (APF), marked by contraction of the midgut body, disappearance of the gastric caeca, and proventriculus **(27, 94)**. Very high autophagic activity is observed in midguts from third instar larvae (Fig. 16.3). This is followed by DNA degradation as measured by TUNEL (Fig. 16.3) during the early pupal stage. The expression of the caspase inhibitor p35 prevents midgut death **(94)**. Additionally, transcription of *rpr, hid, dark*, and *dronc* occurs before AO staining and DNA fragmentation **(27)**. By 12 hours APF, very little cellular structure is left in the midgut and the cytosol is extremely condensed. At this same time, the adult midgut epithelium begins to surround and isolate the larval midgut and it is thought that this barrier to phagocytic cells is why the midgut cells degrade themselves. A recent study using a mutant that blocks autophagy demonstrated that autophagy was required for the onset of DNA fragmentation **(98)**. Surprisingly, this study determined that even without autophagy or DNA degradation, histolysis of the midgut progressed, albeit a little slower **(98)**.

In contrast, programmed cell death in the salivary gland occurs at 10 hours APF, correlating to the second ecdysone pulse. The difference in timing between these two ecdysone-dependent events may reside in their distinct expression of death activators such as *rpr* and *hid*. Cells in the salivary gland detach from the basement membrane surrounding them and are degraded rapidly by 15 hours APF **(99)**. Like midgut histolysis, dying salivary glands

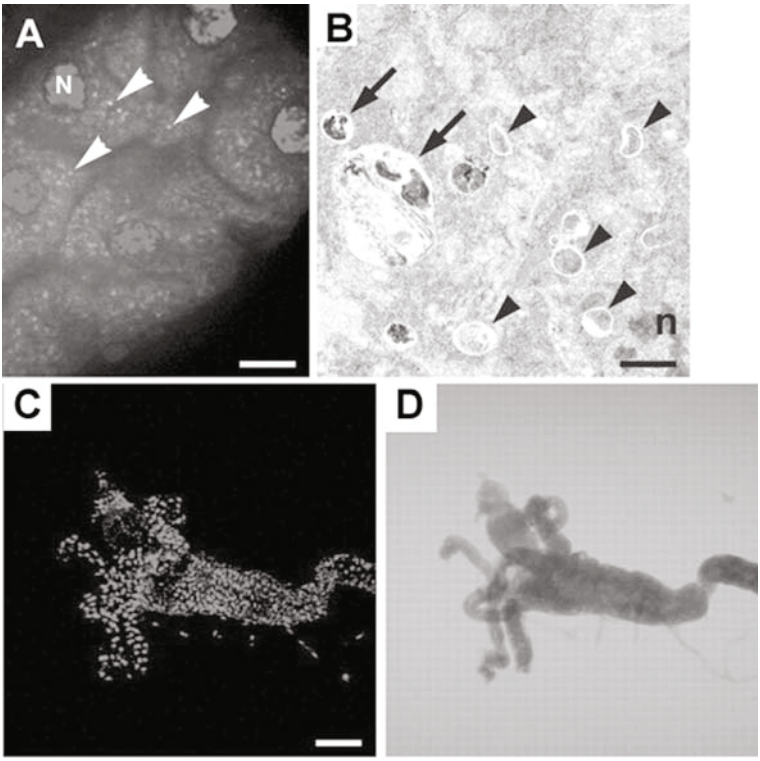

Fig. 16.3 Autophagy during midgut histolysis. (**A**) Autophagy is identified by punctae in midguts labeled with GFP-LC3. A few of the many punctae are indicated by white arrowheads. The nuclei (N) are larger structures in each cell. (**B**) Ultrastructural analysis reveals numerous autophagosomes (*arrowheads*) and autolysosomes (*arrows*) in the larval midgut. (**C**, **D**) The larval midgut displays TUNEL labeling indicative of apoptosis. Bars: (A) 10 μm; (B) 1 μm; (C, D) 200 μm. Reprinted and adapted from **(98)**, with permission

are AO- and TUNEL-positive and contain a massive number of autophagic vacuoles **(100)**. However, the expression of p35 does not completely block salivary gland death, although CC3 and AO staining is blocked **(99, 100)**. A recent study unequivocally demonstrated the essential role for autophagic cell death in salivary glands and identified numerous *atg* mutants in which the process is inhibited **(101)**. Furthermore, the induction of autophagy was capable of destroying salivary glands, independent of caspases; however, *in vivo* the two appear to function in parallel with independent regulation **(101)**.

Examples of Classical Apoptosis

Apoptosis plays an integral part in the development of imaginal discs. Precisely patterned cell death has been reported in the mature larval and young pupal

wing and eye imaginal discs. We focus here on apoptotic events for sculpting the developing wing and notum. The special case of the developing eye deserves its own section.

Larval Wing Disc

Adult wings develop from wing imaginal discs. These discs invaginate from the embryonic ectoderm as simple pouches of epithelium during embryogenesis that grow during the first two larval instars. During the third larval instar, differentiation begins and the wing pouch grows out to form the wing blade during metamorphosis. Based on condensed nuclei and TUNEL staining, a steady average of 1.4% of wing cells are apoptotic throughout the entire second and early third instar larval wing discs **(102)**. The location of these apoptotic cells is not random. In the late larval period, dying cells are found preferentially at the wing/notum border. In the early pupal wing (0–12 hours APF), the majority of dying cells are found at the wing hinge; at 12–20 hours APF, they appear in the pupal wing blade; and at 20–24 hours APF, dying cells are distributed along the periphery of the wing blade and at the wing margin. After 24 hours APF, there is no detectable cell death in the pupal wing. Interestingly, cells in the wing disc often die in small groups, suggesting that death signals are not specific to a single defective cell.

Similar to the embryo, PCD corrects mispatterning in the developing wing **(103, 104)**. The developing wing disc is a superb tissue to study death of cells that initiate the incorrect developmental program because cell fate changes can be readily generated experimentally, and identified easily with molecular markers. When cells are incorrectly misspecified with respect to the dorsal-ventral or anterior-posterior axes, they tend to sort out into their appropriate compartment. When this does not occur, cell death is initiated to remove the cell. Several leucine-rich repeat (LRR) family transmembrane proteins, Fish-lips, Capricious, and Tartan, are involved in this process. These cell surface proteins help a cell decide if it is in the correct place through affinity interactions **(105, 106)**. Cells that are misspecified activate JNK, possibly through activation of the Notch signal transduction pathway. JNK activation is correlated with apoptosis **(107)**. Further experiments are needed to determine how the LRR proteins recognize misspecified cells and how loss of affinity interactions triggers cell death.

The Pupal Notum

The adult notum, the dorsal portion of the thoracic section, develops during pupation and has two types of mechanosensory bristles, machrochaetes and microchaetes **(108–112)**. Each bristle group arises through several divisions from a single sensory organ progenitor (SOP) cell. In the microchaete lineage, the sensory organ progenitor (SOP) cell divides at 17-19 hours APF to form the PIIa and PIIb cells. The PIIa cell matures and divides again at 20–22 hours APF

to form the shaft and socket cells. The pIIb cell divides to form a glial cell and the PIII cell; the PIII cell then undergoes a final division to form the sheath and neuron **(113, 114)**. The glial cell undergoes apoptosis at 22 hours APF that can be blocked by expression of p35 **(115)**. Our laboratory has recently determined that *grim* is required for glial cell death (Wu, J and CBB unpublished). Glial cells fated to die are marked with a decreased amount of DIAP1 protein correlating with studies demonstrating a similar decrease in DIAP1 protein in cells ectopically expressing Grim (Wu, J and CBB unpublished) **(62)**. It is not understood how *grim* is transcriptionally activated in the glial cell, nor is the signaling pathway that leads to *grim*-dependent death understood.

The Developing Eye

The *Drosophila* compound eye is composed of approximately 750 identical units or ommatidia. Each adult ommatidium contains eight photoreceptor neurons, four cone cells, and two primary pigment cells. Between ommatidia lies an interweaving hexagonal lattice composed of interommatidial cells [IOCs; also called interommatidial precursor cells (IPCs) or **2**°/**3**° cells] and mechanosensory bristles (Fig. 16.4 and Color Plate 7). The fly eye is an excellent amplifier of mutation-induced defects: The misplacement of just a few ommatidia can make the entire eye seem jumbled, similar to throwing a stone in a pond. For this reason, a number of mutations that affect cell fate and cell death have been discovered or studied in the eye. The fly eye is a superb example of an epithelium that is patterned or sculpted through cell death and offers the opportunity to study cell death at the level of single cells and within the context of an emerging neuroepithelium.

Cell Death in the Larval Eye Disc

Drosophila retinal development occurs within the eye/antennal imaginal disc. This retinal epithelium is specified by the combined efforts of a number of genes, including *eyeless*, *twin of eyeless*, *sine oculus*, *eyes absent*, and *dachsund*. The loss of, for example, *eyes absent* activity will result in the loss of the eye progenitor cells by cell death, leading to the complete loss of the adult eye **(116)**.

After the imaginal tissue is specified as eye primordia, cells within the eye simply proliferate without commitment to a specific retinal cell fate until the third instar larval stage, when retinal pattern formation begins in a wave of morphogenesis that sweeps across the eye disc from posterior to anterior **(117)**. At the front of this wave is the "morphogenetic furrow," the region in which cells prepare for differentiation by arresting in G1 of the cell cycle. A low level of cell death can be observed anterior to and 10–12 rows posterior to the morphogenetic furrow **(118)**. Within the morphogenetic furrow, a patterned array of

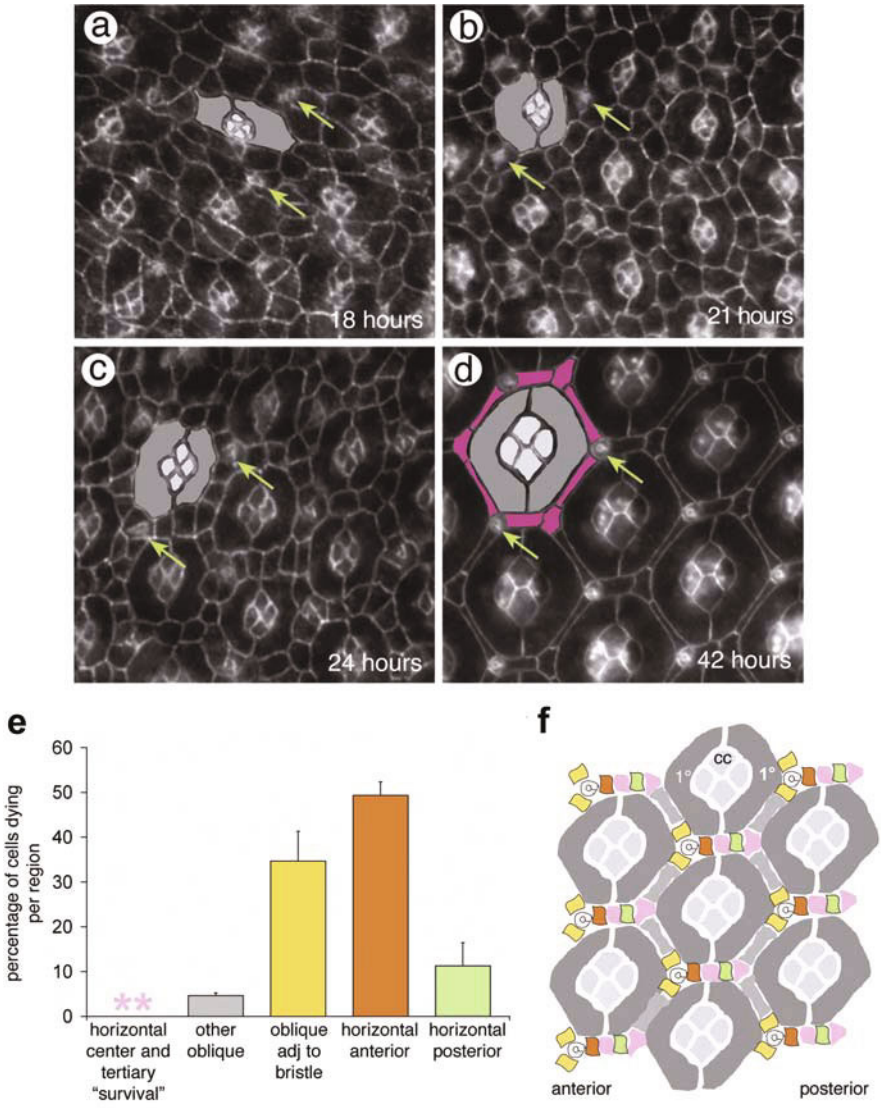

Fig. 16.4 Live imaging demonstrates that cell death in the pupal eye is temporally and spatially regulated. (**A**–**D**) Pupal retinae of developmental ages as shown with cell boundaries outlined in *white*. For clarity, an ommatidium is shaded (primary pigment cells are *dark gray* and cone cells are *light gray*) in each panel and bristle groups are indicated by *green arrows*. IOCs account for the remainder of cells. In (D), the remaining IOCs after death are *colored pink*. (**E**) The percentage of cells observed dying graphed relative to specific regions. The shading correlates to the regions in (**F**). Two pink asterisks indicate that no cells were observed to die in these positions. (F) Schematic of the pupal retina, with each *shaded region* corresponding to a position in which cells will either be more likely to live (*pink and green*) or die (*orange and yellow*). Figure adapted from **(139)**. (*see* Color Plate 7)

cell clusters is established that will eventually become individual ommatidia, and the cell fate of the first neuron, photoreceptor R8, is determined. The cell number in each cluster and spacing between the clusters are controlled by the coordinated action of a number of genes, including *scabrous*, *Notch*, *atonal*, *dEgfr, spitz*, *argos,* and *Star* [for a review, see **(119, 120)**]. In most cases, a mutation or the ectopic expression of these genes, as well as the failure to propagate the morphogenetic furrow, will result in global mispatterning and lead to ectopic cell death.

In a set of elegant studies, Baker and Yu demonstrated that dEgfr is required for cell cycle progression from the G2 phase to the M phase in cells between emerging ommatidia in the larval eye. Ommatidia produce the dEgfr ligand Spitz to ensure survival of most IOCs; those cells farthest from ommatidia often fail to survive **(121)**.

Cell Death in the Pupal Eye

Starting at 22 hours APF, IOCs and the bristle group (composed of four cells) begin to rearrange themselves with the goal of forming an invariant hexagonal lattice between the ommatidial array (Fig. 16.4). In the end, each ommatidium will share nine IOCs and three bristles with its neighbors. This process involves extensive cell movement and extensive programmed cell death: About 2,000 excess IOCs in the pupal retina will be removed **(122)**.

Many of the cell death regulatory factors discussed above are active in the pupal retina as well. Blocking caspase activity, either by ectopic expression of the caspase inhibitor p35 or by overexpression of DIAP1 or DIAP2, leads to too many surviving IOCs within the lattice **(123–127)**. The initator caspase *dronc* is required for IOC apoptosis **(7, 128)**, as is *dark* **(128)**. The loss of *drice* only partially blocks IOC death, indicating that *drice* functions redundantly with other effector caspases **(11)**. Like other examples of PCD in the fly, neither Bcl-2 protein is involved in this process **(44)**. Deletion of one of the two cytochrome c genes, *cyt c-d*, significantly delays IOC death **(128)**.

Prior to cell death, IOCs are initially arranged in multiple layers between ommatidia and then rearrange so that they are in an end-to-end configuration (Fig. 16.4). This distinct change in epithelial patterning, or sorting, is an important first step in the cell death process **(118, 129)**. Recent data suggest that cell-to-cell adhesion mediated by two immunoglobulin family members, Roughest and Hibris, as well as adherens junctions regulates IOC sorting **(130–132)**; for a review, see **(133)**. A small number of IOCs are removed by PCD during this time in a process dependent on *wingless* signaling **(134)**. After the IOCs complete the sorting step, the major phase of IOC death begins. Signaling through the dEgfr/Ras pathway promotes IOC survival **(135–138)**, in part though negative regulation of Hid **(59, 60)**. Cell ablation studies revealed

that primary pigment cells provide a "life" signal to the IOCs, likely through expression of the dEgfr soluble ligand Spitz **(135, 139)**. The Notch signaling pathway counteracts the dEgfr/ras pathway by promoting IOC death **(135, 139, 140)**. At this point, it was unclear how these two signaling pathways could precisely remove the right number of IOCs to leave the precise lattice seen in the adult.

To begin to understand this problem, our laboratory was interested in understanding whether IOC death is spatially regulated. Through visualization of retinae in living pupae, we demonstrated that IOC death occurs in stereotypical regions between ommatidia that we termed "death zones" **(139)** (Fig. 16.4). Cells in the death zone were more likely to die in response to a brief interruption in dEgfr signaling than IOCs in other regions. Together with molecular data, this led to the suggestion that Notch dampens dEgfr survival signaling to allow an unknown death signal propagated from specific regions within the primary pigment cells to remove IOCs in the death zone **(139)**. The live visualization studies clearly identified survival positions for IOCs, as would be required to prevent excessive death **(139)**. These studies demonstrated that IOCs in the death zone die and are replaced by neighboring IOCs that will also die until only IOCs in survival positions remain.

Only a few studies have investigated the transcriptional regulation of IOC cell death. The Runx DNA-binding protein Lozenge is required for IOC death and transcriptionally regulates Argos and Klumpfuss, both of which promote IOC apoptosis **(141, 142)**. A very recent finding is that mutations in *Trithorax-like (Trl)* and *lola-like/batman (lolal)* genes, which share characteristics of *polycomb-Group* and *trithorax-Group* genes, block IOC death nonautonomously **(143)**.

Late in pupal development, the row of stunted ommatidia found at the outer rim of the retina, thought to aid normal photoreceptor axonal pathfinding and ensure straight ommatidial rows, is removed through apoptosis. This process is regulated by *wingless* **(144)** and requires genes in the *H99* region, *dronc* and *dark* **(128, 144)**.

Cell Death in the Newly Emerging Adult

At the end of metamorphosis (about four days APF), the adult fly expands its ptilinum to push through the pupal case (eclosion) and emerge with unexpanded wings and long, thin, and relatively unpigmented bodies. Cell death is observed in muscles and neurons of the newly emerged adult. Many of the abdominal muscles used for eclosion and wing-spreading behavior die by 12 hours after eclosion **(145)**. Most of the ptilinal muscles, a special set of muscles involved in retracting the ptilinum into the head capsule, die and are absorbed within 24 hours after eclosion **(146)**. Cell death of abdominal and metathoracic neuromeres is triggered after

eclosion and is regulated by the level of ecdysone **(147)**. n4 neurons, a subset of ventral CNS type II neurons, disappear within 24 hours of eclosion, have condensed, shrunken nuclei **(147)**, and accumulate *rpr* and *grim* (but not *hid*) transcript **(148)**.

At eclosion, an adult fly's wings are folded and opaque, but shortly thereafter the wings expand. Within the first hour after eclosion, the two halves of the wing epidermis fuse and the cells die by apoptosis (as determined by condensed nuclei, inhibition by p35, and TUNEL labeling) with significant numbers of vacuoles suggestive of autophagy, leaving the wing transparent **(149)**. Only the wing vein cells remain alive.

Degeneration in Adults

Adult fly cells, similar to our own, retain the capacity to undergo apoptosis following various stresses. The life span of a terminally differentiated cell is dependent on organelle health, in particular the mitochondrion. High-energy-demanding cells such as neurons and muscle cells rely heavily on mitochondrial function; thus, these cell types are the first to suffer from mitochondrial dysfunction. Defective mitochondria are usually marked by a decline in mitochondrial membrane potential ($\Delta\psi_m$), respiratory defects, decreased ATP production, and an increase in reactive oxygen species (ROS). Disruption of any of the many genes that ensure mitochondrial integrity and stability, such as *parkin*, *PINK1,* and *OPA1,* to list a few, result in cell death **(150–153)**. Continual trophic support is also important to many cells, especially in the nervous system. This has been demonstrated by the mutual requirement for connections between photoreceptor neurons in the eye and their target neurons in the optic lobes of the brain. The failure to establish and maintain proper connections leads to loss of the optic lobes [e.g., **(154)**] and eventual degeneration of the photoreceptor neurons themselves **(155, 156)**.

The mechanisms that regulate cell death are an increasingly hot topic in studies of phototransduction mutants; these studies have particular relevance to a number of human diseases leading to blindness such as *retinitis pigmentosa*. Mutations in several components of the rhodopsin signaling pathway and Wnt signaling pathway can lead to apoptotic cell death; these mutations mirror mutations in human genes associated with blindness to a remarkable degree [reviewed in **(157–160)**]. Wnt signaling is proposed to play a prosurvival role during photoreceptor injury **(161, 162)**. Significantly, the degeneration observed in some of these mutations can be rescued by expressing the caspase inhibitor p35 in the eye with the result that vision appears to be fully restored **(163, 164)**. The links between mutations that alter light-mediated signaling and the triggering of the apoptotic machinery remain mysterious.

Although *Drosophila* is often thought of as a great model for studying development, aging and degeneration of differentiated cells readily occur in

flies as well, fueling a growing interest to study adult human diseases in flies. The ability to target genes—including disease forms of genes—to specific fly tissues allows us to take advantage of the myriad of fly tools to better dissect the underlying basis of a pathogenesis. These include Parkinson's disease **(165)** and polyglutamine repeat-based diseases such as Spinocerebellar ataxia type 1 and type 3 and Huntington's disease **(166–168)**. Once established, these models can be subjected to genetic screening, and an increasing wealth of new factors are being discovered that participate in the cell death process [recent reviews include **(169–171)**].

Compensatory Proliferation

Apoptosis and proliferation are carefully balanced to maintain tissue homeostasis. It has long been known that excess proliferation per se can induce apoptosis through Myc signaling **(172, 173)**. The converse, that excess apoptosis can induce compensatory proliferation in neighboring cells, has recently been shown. The first hint of this was that irradiated *Drosophila* larvae exhibit massive cell death but yield relatively normal adult structures **(174)**. Furthermore, ectopic cell death in wing discs showed that cells adjacent to the apoptotic cells undergo compensatory proliferation **(102)**. Ryoo et al. examined two possible models for this phenomenon **(175)**. In the "active" model, dying cells signal their neighbors to proliferate. In the "passive" model, the empty space created by the loss of a cell is filled by neighboring cells without cell-to-cell signaling (Fig. 16.5). By expressing p35 to inhibit caspases, "undead" cells were generated that were technically apoptotic but not degraded. These "undead" cells actively signaled their neighbors to proliferate, demonstrating that dying cells can induce proliferation presumably to replace themselves. Further experiments demonstrated that this process depends on Dronc and Dp53 to induce the secretion of Wingless and Decapentaplegic that lead to proliferation in neighboring cells **(175–179)**.

The phenomenon of compensatory proliferation has also been studied in the eye imaginal disc. Cells in the anterior section of the eye disc, like the wing disc, are actively proliferating. In this region, compensatory proliferation is induced by extensive apoptosis in much the same way as in the wing disc **(180)**. However, in the posterior section, behind the morphogenetic furrow, the cells have ceased to proliferate and have begun differentiating. In these differentiating cells, compensatory proliferation depends upon the effector caspases Drice and Dcp1 to induce Hedgehog signaling, causing neighboring differentiated cells to reenter the cell cycle **(180)**. Thus, the mechanism of apoptosis-induced compensatory proliferation is dependent on the developmental potential of the affected tissue (Fig. 16.5).

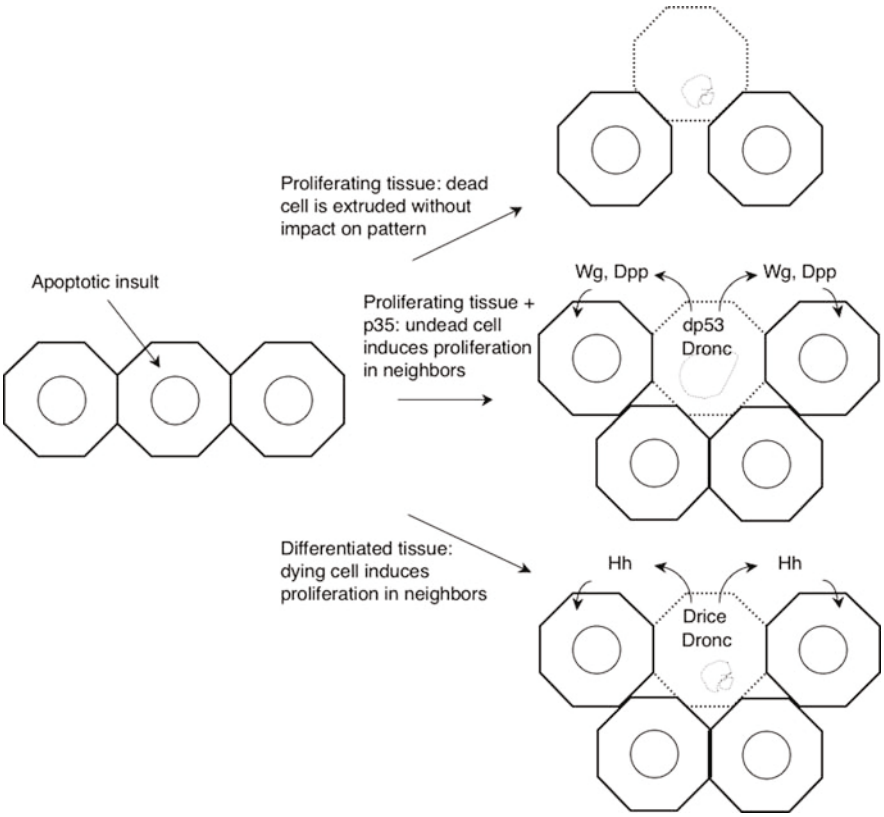

Fig. 16.5 Compensatory proliferation signals differ between proliferating and differentiating tissue. When a cell in an epithelium receives an apoptotic stimulus, it can either (1) *top*: die and be extruded as seen in proliferating wing disc tissue, (2) *middle*: when the dying cell in a proliferating tissue is protected from death by the co-expression of p35, Dronc and p53 make such cells become stable sources of Dpp and Wg to drive proliferation of neighboring cells, and (3) *bottom*: die and induce proliferation of neighboring cells through Dronc, Drice, and Hh, as seen in differentiated eye tissue. Figure adapted from **(195)**

Demolishing or Remodeling? The Apoptotic Machinery in Nonapoptotic Processes

We usually think of apoptosis as an all-or-nothing phenomenon: once caspases are activated, the cell is fated to die. However, the apoptotic machinery has been shown to be involved in many nondeath situations, usually involving the terminal differentiation or remodeling of cells. The mechanisms regulating these processes are largely unknown.

Spermatogenesis. *Drosophila* spermatids have a standard round shape, which changes into a thin, needle-like shape as they mature into sperm, a process called *individualization* **(181, 182)**. Individualization involves the elimination of

the majority of spermatid cytoplasm and organelles in an apoptosis-like process that requires cyt *c*, Hid, DIAP1, Dark, and the caspases Dredd, Drice, Dronc, and Dcp1 **(183–185)**. In this system, caspase activation does not lead to spermatid death, but instead may help break down the unneeded cellular structures.

Border cell migration. At stage 10 of oogenesis, a small group of follicle cells, called *border cells*, migrate between the nurse cells and settle at the border between nurse cells and the oocyte. DIAP1 is involved in border cell migration in a manner that does not require its function as an E3-ligase, nor does the loss of DIAP1 in border cells cause apoptosis as it does in other cells. These data suggest that DIAP1 plays a nonapoptotic role in border cell migration **(186)**.

Dendrite pruning. During metamorphosis, neurons of the larval peripheral nervous system undergo pruning of their dendrites, in preparation for remodeling into an adult nervous system **(187, 188)**. Similarly, in the mushroom body, extensive pruning of larval axons occurs **(189)**. Both of these processes require the ubiquitination (and degradation) of DIAP1 through caspase activation **(190, 191)**. In the case of dendrite pruning, caspase activity is confined to the degenerating dendrites of pruning neurons **(190)**.

Specification of neural precursors. The cellular level of DIAP1 in neural precursor cells is finely regulated by the IKK **(192, 193)**. Decreasing the level of DIAP1 leads to the activation of a small amount of Dronc and Drice, which in turn cleave and activate Sgg46 **(194)**. This results in altered Wingless signaling and changes the fate of a neural precursor away from the sensory organ precursor (SOP) fate.

Immune response. The *Drosophila* immune system defends against infection by making antimicrobial peptides specific for the different pathogens. Exposure to Gram-negative bacteria causes activation of the apical caspase Dredd in immune cells. Dredd then cleaves and activates the transcription factor Relish leading to transcription of the antibacterial peptide diptericin **(57)**.

Summary

Drosophila has firmly placed itself as an important contributor to the cell death field. The ability to use genetics has made *Drosophila* an important tool both for identifying new death factors and as a testing site to determine if interactions observed *in vitro* or in cultured cells can be verified in normal developing tissues. One of the most important contributions made by *Drosophila* researchers to the study of cell death are investigations into how spatially regulated PCD is used to pattern developing tissues and correct mispatterning events. Recently, cell death work in *Drosophila* has been extended to include a more direct assault on specific questions of human disease. These studies represent the next logical step in utilizing the remarkable features of the fruit fly.

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Part III
Apoptosis in Mammalian Physiology and Pathogenesis

Chapter 17
Cell Death: Defining and Misshaping Mammalian Embryos

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Abstract Cell death has long been known to be a normal part of embryonic development, typically sculpting the body, removing vestigial or sexually incongruent tissues, generating lacunae and separation of tissue layers, and playing other important roles in morphogenesis. The germ line manifests considerable death of supporting cells, presumptively defective cells, and oocytes that may initiate but not complete maturation, and the differentiation of spermatocytes involves apoptosis-like activity. Most of the deaths are apoptotic. Although in nonmammalian embryos one does not encounter nonpathological deaths before the maternal-zygotic transition, in mammalian embryos spontaneous cell death begins at the end of compaction. Many embryonic defects are associated with abnormal patterns of cell death. However, it is usually not clear whether the abnormality is a cause or result of the defect.

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A Bit of History

Cell death has been recognized as an important component of embryonic development as long as cell death itself has been acknowledged. The great 19th-century embryologist W. Vogt saw cell death in embryos in 1848, and by 1885 Flemming documented the obvious: that the loss of tadpole structures during metamorphosis was cell death. Throughout the early 20th century, numerous researchers examined the death of larval muscles in metamorphosing insects, and by the mid 20th century, Levi-Montalcini and Hamburger had demonstrated that the larger sympathetic ganglia in the shoulder and hip girdle of chicks resulted from a greater dieback of previously born neurons in nonlimb

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ganglia, thus launching their search for nerve growth factor on an issue of embryonic cell death **(1)**. Nearly simultaneously, John Saunders and Edgar Zwilling, studying the mechanisms of differentiation of chick limbs, noted that there were patches in the limbs, notably in the interdigital spaces, the axilla of the limbs, and (as previously noted by Dame Honor Fell) in the chondrogenic regions of the limbs **(2, 3)**. These studies would later lead to experiments that demonstrated the control of cell death in limbs. Meanwhile, Glücksmann laboriously catalogued the numerous instances of cell death in embryos and throughout life, attempting to group them according to their biological purpose **(4–6)**. While this classification may seem artificial today, he clearly described the death of embryonic organs as a normal biological process.

Meanwhile, in very different fields, other developmental biologists were acknowledging the importance of cell death in embryonic development. Immunologists were beginning to understand that the variety and specificity of the immune system derived from a massive overproduction and massive death of thymocytes **(7)**. Endocrinologists recognized the dual sexuality of early vertebrate embryos, with sexual differentiation including the degeneration of the Mullerian system in males and the Wolffian ducts in females. Insect physiologists defined programmed cell death. And, finally, the genetics of apoptosis were first defined for the embryonic development of the worm *Caenorhabditis elegans* **(8–11)**.

Thus, while the widespread appearance of cell death as a developmental process was readily acknowledged, acceptance of the idea that it was a controlled process came relatively late. This was surprising: As one of us argued many years before, if a developmental cell death can be predicted, it is a genetic trait as much as color, shape, or number of spines is a genetic trait. The demonstration that death was controlled came from two sets of experiments. In chick embryos, Saunders and his co-workers demonstrated that the cells destined to become part of the "posterior necrotic zone" (an axillary zone in which cells die) would die on schedule when explanted; but these same cells were not mortally wounded, as they could heal into the back of the chick and survive and differentiate if transplanted rather than explanted **(2, 3)**. In metamorphosing insects, Lockshin and Williams documented a sequence of clearly biological steps, subject to physiological regulation, that led to the death of larval muscles **(12–16)**. Finally, Horvitz, Sulston, Brenner, and colleagues demonstrated that the death of 131 cells in an embryonic nematode was completely predictable and that the deaths of these cells (12% of the total cells in the embryo) were controlled by a limited number of genes; their genetic pursuits ultimately led to the identification of the first major gene for apoptosis proteases **(17)**.

Cell Death in the Germ Line

Cell death and cell death-like processes are clearly important in the differentiation of gametes. There is ample evidence, both from observation and from genetic manipulation, that apoptosis operates during gametogenesis as it does

elsewhere to eliminate defective cells, in this case, germ cells (oocytes and spermatocytes) suffering chromosomal or other damage **(18–22)**. There is also evidence that the substantial loss of most of the oocytes that were originally formed in the mammalian embryo is through controlled apoptosis. Since the administration of excess gonadotrophins can produce superovulation, the primary assumption is that the attrition of oocytes derives from their initiation of maturation but failure to acquire adequate gonadotrophin, but the mechanism remains mysterious **(23)**. Ultimately, corpora lutea undergo apoptosis similar to the death of follicle cells and nurse cells in invertebrates. A very interesting and currently incomplete story is that of sperm individualization. All spermatocytes begin life as syncitial cells with conventional amounts of cytoplasm. They are ultimately separated and differentiated when a contracted ring of actin moves from the nucleus or head of the sperm toward the distal end of the tail, squeezing away the bulk of the cytoplasm into a "waste bag" and leaving only the sperm tail. The waste bag is destroyed in an apoptosis-like process, and it now appears that caspases are active in the spermatocyte and necessary for the proper maturation of the sperm **(21**, **24)**. Since the most advanced studies have been conducted in *Drosophila*, the equivalence of the caspases (initiator or effector) and the targets of their activity have not been determined. Nevertheless, several types of terminal differentiation in mammals appear to involve "partial apoptosis." For instance, the elimination of organelles from lens fibers and the differentiation of keratinocytes both involve components of the apoptosis machinery even though the destruction of the cell is not complete **(25**, **26)**.

Cell Death in the Cleaving Embryo

In nonmammalian vertebrate embryos, apoptosis is typically not seen prior to gastrulation, and some authors argue that embryos prior to the maternal-zygotic transition (MZT, the period during which the embryo begins to make its own messenger RNA and cleavage becomes asynchronous, typically between the 8th and 12th divisions – 256 to 4,096 cells) cannot undergo apoptosis. The failure is typically attributed to either the blockage of apoptosis machinery or the absence of some component of the machinery. In our hands, the failure appears to be an artifact of aquatic embryos, especially freshwater embryos like zebra fish. Pre-MZT embryos exposed to toxins activate caspase-3 in a manner equivalent to post-MZT embryos, but the cells lyse almost immediately thereafter, whereas the cells in older embryos survive two more hours and thus can manifest the typical morphology and biochemistry of apoptosis. The mechanism for this differential fragility is under investigation **(27)**.

Mammalian embryos, having the requirement of creating a placenta and having interpolated into their development the compaction and expansion of the blastocyst, are quite different. Zygotic gene activity begins as early as the two-cell stage, and the earliest patterned or predictable death occurs as the

compacted blastocyst begins to expand, when one or two cells die at the junction of the inner cell mass and the trophoblast. Caspase activity appears to be important to the embryo, as exposure of embryos to a pan-caspase inhibitor between the two-cell and four-cell stage leads to a failure of expansion, with cell death beginning at the ICM-trophoblast junction and finally engulfing the entire embryo **(28)**.

Cell Death During Organogenesis

For technical reasons, most studies of cell death in vertebrate embryos have been conducted using frog, chicken, or fish embryos. In these, the story is well known. In all vertebrate embryos, organogenesis involves considerable amounts of cell death. Many of the deaths are scattered throughout the embryo; whether they are highly predictable is an open question. However, in specific regions, it is apparent that morphogenesis involves both the burgeoning of rapidly growing tissues and the death of others to generate separations or lacunae. Most separations of tissue layers, such as between the parietal and visceral parts of the somitic musculature, and most appearances of lacunae, such as the openings of the bronchii and alveoli, the differentiation of the cardiac chambers, and the glomeruli, to some extent involve cell deaths **(26)**. In the nervous system, the immune system, and perhaps elsewhere, a common embryonic solution to establishing exquisite sensitivity is to overproduce cells and select for the most appropriate connections or types of antibodies. While, at first glance, this would appear to be extraordinarily wasteful, it is very conservative from the standpoint of DNA. For instance, to produce one billion cells with specific connections or antibodies would require at least two billion genes, one for each receptor and ligand or each chain of an antibody. To produce 10 billion cells and select the most appropriate one billion would require 33 or 34 cell divisions plus whatever general markers of appropriateness (e.g., ability to capture nerve growth factor from a contacted target cell would achieve the purpose), a substantially reduced genetic investment, and an investment that could be readily generalized for use and reuse. Thus, in both the differentiating nervous system (sympathetic and sensory ganglia) and the differentiating immune system, between 50–90% of the cells that are born die. In the nervous system, it appears that while many neurons make synaptic contact with target organs, only a few make successful contacts and acquire nerve growth factor (NGF) from the target. As is apparent from the study of PC12 cells that have differentiated into neurons by the application of NGF, which is then withdrawn, NGF acts as a trophic factor for the neurons; in the absence of NGF, the cells slowly atrophy until they finally consume their mitochondria and, lacking further means of respiring, die **(29, 30)**. In the immune system, if differentiating thymocytes are overstimulated (anti-self-thymocytes), they are destroyed by Fas-Fas ligand interaction, while thymocytes that fail to

encounter any potential antibody are similarly destroyed (death by neglect) **(31)**. In both instances, the huge initial population is whittled down to an appropriate number.

Cell deaths are commonly seen in many other areas in which morphogenesis occurs: in the separation of the lens from the cornea; in the formation of olfactory pits; in the formation of the cloaca in fish; along the margins of the somites; and between the digits and in the axilla of developing limbs **(32–37)**. In the formation of the lens, the nuclei of the lens fiber cells migrate; as they are migrating, they fragment their DNA and eventually disappear, giving rise to the clear lens fiber cells. This type of programmed cell death can also be called *nuclear apoptosis* **(34)**. Primarily because of ease of access, these tissues have received the most attention, but there are undoubtedly many other instances of cell death in the embryo. Earlier researchers had identified as a major element of sex differentiation the death of the Mullerian ducts in males and the Wolffian ducts in females. The death of the Wolffian ducts appears to be a typical hormonoprivic loss, perhaps similar to the death of neurons deprived of NGF, but the Mullerian ducts respond negatively to the testis-derived Mullerian Inhibitory Substance (MIS). Some effort was expended to determine how MIS functioned, but full details are not forthcoming **(23)**. In other situations in which cell death is commonly observed, the fusion of lateral plates along the midline of the forming face and most likely elsewhere, including perhaps the closing neural tube, appears to be secured by the death of cells along the midline, with a resulting greater adhesivity generated by unknown means, and consequent fusion of the cells from the two sides.

In brief, cell death, usually apoptotic, is a common and presumptively necessary component of morphogenesis. However, experimental access at the tissue or organ level is normally difficult, especially in mammalian embryos, and most of the reports of cell death have been descriptive. The consequences of the deregulation of cell death have been explored by the use of mutations or, occasionally, gross interference with effector caspases.

Consequences of Deregulation of Cell Death

We are now well aware of the occurrence of cell death in embryos, but the questions of interest are now how important it is, what happens when it inappropriately fails to occur or occurs too extensively, and how it is controlled.

In the simplest of situations, it is not obvious that cell death is very important. For instance, the genetic prevention of all the embryonic cell deaths in *C. elegans* is not obviously a problem. The worms live and move about normally, though they may reproduce a bit more slowly. Preventing the death of the intersegmental muscles in moths likewise does not seriously interfere with their lives, though their lives as adults are quite short (approximately one week) and prevention of the death of the muscles is achieved by pharmacological means that themselves

are somewhat toxic. We have, however, little ability to judge the subtleties of the lives of worms or moths.

As noted earlier, most vertebrate embryos do not manifest cell death prior to the MZT. This observation has given rise to the assumption that there is something special about this transition, perhaps with inhibitors of apoptosis existing prior to the transition or the machinery of apoptosis having to be synthesized by the zygote **(38, 39)**. As far as we can tell, this is an artifact: Pre-MZT zebrafish embryos contain caspase-3 and can activate it quite efficiently when the eggs are exposed to various toxins, but the cells of these freshwater embryos lyse very shortly after caspase-3 is activated and do not have the time to develop the classic characteristics of apoptosis. Later, post-MZT, embryos do not lyse for at least two hours after activating caspase-3 and therefore have ample opportunity to manifest morphological and biochemical characteristics of apoptosis. The difference appears to reside in the fragility of the pre-MZT cells. Whether this fragility derives from the greater size of the cells and the consequent decreased relative strength of the cell membranes or from some more profound biological or biochemical cause has not been determined **(27)**.

In vertebrate embryos, many morphological and developmental anomalies involve the deregulation of the cell deaths that occur during organogenesis—though, of course, in most of these instances, one can argue that the mechanism of the anomaly involves cell death, without arguing that the problem with cell death is primary or causal. For instance, an early difference between the differentiation of chicken feet and duck feet is a much lower cell death in the interdigital regions of the duck feet, in which the interdigital regions are destined to form the webbing. A mutation called Hammertoe in mice produces a similar lack of cell death, resulting in webbing of the feet with disastrous results for the anatomical layout of the feet. Exposing the mice *in utero* to excess retinoic acid, which increases cell deaths throughout the embryo, increases the number of deaths within the interdigital region and partially or nearly completely rescues the defect. In this case, the problem appears to derive from the threshold of response at which cell death occurs, since the deaths reappear in the pattern in which they were supposed to have occurred. Likewise, cleft palate and hare lip appear to derive from a failure of cell deaths where the left and right facial plates meet, but the reason for the failure of deaths has not been determined.

Likewise, failure of cell death in the immune system, due to lack of Fas or Fas ligand, appears at first to be inconsequential, but later in life the affected mice develop autoimmune disease and symptoms resembling lupus erythematosis. This result is obtained for knockout of both Fas (the lpr mouse) and Fas ligand (the gld mouse). Thus, there are no profound morphological consequences, although ultimately the persistence of self-reactive thymocytes becomes a problem. Similarly, the knockout or knockin of presumptively even more generalized regulators of cell death, such as Bcl-2 or Bbcl-xL, frequently results in no immediately obvious phenotype. Most researchers consider that redundancy of function and compensatory activities adjust the development of the embryo, so that disruption of the presumptively important gene has only minor

consequences. Such an argument obtains, for instance, the knockout of Myo-D, the major gene committing cells to muscle differentiation. Overlapping and redundant functions of related genes compensate, so that the resulting embryo is only slightly abnormal.

When cell death is severely disrupted, as when the initiator caspase-9 or the nonredundant regulator apaf-1 are knocked out, the resulting phenotype is lethal, in which the embryos show failure of neural tube closure and apparently severe overgrowth of the brain **(40–42)**. However, it is not clear that this phenotype results from failure of cell death. There is considerable, perhaps normal, cell death in the brain, although the cells do not necessarily look apoptotic; and the spinal cord, in which likewise large amounts of cell death should occur, is of normal size. Thus, the phenotype is not necessarily a direct result of the failure of cell death in the brain **(43)**.

One limitation of studying cell death in mammalian embryos is that the physiological significance of cell death will be appreciated only *in vivo*, limiting most efforts, for technical reasons, therefore to observations of normal and mutated embryos. A second limitation is that, as described earlier, redundancy and compensation can easily obscure the importance of a gene. A severe limitation for cell death research is that normal embryonic deaths are generally not decided by direct control of the cell death effector machinery. Rather, a combination of circumstances triggers cell death by creating a metabolic constellation that initiates (usually) the intrinsic or mitochondrial pathway to apoptosis. Efforts to override, knock out, or knock in this pathway may result in aberrations in the intrinsic pathway. However, the circumstances that initiated the death are still operative, and most frequently, the death will still occur, though perhaps not by apoptosis, or the cell will survive but with minimal functionality. Thus, the genetic manipulation will produce no measurable phenotype. The other consideration is that, for most genetic aberrations of cell death that produce viable phenotypes, the phenotype is damage to a specific organ or tissue, as seen in Hammertoe, brachyury, cleft palate, and many similar mutations. In these cases, while the basis of the phenotype may be abnormal cell death, it is obvious that the problem lies in the invocation of the cell death process rather than in the machinery of cell death. This is quite different from what is seen in embryos that can be observed, such as those of the nematode *Caenorhabditis* and the fruit fly *Drosophila*. In these embryos, knockout of the primary cell death-suppressing genes leads to massive and generalized cell death and early death of the embryo. In mutations such as Hammertoe, the problem is with cell deaths in a particular location and pattern, and the pattern can be restored experimentally. In this case, the function of the cell death machinery has not been lost; the regulation of the machinery is hampered or the signaling is altered by the genetic mutation. Thus, the issue is either the weakness of the pattern or an increased robustness of the cells within the region of expected death. As is the case with most of these questions, the question ultimately returns to the biological means by which the command to die is issued—the deprivation of nutrients or oxygen, the production of a specific

component dependent on a growth factor, adherence to a specific matrix or substratum, a lack of congruence between growth stimuli and metabolic capability to grow, or other unknown mechanisms—as opposed to the mechanics of cell death itself **(44)**. Thus, as always, the question returns to the mechanisms by which cells communicate with each other.

Because of gross deformations or compensatory activities that nullify any potential phenotype, it is often difficult to interpret the significance of knockout or knockin mutations. However, some insight may be obtained by the phenotypes that are seen. From a vast list of descriptions of the results of these manipulations, Table 17.1 summarizes some relevant descriptions of knockout mutations in mice. For brevity, the references given are reviews, and knockin or upregulating mutations have not been included. The references should be consulted for further information.

Table 17.1 Phenotypes of knockouts of some effectors of apoptosis

Gene	Function	Embryonic phenotype	Adult phenotype	Reference
CASPASES				
Casp1	Activator of interleukin-1	Normal	Poor inflammatory response	**(45, 46)**
Casp2	Initiator caspase	Normal; loss of facial neurons	Oocytes resistant to induced apoptosis	**(45, 47, 48)**
Casp 3	Effector caspase	Perinatal lethal (dep. on genotype, 3–5 weeks)	Severe brain overgrowth; reduced apoptosis of T cells; failure of granulosa atresia	**(45–47, 49)**
Casp6	Effector caspase	Normal	Normal	**(45–47)**
Casp7	Effector caspase	Embryonic lethal; mild nonlethal antiapoptotic phenotype	N/D; survivors perhaps compensated by caspase-3	**(45–47, 50)**
Casp8	Initiator caspase	Embryonic lethal (E 12.5)	Impaired cardiac muscles; abdominal hemorrhage; hyperemia	**(45–47)**
Casp9	Initiator caspase	Embryonic lethal (98%)	Severe forebrain overgrowth	**(45–47)**
Casp11		Normal	No IL-1β & IL-18 processing; decreased prenatal oocytes	**(46–48)**
ADAPTORS				
FADD	Adaptor between casp8 and death receptors	Embryonic lethal	Impaired cardiac muscles; abdominal hemorrhage; hyperemia	**(47)**

Table 17.1 (continued)

Gene	Function	Embryonic phenotype	Adult phenotype	Reference
FLIP	Caspase-8 inhibitory protein	Embryonic lethal (heart)		**(51)**
Apaf-1	Component of apoptosome (casp9)	Delayed removal of interdigital webs		**(47)**
BCL-2 FAMILY MEMBERS				
Bcl-2	Antiapoptotic	50% die 1–6 weeks after birth (neuronal depletion)	Sensitive to apoptotic stimuli; rapid loss of immune cells after birth; growth retardation; polycystic kidneys	**(46, 52, 53)**
Bcl-x	Antiapoptotic	Lethal E13; massive apoptosis of neurons & hepatic hematopoietic cells		**(46, 52, 53)**
Bcl-w	Antiapoptotic	Viable	Male sterility	**(53)**
A1	Antiapoptotic	Viable	Increased neutrophil apoptosis; hair loss	**(53)**
Mcl-1	Antiapoptotic	Perimplantation lethality; decreased B & T lymphocytes		**(53)**
Diva/Boo	Antiapoptotic	Viable	Viable	**(53)**
Bak	Proapoptotic	Viable	Viable	**(53)**
Bax	Proapoptotic	Inc Purkinje & retinal precursors; hyperplastic thymocytes & B cells	Increases in granulosa cells, some neurons, lymphocytes; atretic ovaries; disordered seminiferous tubules	**(46, 52, 53)**
Bad	Inactivator BH3-only	Viable	B-cell lymphoma; testis defects	**(53)**
Bak	Inactivator BH3-only	Viable	Viable	**(53)**
Noxa	Inactivator BH3-only	Viable	Viable; slightly resistant to toxic cell death	**(53)**
Hrk	Inactivator BH3-only	Viable	Viable	**(53)**
Bad	Activator BH3-only	Viable	Thymocytes resistant to receptor-mediated apoptosis	**(53)**

Table 17.1 (continued)

Gene	Function	Embryonic phenotype	Adult phenotype	Reference
Bim	Activator BH3-only	Viable, but low birth numbers	Hypertrophy of immune system; autoimmunity	**(53)**
Puma	Activator BH3-only	Viable	Lymphocyte-resistant	**(53)**
Bid	Activator BH3-only	Viable	Resistance to death receptor-mediated apoptosis in the liver	**(46)**
METABOLIC INTERACTORS (INTRINSIC PATHWAY)				
p53	Initiator of DNA-damage initiated apoptosis; tumor suppressor	Viable	Increased sarcomas; decreased longevity	**(54, 55)**
Mdm2, MdmX	E3 ubiquitin ligase, negative regulator of p53	Lethal unless in p53-null		**(56)**
Cytochrome c	Respiratory pigment; when in cytoplasm activates caspase-9	Midgestation lethal, defective oxidative phosphorylation	–	**(46)**
Apaf-1	Apoptosis-activating factor in apoptosome	Perinatal death; defective neuronal apoptosis, forebrain overgrowth		**(46)**
RECEPTORS AND LIGANDS (EXTRINSIC PATHWAY)				
Tnfr I (p55)	Cell death receptor	Viable	Defective lymphoid organogenesis	**(46)**
Tnfr2 (p75)	Cell death receptor	Viable	Resistance to TNF-α-mediated cell death	**(46)**
FasL (gld)	Cell death receptor	Viable	Lymphoproliferation, autoimmune disease	**(46)**
Fas (lpr)	Cell death ligand	Viable	Lymphoproliferation, autoimmune disease	**(46)**
Fadd	Death receptor adaptor	Embryonic lethal E12.5 (cardiac development)		**(46)**
Traf-2,-3	Death receptor adaptor	Postnatal runting & death (T- & B-cell precursor development)		**(46)**

Table 17.1 (continued)

Gene	Function	Embryonic phenotype	Adult phenotype	Reference
Traf-4-6	Death receptor adaptors?	Quasi-normal	Abnormal trachea, lymphocyte activation, bones	**(46)**
Rip	Death receptor adaptor	Postnatal runting & death; apoptosis in lymphoid & adipose		**(46)**
Nemo	Death receptor adaptor	Male embryonal death; hepatic apoptosis	Female pigment problems; failure of NF-κ activation	**(46)**
RelA	Death receptor adaptor	Embryonal death; hepatic apoptosis; failure of NF-κB activation		**(46)**
TWEAK	Weak inducer of apoptosis (receptor-mediated)	Viable	Increased natural killer cells	**(57)**
TRAIL	Receptor ligand	Viable	Enhanced innate immune responses	**(57)**
INHIBITORS AND OTHERS				
Casper	Apoptosis inhibitor	Embryonic lethality E10.5 (cardiac)		**(46)**
Survivin	Apoptosis inhibitor	Embryonic lethality		**(46)**
XIAP	Apoptosis inhibitor	Viable	Viable (c-IAP-1 & -2 compensate)	**(46)**
SMAC/ Diablo	Apoptosis inhibitor	Viable	Viable	**(46)**
AIF	Apoptotic effector	Viable	Late-onset cerebellar & retinal degeneration	**(46)**
Cathepsin B	Lysosomal enzyme	Viable	Resistance to TNFR-mediated hepatic apoptosis	**(46)**

Conclusions and Summary

Cell death has long been recognized as an important component in the shaping and sculpting of embryos, and it is now apparent that cell death and cell death-like processes are likewise major aspects of gametogenesis. In most embryos, there is little or no cell death prior to the maternal-zygotic transition, but in mammalian embryos, characteristic deaths of one or two cells occur at the end

of compaction and appear to be necessary for the separation of the trophoblast from the inner cell mass. Considerable sculpting of the embryo occurs by cell deaths during organogenesis, and appropriate cell numbers, especially in the CNS and in the immune system, are generated by the massive overproduction of cells and the selection of a few, with the deaths of the rest. The complete disruption of the cell death machinery is lethal, but many mutations of the regulatory machinery yield only modest or no phenotypes, indicating substantial redundancy and compensation of regulatory mechanisms. Most of the deaths are apoptotic and are identified by techniques used to recognize apoptosis. Aberrant cell deaths that produce known phenotypes are typically localized, indicating that the mechanism of activating a programmed death in a specific region is aberrant rather than the mechanism of death. These results lead us to conclude that we need to know much more about the conversations among cells that lead cells to commit suicide.

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Chapter 18
Matters of Life and Death in the Immune System

Christopher P. Dillon and Douglas R. Green

Abstract Apoptotic function in the immune response is a matter of life and death for an organism. A robust immune response is vital for the survival of any organism fighting infection. In order for an effective immune response to be launched, the apoptotic mechanisms used to shape that response have to function properly. Many more immune cells are generated than required in order to produce a few that can effectively discriminate between and respond to foreign antigens within a vast pool of self peptides. These cells undergo a gauntlet of apoptotic checkpoints during and after development that ensure they are able to fully respond to challenges. Subsets of immune cells even use these same proapoptotic pathways to control their targets. Indeed, understanding matters of life and death in the immune system really means understanding how apoptosis shapes almost every aspect of the immune response.

Keywords Apoptosis · Bcl-2 family · Immunology · Lymphocyte · Myeloid

Introduction

Apoptosis, or programmed cell death, is an important physiological process that plays an essential role in the immune system. During lymphocyte development, a selection process utilizing apoptosis ensures that only cells with functional, but not harmful, antigen receptors are permitted to develop. In an immune response to foreign pathogens, pathogen-specific cells exponentially expand in order to eliminate the invader, but then these cells must be culled via apoptosis to allow responses against future pathogens. Not only does apoptosis regulate the homeostasis of lymphocytes and innate immune cells, but it is also utilized by certain immune cells as a mechanism to eliminate

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foreign antigens. This chapter discusses the various roles of apoptosis in immune system development, function, and response.

Apoptosis Pathways

As reviewed earlier in this book, two distinct pathways of apoptosis have been characterized on a molecular level, termed the *extrinsic* and *intrinsic* apoptosis pathways. The extrinsic pathway involves death receptors on the cell surface, which cluster together when engaged by extracellular ligand and recruit the adaptor molecule Fas-associated protein with death domain (FADD). This complex recruits and activates caspase-8, which, in turn, directly or indirectly activates effector caspases and causes cell death. In contrast, the intrinsic apoptosis pathway proceeds via mitochondria in response to intracellular signals such as DNA damage, ER stress, and growth factor withdrawal. These signals upregulate the proapoptotic BH3-only family members such as Bid and Bim, which oppose the antiapoptotic activity of Bcl-2 family members such as Bcl-2 and Bcl-xL to activate Bax and Bak, resulting in mitochondrial outer membrane permeabilization (MOMP). The release of cytochrome c following MOMP activates caspase-9 by the Apaf-1 apoptosome, causing effector caspase activation and cell death. Apoptosis in the immune system is regulated by both the extrinsic and intrinsic pathways, as discussed in this chapter. The role of a novel pathway of apoptosis in which caspase-1 is activated through the pyroptosome in macrophages is also discussed.

T-Cell Development

Developing thymocytes undergo a series of checkpoints to ensure their development into functional nonself-reactive T cells. Failure to pass these checkpoints can lead to thymocyte apoptosis at any one of four stages: prior to T-cell receptor (TCR) rearrangement, during defective rearrangement, failure of positive selection, and negative selection (Fig. 18.1). In each maturing thymocyte, the TCR genes undergo a random recombination of multiple gene segments to generate a receptor with unique antigen specificity. This diversity of receptors is further augmented by the inclusion of additional bases known as P- and N-nucleotides at the junctions between these segments. Since these modifications can disrupt the reading frame and cause truncated proteins to be produced, only one third of the recombined receptors are expressed on the cell surface. However, a thymocyte must have successful signaling through these recombined receptors to pass to the selection phase of T-cell development.

The selection phase serves two purposes: (1) to identify cells capable of recognizing peptides presented on self-major histocapability complex (MHC) proteins and (2) to eliminate cells that recognize self-peptide. During positive

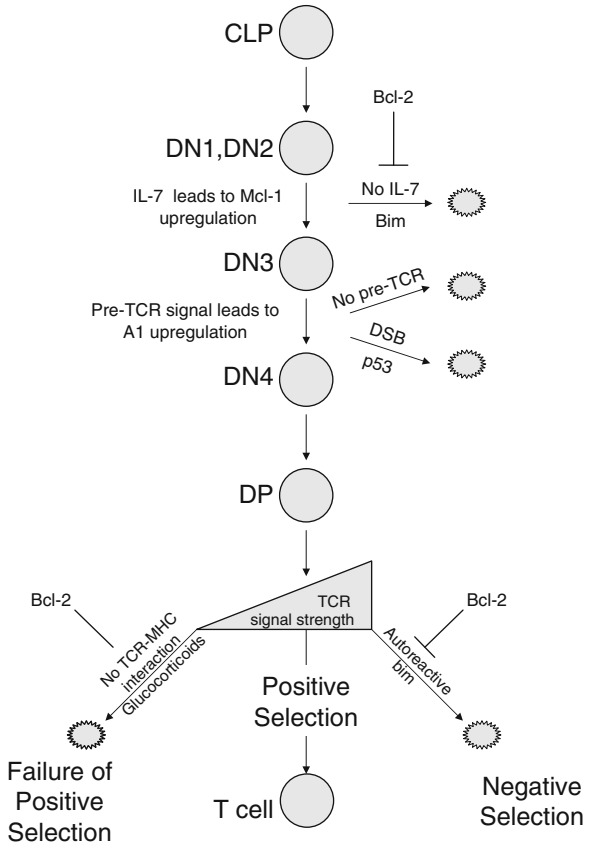


Fig. 18.1 Schematic of the apoptotic checkpoints in T-cell development. Apoptosis can occur during four key checkpoints in development: before rearrangement of the T-cell receptor, during recombination of the receptor when double-strand breaks (DSB) fail to resolve or when a functional receptor cannot be expressed, due to failure of positive selection, or due to negative selection. CLP: common lymphoid progenitors; DN: double-negative thymocytes; DP: double-positive thymocytes

selection, cells that recognize self-MHC receive survival signals, while cells that bind too strongly to self-peptide/MHC complexes are actively eliminated through apoptosis in negative selection. Negative selection is limited to the deletion of cells reactive to self-antigens that are presented in the thymus. The autoimmune regulator gene (AIRE) increases the efficacy of negative selection by driving expression in the thymic medullary epithelial cells of genes normally expressed in peripheral tissues **(1, 2)**. Approximately 95% of developing thymocytes fail to mature, but rather undergo apoptosis at various stages of development.

Cytokines regulate thymocyte apoptosis prior to TCR rearrangement through the activation of prosurvival signaling. Following the differentiation of common lymphoid progenitors (CLPs) in the thymus, IL-7, a prosurvival factor, acts

on cells during the first stages of commitment to the T-cell lineage **(3)**. Signaling from IL-7 upregulates the antiapoptotic Bcl-2 family member Mcl-1 and counteracts a Bim-dependent proapoptotic program **(4, 5)**. Accordingly, ablation of IL-7 causes a block in development that can be rescued by exogenous Bcl-2, suggesting the importance of the intrinsic apoptosis pathway at this stage **(6, 7)**. A lack of prosurvival signaling in early thymocytes derived from CLPs likely acts to eliminate cells that are not fully committed to the T-cell lineage.

The next important checkpoint in the development of thymocytes is the rearrangement of the TCR. Failure to resolve double-strand breaks induced by recombination leads to p53-dependent apoptosis **(8, 9)**. However, successful resolution of the double-strand breaks does not necessarily lead to survival. The engagement of a productively recombined receptor on the cell surface is required for prosurvival signaling. For example, signaling through the beta chain/pre-TCR alpha complex enables the survival of thymocytes to the CD4/CD8 double-positive stage, where positive selection occurs, through the upregulation of the antiapoptotic Bcl-2 family member A1 **(10)**. Interestingly, the transgenic expression of Bcl-2 in recombination-deficient thymocytes cannot rescue these cells during development **(11)**, suggesting either that only certain proapoptotic BH3 family members play a role at this stage or that additional processes control progression and/or survival. FADD might also play a role at this checkpoint, although it appears that this role is not dependent on the engagement of Fas **(12)**. Cells that receive prosurvival signaling through the engagement of productively recombined T-cell receptor proceed to the selection phase.

Apoptosis in the selection phase is important in molding the T-cell repertoire. During the process of positive selection, cells that fail to recognize self-MHC undergo "death by neglect" **(13)**. Interactions with thymic cortical epithelial cells through the TCR and other surface molecules drive transcriptional programs that lead to the upregulation of Ras and ERK signaling and consequently cell survival **(14–16)**. However, it is unclear if death in the absence of positive survival signaling is dependent on the intrinsic apoptosis pathway. Glucocorticoids have been implicated as the potential mediator of "death by neglect" **(17)**, as Bcl-2 inhibits thymocyte death induced by these steroids **(18)**. The ectopic expression of Bcl-xL or Bcl-2 increases the number of thymocytes compared to wild-type animals; however, it is unclear whether this reflects an inhibition of glucocorticoid-induced apoptosis **(19)**. Further work is required to fully elucidate the mechanisms of cell survival engaged during positive selection. However, by eliminating thymocytes that cannot bind self-MHC through apoptosis, this checkpoint ensures that only cells capable of responding to antigen-presenting cells when they reach the periphery survive to the next stage of development.

Negative selection functions as the primary checkpoint during T-cell development to prevent the onset of autoimmune disease. Disruption in the process regulating the elimination of autoreactive thymocytes can allow potentially destructive T lymphocytes to be released to the periphery. The use of genetically

modified animals has helped to elucidate the role of specific apoptotic pathways in this process. The ablation of Bim in mice results in an increased number of thymocytes that escape negative selection and may drive the development of autoimmune disease, although additional regulatory mechanisms appear to prevent the escape of these surviving cells into the periphery **(20)**. While the impact of other BH3 family members is less striking, they may also play a role in this process. Elimination of PUMA in combination with Bim deficiency provides additional protection against various inducers of cell death in thymocytes, including growth factor withdrawal **(21)**. The ablation of Bax and Bak, the downstream effectors of this pathway, strongly phenocopies the Bim knockout, further supporting the idea that Bim is a major effector of negative selection **(22, 23)**. Interestingly, the transgenic expression of antiapoptotic molecules such as Bcl-2 does not yield as strong a phenotype as the ablation of Bim, suggesting that additional antiapoptotic mechanisms may also operate during negative selection **(18, 19, 24)**.

The intrinsic pathway may also be activated during negative selection through the orphan receptor Nur77, along with its close relative Nor1 **(25–29)**. Upon stimulation with a death signal, Nur77 is exported from the nucleus, binds to Bcl-2, and appears to convert the latter from an antiapoptotic to proapoptotic form by exposing the BH3 domain **(30, 31)**. The first evidence for Nur77 involvement in this checkpoint was inefficient clonal deletion in animals that expressed a truncated transgenic version of Nur77 **(29)**. However, since Nur77 knockout animals do not have any defect in T-cell development, its role in T-cell development has been debated. Nor1 is thought to compensate for the loss of Nur77, enabling negative selection to proceed **(31)**. To date, animals in which both of these genes have been eliminated have not been generated.

While the intrinsic apoptosis pathway plays a role in negative selection, experiments suggest that the extrinsic pathway does not influence this checkpoint. No significant differences in negative selection are seen by the targeted ablation of Fas, FADD, or caspase-8 in mice **(32–34)**. The role of TRAIL, another death receptor ligand, in thymocyte development remains controversial. Experiments by several groups using three experimental models of negative selection have yielded opposing results regarding the role of TRAIL in this process. While TRAIL knockout mice are susceptible to collagen-mediated arthritis, aged animals do not develop spontaneous autoimmunity, leaving open the question of whether TRAIL is essential for negative selection **(35, 36)**. Further investigation using T-cell-specific ablation of TRAIL or the TRAIL receptor will be required to clarify its role in the development of T cells.

Negative selection is the key checkpoint in central tolerance, eliminating autoreactive cells through apoptosis before they can egress from the thymus to the periphery and contribute to autoimmunity. As we now discuss, apoptosis continues to play a role in the function of immune cells once they have completed development.

Peripheral Maintenance and Tolerance

Once thymocytes develop into single positive T cells and egress into the periphery, they are maintained in a naïve state until an encounter with a specific antigen. The survival of naïve T cells appears to be dependent on inhibition of the intrinsic apoptosis pathway. The balance between Bim and Bcl-2 is important for maintaining naïve cells in peripheral immune organs. The number of naïve T cells in animals deficient for Bcl-2 is low; however, the cell numbers are restored to normal levels when both Bim and Bcl-2 are ablated **(37)**. Bcl-2-deficient T cells cultured with cytokines *ex vivo* have a survival defect **(37)**, which can be replicated *in vivo* by neutralizing IL-7 with an antibody in thymectomized mice, suggesting that the survival role of IL-7 in these cells is to upregulate the expression of Bcl-2 **(37)**. While the neutralization of IL-7 affects the survival of both CD4 and CD8 cells, CD8 cells are much more sensitive to the loss of prosurvival signaling. Similar to thymocytes prior to TCR rearrangement, engagement of the intrinsic pathway in the absence of prosurvival cytokine signaling likely serves as a way to limit the size of the naïve T-cell niche when the pool of naïve T cells exceeds the limiting amounts of IL-7.

In contrast to the intrinsic pathway, the extrinsic pathway plays a role in peripheral T-cell apoptosis following antigen encounter. This pathway primarily serves to induce tolerance in chronic immune reactions resulting from either persistent infection or autoimmune responses. Evidence for this role comes from mice mutated for Fas, which develop a severe systemic autoimmunity with presentation of symptoms such as lymphadenopathy (enlarged lymph nodes), accumulation of aberrant T cells, and immune complexes that mimic the phenotype of systematic lupus **(38, 39)**. Similarly, mutations in Fas are also found in human patients with autoimmune lymphoproliferative syndrome (ALPS) **(40)**. The clinical symptoms in both of these cases are believed to result from a failure to control peripheral T-cell immune responses. Consistent with this hypothesis, Fas and its ligand FasL are also implicated in activation-induced cell death *in vitro* **(41–43)**, a model where cells are restimulated through the TCR after prior activation, which is thought to resemble the apoptosis induced by a persistent immune response. In another model of peripheral T-cell apoptosis, the superantigen-mediated deletion of T cells, cell death depends at least partially on Fas and FasL **(44)**. While Fas and FasL do not appear to play a role in T-cell development, they appear to be critical in limiting the immune response in cases of chronic stimulation.

Recent evidence suggests that the proper function of both intrinsic and extrinsic apoptotic pathways is essential to prevent the development of autoimmune disease. When Fas mutant mice are crossed to mice deficient for Bim, a more severe autoimmune phenotype is seen than in either of these mutated mice individually. There is debate over whether this difference results from the prolonged life span of activated antigen-presenting cells **(45)** or the combined

loss of Bim-regulated negative selection and Fas-regulated peripheral tolerance in T cells **(46, 47)**. However, it is likely that both of these mechanisms contribute to the phenotype of the animal. The combined regulation by both the intrinsic and extrinsic pathways demonstrates the importance of both central tolerance and peripheral tolerance in preventing autoimmunity.

T-Cell Memory

Memory T cells are long-lived cells capable of producing a rapid response upon a second challenge with the same antigen. These cells, whose precise origins are unclear, remain after the initial immune response is resolved, while the majority of effector cells are eliminated through apoptosis. They may be formed early in the immune response during the first few cellular divisions following antigen exposure or they may arise from effector cells that survive the contraction phase. In either case, apoptosis plays an important role in regulating memory T-cell homeostasis. The formation of memory cells can occur in the absence of Bcl-2, although such cells display reduced competitiveness and survival when adoptively transferred into wild-type mice **(37)**. Bim plays a particularly important role in CD8 memory cells, especially during viral infection **(48–50)**. The ablation of Bim increases the number of memory cells present immediately after an immune response; however, these cells undergo slow attrition, eventually resulting in near normal numbers of memory cells, suggesting that the survival of memory cells is dependent on additional pathways **(37)**. The downregulation and conversion of CD8 effector cells into memory cells is not dependent on Fas or other death receptors *in vivo,* as CD8 contraction appears normal following viral infection in mice where Fas is mutated **(51, 52)**. However, other components of the extrinsic pathway might play a role in survival of CD8 cells post-antigen encounter. CD8 cells activated in the absence of CD4 cells are prone to apoptosis in a TRAIL-dependent manner upon secondary activation **(53–56)**. However, the presence of CD4 cells is only required during the initial activation of the CD8 cells and not during the restimulation. This observation remains controversial, as other groups using a variety of viral infections have shown that TRAIL plays either no role or a more limited role in CD8 memory cell survival **(53, 57)**. Apoptosis therefore plays an important role not only in the maintenance of memory cells, but also in full and productive secondary responses by generating space in the immunological niche through the elimination of cells that do not receive complete activation signals.

CD8 Effector Function

In addition to removing autoreactive T cells, apoptosis is an important mechanism by which certain classes of T cells perform their function. CD8 T cells, or cytotoxic T lymphocytes (CTLs), recognize cells infected with intracellular

pathogens and eliminate these cells via apoptosis. While CTLs can induce apoptosis in target cells by engaging death receptors such as Fas, it is believed that the release of granzyme B and perforin is the main pathway by which target cell killing is induced **(58)**. Granzyme B and perforin are released in secretory vesicles called granules at the immunological synapse between the CTL and its target **(59)**. Perforin creates pores in cellular membranes enabling the protease granzyme B to gain access to the target cell cytosol **(58, 60, 61)**. The cytotoxic effect of CTLs is greatly reduced in the absence of perforin, demonstrating the importance of these pores **(62, 63)**. Once granzyme B is released into the target cell cytosol, it initiates apoptosis primarily through the cleavage and activation of effector caspases, such as caspase-3 **(64, 65)**. It can also directly cleave other targets important for apoptosis as well, such as the inhibitor of caspase-activated DNAse (ICAD), lamin B, and DNA repair enzymes **(66, 67)**, although the ability of caspase inhibitors to block granzyme B-induced apoptosis suggests that such direct activity of granzyme B is not a major mechanism of its effects. Granzyme B also cleaves Bid, thereby engaging the mitochondrial pathway of apoptosis **(68–72)**. However, recent studies have shown that while human granzyme B effectively cleaves Bid, murine granzyme B is poorly active in this regard **(73)**.

Other additional granzymes also participate in triggering cell death, apoptotic or nonapoptotic. Granzyme A does the latter, through cleavage of substrates unrelated to the apoptotic process **(74)**. Another family member, granzyme H, appears to have proapoptotic activity and can cleave a number of relevant substrates, including Bid, to trigger apoptosis **(75)**. In any case, apoptosis induced by CTLs reduces the spread of infection from cell to cell and plays a vital role in maintaining a healthy organism.

Regulatory T-Cell Function

Regulatory T cells, or Tregs, as one of the primary regulators of peripheral tolerance, can limit autoimmune and chronic inflammatory diseases such as diabetes, asthma, and inflammatory bowel disease through a number of mechanisms that inhibit the activity of CD4 and CD8 effector cells **(76)**, including apoptotic mechanisms (Fig. 18.2). Tregs can inhibit their targets through cytotoxic mechanisms similar to those of CTLs. Transcriptional profiling shows an upregulation of granzyme B in regulatory T cells and Tregs from granzyme B-deficient animals are less suppressive that wild-type Tregs **(77–79)** . Alternatively, Tregs are able to regulate the survival of their target cells through the deprivation of cytokines, thus preventing the upregulation of important survival molecules **(80)**. In this regard, Tregs express high levels of CD25, which allows these cells to capture local IL-2. In some instances, Tregs inhibit their target cells through TRAIL, indicating that death receptors also play a role in Treg function **(81)**. Tregs can also inhibit target cell proliferation through

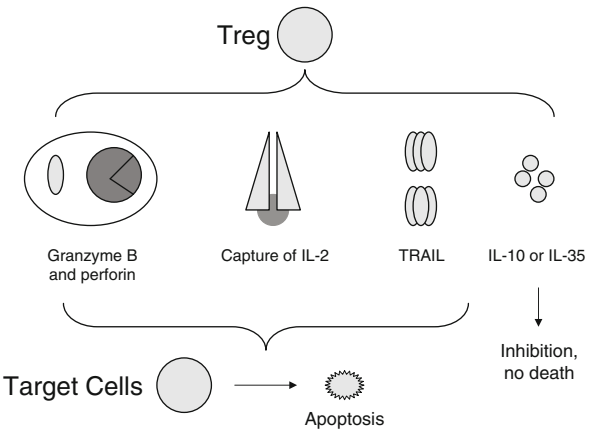


Fig. 18.2 Regulatory T-cell inhibition of target cells. Tregs can induce apoptosis through secretory vesicles containing granzyme B and perforin, deprivation of prosurvival cytokines, and ligation of death receptors through TRAIL. Tregs also release inhibitory cytokines like IL-10 and IL-35, which do not lead to apoptosis

nonapoptotic pathways such as the production of inhibitory cytokines like IL-10 and IL-35 **(76)**. Regardless of the specific mechanism used by Tregs, they provide another key checkpoint in addition to negative selection and Fas-mediated apoptosis to limit potentially destructive immune responses.

B-Cell Development

B-cell development parallels T-cell development with a number of checkpoints to ensure the production of functional nonself-reactive cells. To successfully complete development, B cells require prosurvival cytokine signaling and must survive a selection process that eliminates autoreactive cells through apoptosis (Fig. 18.3). In a mechanism similar to that of IL-7 signaling in thymocytes, signaling induced by the TNF family member BAFF is very important for the survival of developing B cells. Engagement of the BAFF receptor upregulates antiapoptotic Bcl-2 family members in an NF-κB-dependent manner **(82–84)**, and the loss of BAFF leads to a block in B-cell development at the transition to

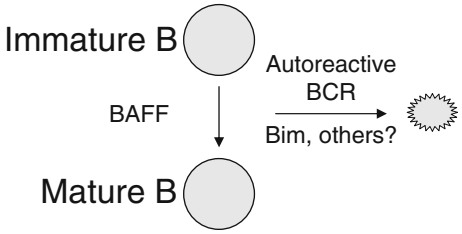


Fig. 18.3 Checkpoints during B-cell development. Survival of B cells from the immature to mature stage is mediated by BAFF. Cells expressing an autoreactive B-cell receptor are eliminated through Bim and potentially other BH3 family members

mature B cells. The transgenic expression of BAFF in mice results in an increase in the number of autoreactive B cells and the onset of autoimmune phenotypes similar to lupus **(85)**. The ectopic expression of antiapoptotic Bcl-2 family members in BAFF-deficient cells should clarify whether the block in B-cell development is dependent on apoptosis in a manner similar to A1 expression in T-cell development.

Similar to T cells, B cells that express autoreactive receptors are eliminated during development via apoptosis. Immature B cells that encounter and bind self-antigen in the bone marrow undergo apoptosis in a process analogous to the negative selection of T cells **(86)**. Evidence for the role of apoptosis in B-cell selection comes largely from the use of the transgenic hen egg lysozyme (HEL) system. The systematic expression of HEL leads to deletion of maturing B cells expressing the transgenic B-cell receptor **(87)**. Complete deletion depends on Bim, as deficiency of this molecule increases the number of autoreactive B cells **(88)**. In contrast to T-cell development, an additional deficiency in PUMA does not lead to increased numbers of autoreactive cells compared to the loss of Bim alone **(21)**. The loss of both Bax and Bak leads to an increase in the number of developing B cells, and those cells are resistant to BCR-induced death *in vitro*, highlighting the importance of the intrinsic pathway during B-cell development **(89)**. The fact that the phenotype of mice with B cells doubly deficient of Bax and Bak is more striking than those in which Bim alone is ablated suggests that proapoptotic BH3 molecules other than Bim likely play a role in B-cell development.

While peripheral B cells are susceptible to elimination through the engagement of the Fas/FasL pathway, the extrinsic pathway is not required for apoptosis during B-cell development. Compared to control mice, B cells from MRL/lpr mice, which express a defective form of Fas, undergo the normal deletion of autoreactive cells **(90)**. Similarly, *in vitro* systems that mimic B-cell development show no role for Fas, FADD, or caspase-8 **(91)**. Normal B-cell development is also seen in mice lacking TNF and TRAIL **(92)**. While the extrinsic pathway does not have an effect on B-cell development, apoptosis through the intrinsic pathway is essential for the elimination of autoreactive cells and the prevention of autoimmunity and therefore will remain an area of active research for some time.

Apoptosis in Myeloid Cells

The life span of myeloid cells, including granulocytes, mast cells, macrophages, and dendritic cells, is regulated by apoptotic mechanisms, involving both intrinsic and extrinsic pathways. Granulocytes, particularly neutrophils, are among the earliest-responding innate immune cells whose role is to limit pathogen expansion during the early immune response. The life span of these cells is limited to prevent "cytokine storm," or excessive inflammation, from causing

tissue damage and can be modulated through prosurvival cytokines. Granulocyte life span is extended upon stimulation with proinflammatory cytokines, which upregulate the antiapoptotic Bcl-2 family members A1 and Mcl-1 **(93, 94)**. Granulocytes from animals lacking one of the isoforms of A1 have reduced survival from cytokine withdrawal *ex vivo* compared with wild-type cells **(95)**. In contrast, some components of the extrinsic pathway do not play a role in granulocyte death, as mice in which Fas or FasL has been mutated have near normal numbers of these cells **(96)**. However, TRAIL regulates human neutrophil survival and might be responsible for the neutropenia found in lupus patients **(97–99)**. The innate immune response can also be regulated by mast cells, which release granules of proinflammatory molecules upon pathogen encounter. Similar to granulocytes, mast cell survival is also regulated by A1, which is upregulated upon ligation of the mast cell immune receptor FcϵR1. It is not clear which proapoptotic signals A1 blocks, as Puma-deficient cells have similar survival to wild-type cells after FcϵR1 ligation despite having a significant survival advantage following growth factor withdrawal **(100)**. Mice lacking an isoform of A1 have a reduced allergy response but do not have a clinical loss of mast cells, suggesting that at least this A1 isoform does not play a role in homeostasis in these cells **(101)**. Therefore, other Bcl-2 family proteins probably regulate mast cell homeostasis.

Both the intrinsic and extrinsic pathways control the survival of macrophages, innate immune cells whose primary role is phagocytosis. The antiapoptotic Bcl-2 family members A1 and Mcl-1 are upregulated during inflammatory conditions, providing a survival signal for macrophages. Mice overexpressing Bcl-2 or suffering from an ablation of Bim have an excess number of macrophages **(102, 103)**. Macrophage numbers are also increased in lpr mice, suggesting a role for Fas in macrophage maintenance **(104)**. In fact, following differentiation from monocytes, macrophage lifespan is controlled by a viability switch in which proapoptotic Fas signaling is opposed by the expression of FLICE-inhibitory protein (FLIP), which prevents apoptosis by blocking caspase-8 activation downstream of death receptor ligation **(105)**.

Macrophages appear to have a third apoptotic pathway separate from either the intrinsic or extrinsic pathways. When stimulated with a number of bacterially derived compounds, macrophages can activate the inflammatory caspase-1 through a complex known as the inflammasome, which contains Nalp proteins and another adaptor, ASC. Recent evidence suggests that activation of caspase-1 in this context can lead to apoptosis, which has been termed *pyroptosis* **(106–109)**. However, the exact nature of the activating structure for caspase-1 in pyroptosis is unclear. In some cases, proinflammatory stimuli trigger the formation of the pyroptosome, a superstructure containing the adaptor protein ASC but not Nalps, which serves to recruit and activate caspase-1 in an apoptotic manner **(110)**. In contrast to apoptosis induced through either the intrinsic or extrinsic pathway, this form of cell death is proinflammatory and is likely the cause of certain autoinflammatory diseases such as PAPA syndrome where activating mutants in intracellular signaling casecades initiate apoptosis

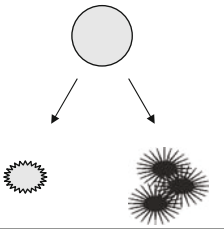
through this pathway **(111)**. Given the importance of the macrophage as a first responder for the immune system, a precise understanding of all apoptotic mechanisms in these cells will be required for effective therapy.

Dendritic cells (DCs) regulate other immune cells through the presentation of antigen and secondary signals required to productively activate them for an immune response. DCs have an internal clock based on the intrinsic apoptosis pathway that regulates their life span, which is critical as cells that survive continue to stimulate immune responses. This molecular timer upregulates Bim upon stimulation through Toll-like receptors or the maturation of the dendritic cell **(112)**. This proapoptotic signaling is counterbalanced by the expression of Bcl-xL and Bcl-2 induced by both the Toll-like receptors and CD40L **(113, 114)**. Adoptive transfer experiments suggest the lengthened life span of dendritic cells due to the loss of Bim leads to autoimmunity, as indicated by an increase in antinuclear antibody production **(112)**. Since Bim loss also leads to increases in both T- and B-cell survival, tissue-specific ablation of Bim in each individual subset will be required before its precise role can be defined.

The extrinsic pathway also plays a role in determining dendritic cell life span. Dendritic cells from animals that transgenically express the baculoviral caspase inhibitor p35 specifically in DCs are resistant to Fas-induced death and accumulate with age, resulting in increased T-cell proliferation when the animals are immunized with peptide **(115)**. These mice have an accumulation of antinuclear antibodies, as well as deposition of IgG into the glomeruli of kidneys, compared to nontransgenic controls. When these mice were crossed into the autoimmune-prone MRL strain background, a chronic lymphoid activation was found similar to that in lpr mice **(115)**. The ability to modulate dendritic cell life span through apoptosis will be an important therapeutic goal, given the increasing amount of evidence on how this life span influences the strength and duration of the immune response.

Apoptotic Cells Induce Tolerance

Apoptosis is important for the elimination of unnecessary or defunct cells. Given that the elimination of these cells is routine, an immune response against these cells would be counterproductive. Thus, it is critical that apoptotic cells induce tolerance, or the lack of an immune response. In comparison to the orderly dismantling of cells that takes place in apoptosis, necrosis is a damaging process that releases danger signals that activate antigen-presenting cells. This form of cell death often occurs in cells infected with intracellular pathogens and provides the second signal, such as engagement of the Toll-like receptors, required to initiate a productive immune response. In the absence of this second signal, the immune system does not respond to the foreign antigen and is thus tolerant. However, it was unclear whether this lack of second signal was the only way for antigen-presenting cells to distinguish between apoptotic and necrotic cells. Recent work has begun to detail an additional mechanism behind the differences in initiating an immune response by these two forms of cell death (Fig. 18.4). In apoptosis, caspases cleave



	Apoptosis	Necrosis
Context	Normal cell turnover	Infection
Immune Response	Tolerizes	Stimulates
HMGB1 status	Oxidized	Reduced

Fig. 18.4 Comparison of effects of apoptosis and necrosis on the immune response

p75, a mitochondrial component of complex 1, leading to the production of reactive oxygen species and the oxidation of HMGB1 **(116)**. The importance of the oxidation of HMGB1 as a signal for tolerance was demonstrated experimentally by treating necrotic cells with H_2O_2 and showing that they induce tolerance rather than an immune response **(116)**. It is likely that other signals from apoptotic cells play an important role in modulating the activation of antigen-presenting cells, since in some cases, apoptotic cells can induce immunity instead of tolerance **(117)**. Understanding the full range of these signals will provide important insights for therapy.

Nonapoptotic Roles for Apoptosis Genes in the Immune System

Several apoptosis-related genes appear to have nonapoptotic roles in the functioning of the immune system. These genes appear to be important for the survival, proliferation, or activation of immune cells. Some of these genes have been previously shown to have nonapoptotic roles in other tissues, such as caspase-8, where animals homozygous for the deletion of caspase-8 die between E10.5 and E12.5 from weakened muscles in the development of the heart **(118)**. While T cells deficient for caspase-8 appear to develop properly, they fail to proliferate in response to stimulation with CD3 and CD28 **(34)**. Recent work has suggests that caspase-8 forms a complex with Carma and Malt-1 that activates NF-κB, leading to activation of the cell and proliferation **(119)**.

FADD is another molecule in the death receptor pathway that appears to have a nonapoptotic role in the immune system. The ablation of FADD or the expression of the dominant-negative FADDdd decreases the number of developing T cells in the thymus compared to control animals **(120**, **121)**, suggesting that FADD plays an important role in thymocyte survival. Molecules in the intrinsic pathway may also have nonapoptotic roles in immune cells. The overexpression of Bcl-2 or the lack of Bax and Bak appears to inhibit the proliferation of both developing thymocytes as well as mature T cells **(122–124)**, probably due to ER effects and calcium homeostasis. While the apoptotic role of these molecules is quite well defined, ongoing work is focused on the exact mechanisms by which these molecules effect their nonapoptotic functions.

Concluding Remarks

The adaptive immune system depends on the generation of functional immune receptors capable of recognizing foreign antigen in the context of self-MHC. A series of rigorous checkpoints prevents autoimmunity and ensures the full productivity of the immune system. Apoptosis shapes the immune receptor repertoire during development, prevents reactions against self, and is an important mechanism by which some cells effect their function. The system is further complicated by complex interactions between different immune cell types that each rely on apoptosis in different ways to determine their life span. This added complexity makes understanding the role of apoptosis both more difficult and more crucial, as there is an increasing emphasis on manipulating apoptosis as a therapeutic tool against autoimmunity and cancer that will make research in this area vital for years to come.

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Chapter 19
Cell Death in the Hematopoietic System

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Abstract The mammalian hematopoietic system is prodigious: billions of blood cells are generated every day to maintain the supply of oxygen to the tissues, repair damaged vasculature, and resist infection. It is becoming increasingly clear that the intrinsic apoptosis pathway, under the control of the Bcl-2 family of proteins, plays many essential roles in the development and function of hematopoietic cells of most, if not all, of the various lineages. It promotes survival, regulates key aspects of maturation and function, and ultimately triggers the timely demise of functionally expended cells. In this chapter, we review the role of the intrinsic pathway in mediating the life and death of the non-lymphoid hematopoietic lineages.

Keywords Hematopoietic stem cells · Progenitor cells · Neutrophils · Macrophages · Megakaryocytes · Platelets · Erythrocytes · Bcl-2 family proteins · Apoptosis

Introduction

Hematopoiesis is the process by which hematopoietic stem cells (HSCs) move through multiple rounds of division, proliferation, commitment, and differentiation to produce mature blood cells of the various specialized lineages (Fig. 19.1 and Color Plate 8). The most primitive HSCs, so-called long-term repopulating HSCs (LT-HSCs), can sustain hematopoiesis throughout life **(1)**. LT-HSCs produce short-term (ST-) repopulating HSCs, which then generate multipotent progenitors (MPPs) **(2)**. While ST-HSCs and MPPs cannot self-renew on a long-term basis, they still possess the ability to generate the full lymphoid-myeloid spectrum of hematopoietic cells. In the classical model, the

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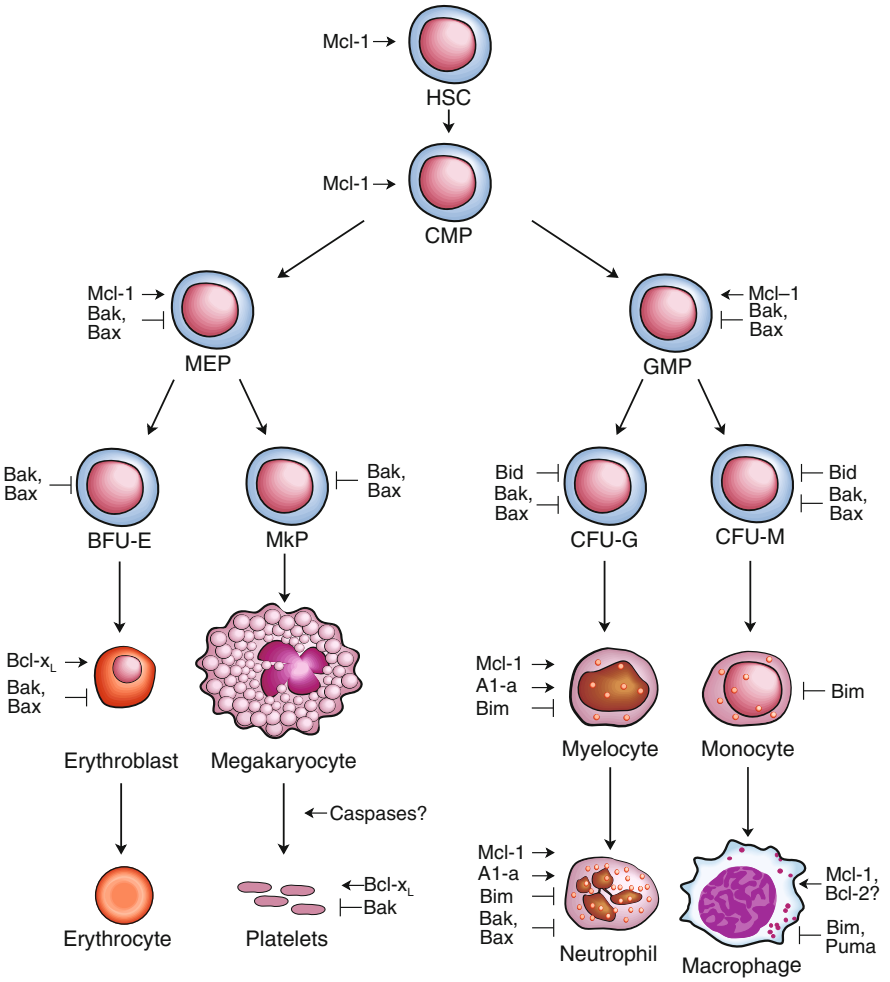

Fig. 19.1 Bcl-2 family proteins regulate multiple aspects of hematopoiesis. Schematic representation of the non-lymphoid hematopoietic hierarchy. Proposed pro-apoptotic (⊥) and anti-apoptotic (↑) functions for Bcl-2 family members are indicated. Hematopoietic stem cell (HSC), common myeloid progenitor (CMP), megakaryocyte/erythroid progenitor (MEP), granulocyte/macrophage progenitor (GMP), burst-forming unit-erythroid (BFU-E), megakaryocyte progenitor (MkP), colony-forming unit granulocyte (CFU-G), colony-forming unit macrophage (CFU-M) (*see* Color Plate 8)

first restriction of lineage potential occurs when MPPs give rise to common lymphoid progenitors (CLPs) **(3)** and common myeloid progenitors (CMPs) **(4)**. The latter go on to produce granulocyte/macrophage progenitors (GMPs) and megakaryocyte/erythroid progenitors (MEPs), which then generate committed unipotential granulocyte, macrophage, megakaryocyte, and erythroid progenitors. A defining feature of this model is the early and strict separation of

the lymphoid and myeloid branches of the hematopoietic system. Recent evidence, however, indicates that the reality may be more complex. Several studies have suggested that the loss of megakaryocyte/erythroid potential is an early step in HSC development and that rather than a CLP, HSCs (or MPPs) produce lymphomyeloid primitive progenitors (LMPPs), which retain granulocytic potential **(5–7)**. In addition, it appears that the earliest thymic T-cell progenitors, which exhibit no B-cell potential, can generate macrophages and granulocytes **(8, 9)**. Regardless of the debate surrounding their ontogeny, it is clear that for each hematopoietic lineage, the balance between production and destruction dictates the steady-state number of mature cells. Apoptotic regulators—in particular, members of the extended Bcl-2 family of proteins—facilitate both aspects (Fig. 19.1). Increasingly, evidence from the hematopoietic system supports the ideas that cellular life span is genetically predetermined and that cells are intrinsically programmed to die by default in the absence of external influences **(10)**. In this chapter, we review the role of the intrinsic apoptosis pathway in mediating the life and death of the non-lymphoid hematopoietic lineages.

Hematopoietic Stem Cells

At steady state, the apoptotic death of hematopoietic stem cells appears to provide a natural constraint on the size of the stem cell compartment. Perhaps the best evidence of this came from early studies demonstrating the protective effects of overexpressing pro-survival proteins in HSCs. Transfection of *Bcl-2* into an interleukin-3 (IL-3)-dependent, multipotent hematopoietic cell line promoted survival in the absence of IL-3 **(11)**, and the systemic overexpression of Bcl-2 in the hematopoietic compartment of mice enhanced progenitor cell survival, leading to an expansion of HSC numbers **(12)**. A similar result was found in the developing embryo, where the ectopic expression of Bcl-2 in aorta-gonad-mesonephros (AGM) hematopoietic stem cells led to an increase in their number and activity **(13)**. This supports the notion that, at steady state, apoptosis limits the size of the HSC pool. However, mice lacking Bcl-2 exhibited no reductions in hematopoietic progenitors **(14–16)**, indicating that it is not an essential mediator of HSC survival. Other pro-survival family members are also dispensable. Hematopoiesis in mice lacking Bcl-w was unperturbed **(17)**. Evidence from chimeras created by injecting *Bcl-x*$^{-/-}$ ES cells into Rag2-deficient blastocysts indicates that HSCs and progenitors lacking Bcl-x_L can support the production of mature cells **(18)**. In addition, the direct pharmacological inhibition of Bcl-x_L, Bcl-w, and Bcl-2 with the small molecule BH3 mimetic ABT-737 did not induce any obvious hematopoietic defects in mice (other than the death of platelets; see ahead) **(19, 20)**. Thus, it appears that these three pro-survival proteins are not absolutely required by HSCs and progenitors. It is unclear what role A1 plays, since there are four *a1* genes in mice **(21)**,

and they have yet to be deleted as a unit. This leaves Mcl-1, which, in contrast to the others, is essential. Mcl-1 is expressed at high levels in HSCs, gradually declining in MPP, CLP, and CMP progenitor populations **(3, 22)**. Opferman and colleagues have demonstrated that the deletion of *Mcl-1* in adult mice causes the rapid death of HSCs and progenitors **(23)**. They crossed a floxed allele of *Mcl-1* to mice expressing Cre recombinase under the control of the endogenous *Mx1* locus, which can be transiently activated in response to the administration of polyinocinic-polycytidylic acid (pI-pC). The conditional deletion of *Mcl-1* from hematopoietic cells was achieved with a single intraperitoneal dose of pI-pC. A profound reduction in both the proportion and total number of HSCs (lineage$^-$ Sca-1$^+$ c-Kit$^+$) and progenitors (lineage$^-$ Sca-1$^-$ c-Kit$^+$) in the bone marrow was evident within seven days. Analysis of bone marrow chimeras confirmed that these effects were cell-intrinsic. Interestingly, more differentiated progenitor populations such as the Ter119$^+$ erythroid series were significantly more resistant to the loss of Mcl-1. In purified populations cultured *ex vivo*, the retroviral transduction of Cre induced apoptosis in more than 90% of HSCs and in 69–81% of CMPs, CLPs, and GMPS from *Mcl-1*$^{f/f}$, but not wild type, mice. Together, these data demonstrate that Mcl-1 is required by HSCs and multipotent hematopoietic progenitors. While the expression of other members of the family, such as Bcl-2, can protect these cells from apoptosis, Mcl-1 mediates their survival at steady state.

If apoptotic death plays a role in regulating the size of the primitive hematopoietic compartment, it might be expected that the deletion of pro-death Bak or Bax, the key cellular executioners, would result in its expansion. This is particularly true of Bak, which is sequestered by Mcl-1 **(24)**. The evidence generated to date is incomplete. Mice lacking either Bak or Bax are viable and ostensibly healthy, with mild lymphoid hyperplasia and increased platelet counts the only reported hematopoietic defects in *Bax*$^{-/-}$ and *Bak*$^{-/-}$ animals, respectively **(19, 25, 26)**. Conversely, a deficiency for both—on an inbred genetic background—results in embryonic lethality. On a mixed background, however, a small proportion of *Bak*$^{-/-}$ *Bax*$^{-/-}$ mice survive to adulthood **(26)**. An analysis of these animals found increased numbers of myeloid colony-forming units (CFU) in the bone marrow, suggesting that the inhibition of the intrinsic apoptosis pathway does indeed promote the expansion of myeloid progenitors. However, no HSC data were presented, so it remains unclear exactly what role Bak and Bax play in mediating HSC homeostasis. The contribution of the pro-apoptotic BH3-only family members is even less well understood. Most have been deleted in mice, with generally mild phenotypic consequences, consistent with there being significant functional redundancy among them. The exceptions tend to be those BH3-only proteins that interact with multiple pro-survival proteins, such as Bim, whose loss results in a range of hematopoietic defects **(27)**. Bim is downregulated in response to cytokine in hematopoietic progenitors isolated from primary bone marrow cultures **(28)**. Nevertheless, the frequency of hematopoietic progenitors in the bone marrow was normal in *Bim*$^{-/-}$ mice, indicating it is not essential for HSC homeostasis **(27)**. Similarly,

the HSC compartment was unaffected by the loss of Bid **(29)**, with no changes in lineage$^-$ Sca-1$^+$ c-Kit$^+$ (LSK) cells, CMPs, GMPs, or MEPs observed. Interestingly, the number of multipotent [colony-forming unit granulocyte erythrocyte monocyte macrophage (CFU-GEMM)] and macrophage progenitor [colony-forming unit macrophage (CFU-M)] cells was increased, suggesting that Bid may regulate the later stages of myeloid commitment and differentiation. In support of this, Bid-deficient animals have been reported to develop a disorder resembling chronic myelomonocytic leukemia **(29)**. However, studies of an independently derived knockout allele of Bid found no increase in the rate of hematopoietic malignancy **(30)**. Hrk **(31)**, Bad **(32)**, Puma **(33)**, Bmf **(34)**, Bik **(35)**, and Noxa **(33)** have also been deleted in mice. Since HSC number and function have not been specifically characterized in these animals, it is not possible to exclude a role for them in constraining HSC numbers *in vivo*.

Neutrophils

Neutrophils, often referred to as granulocytes, are polymorphonuclear (PMN) leukocytes that develop in the bone marrow. They are the most abundant white blood cell in the human body, and they carry an arsenal of toxic granule proteins, enzymes, and reactive oxygen species, which are deployed in the fight against invading pathogens. The neutrophil lineage arises from bipotential granulocyte/macrophage progenitors, which, in response to growth factors—primarily granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF)—generate mature neutrophils via committed granulocyte progenitors, promyelocytes, myelocytes, and metamyelocytes **(36)**. After egress from the bone marrow, terminally differentiated neutrophils circulate for 6 to 20 hours before leaving the bloodstream and entering tissues, where, within 1 to 4 days, they undergo constitutive apoptosis and engulfment by macrophages. Aberrantly accelerated death leads to neutropenia, a reduction in peripheral neutrophil numbers, and an increased risk of bacterial and fungal infections **(37, 38)**. The short life span of the neutrophil is extended by activation, which can be induced by a wide range of cytokines and proinflammatory mediators **(39)**. Resolution of inflammatory responses requires the timely apoptotic death of neutrophils—the safe clearance of these potentially dangerous cells is essential to prevent autoinflammation. Neutrophils that fail to undergo apoptosis can inappropriately activate the inflammatory response, causing tissue damage, and evidence suggests this may contribute to disorders such as Crohn's disease and ulcerative colitis **(40, 41)**.

Neutrophils express several members of the Bcl-2 family, including pro-survival Mcl-1 and A1 and pro-apoptotic Bax, Bad, Bak, Bid, and Bik **(42, 43)**. Several studies performed with human cell lines or primary cells indicate that Mcl-1 plays a role in maintaining the survival of neutrophils. For example, the antisense-mediated disruption of Mcl-1 expression has been shown to induce

cell death in primary human macrophages, neutrophils, and myeloid cell lines **(44–46)**. The deletion of *Mcl-1* in mouse neutrophils, by crossing mice harboring a floxed allele with animals expressing Cre under the control of the *LysM* promoter, resulted in an 80% reduction in peripheral blood neutrophil numbers and an 86% reduction in splenic neutrophils **(47)**. The spontaneous apoptosis of Mcl-1-deficient neutrophils *ex vivo* was two- to threefold higher than that of wild-type cells. Although it has yet to be formally proven that the deletion of Mcl-1 does not cause a block in neutrophil development, these data suggest that Mcl-1 is required for neutrophil survival. Interestingly, GM-CSF restored the rate at which neutrophils lacking Mcl-1 underwent spontaneous apoptosis to almost wild type levels. The authors suggested that the mechanism was an elevation in the levels of other pro-survival proteins, such as A1. Evidence from mice lacking *A1-a*, one of the four *A1* genes, supports this hypothesis. A1-a-deficient neutrophils exhibited accelerated spontaneous apoptosis in culture **(48)**. However, the number of peripheral and peritoneal exudate neutrophils in these animals was unchanged. It has also been demonstrated that the transgenic overexpression of Bcl-2 protected neutrophils from apoptotic death in culture but did not affect their numbers *in vivo* **(49, 50)**. Mice reconstituted with = *Bcl-2*$^{-/-}$ bone marrow exhibited no significant decrease in blood neutrophil number **(51)**. Thus, it would appear that multiple pro-survival proteins can mediate changes in neutrophil survival and life span, but only Mcl-1 is essential for neutrophil homeostasis.

The mechanisms responsible for neutrophil death and the role of the intrinsic pathway are yet to be fully elucidated. Mice lacking either Bak or Bax show no changes in peripheral blood neutrophil number **(25, 26)**. In contrast, mice doubly deficient for Bak and Bax exhibited an approximately fourfold increase **(26)**, presumably due to a decrease in the rate of spontaneous apoptosis in the circulation. Whether Bak and Bax also mediate the constitutive or postactivation death of neutrophils that have migrated into tissues is unclear. Certainly, there is little evidence to support a role for the extrinsic pathway in regulating constitutive death, suggesting that Bak and Bax may be the key players **(52–54)**. However, the death that follows activation and phagocytosis of pathogens does appear to involve caspase-8, even if the extrinsic death receptors are not required **(55, 56)**. The response of Bak- and Bax-deficient neutrophils in this regard has not been studied, but data from mice lacking the BH3-only protein Bim implicate the intrinsic pathway in the process. Bim-deficient mice showed a mild increase in peripheral blood neutrophils and a threefold elevation in the spleen **(27)**. The absence of Bim protected bone marrow neutrophils from spontaneous and chemotherapeutic drug-induced death **(51)**. It also protected activated neutrophils, which had been harvested from mice treated with an intraperitoneal injection of casein. These data suggest that the intrinsic apoptosis pathway may indeed contribute to death post-activation and demonstrate that Bim regulates neutrophil survival, both at steady state and in response to stimulation.

Macrophages

The macrophage lineage begins with committed macrophage progenitors in the bone marrow, which differentiate into monoblasts, promonocytes, and, eventually, monocytes. The latter move into the blood stream where they circulate for several days before migrating into the tissues and differentiating into macrophages and dendritic cells. In inflamed tissue, macrophages are responsible for clearing pathogens and dying cells and mediating wound healing. Tissue-resident macrophage populations are diverse and highly specialized. They include osteoclasts, which play a key role in bone formation and resorption; Kupffer cells, liver phagocytes that contribute to clearance of red cells, apoptotic cells, and particulate matter from the portal circulation; microglia, which form the front line of immune defense in the brain; and alveolar macrophages, which clear inhaled organisms and particulate matter from the lung.

Despite considerable evidence implicating Mcl-1 in macrophage survival **(45**, **46**, **57)**, specific deletion of this Bcl-2 family member in mice using Cre recombinase under the control of the *LysM* promoter resulted in no obvious defects in monocyte development or macrophage numbers in the blood, spleen, bone marrow, and peritoneal exudates **(47)**. Mcl-1-deficient peritoneal macrophages showed no increase in apoptosis in response to Toll-like receptor stimulation and had normal levels of proinflammatory cytokine production. Thus, unlike the situation in neutrophils, Mcl-1 is not absolutely required for macrophage survival *in vivo*, suggesting functional redundancy among the pro-survival proteins. Bcl-2 expression was dramatically upregulated in *Mcl-1*$^{-/-}$ macrophages, indicating that it might play a compensatory role. This is supported by evidence that the overexpression of Bcl-2 in the hematopoietic compartment of mice resulted in a significant increase in monocyte and macrophage numbers **(58)**. However, no defect in these cells has been reported in mice lacking Bcl-2 [or Bcl-w **(17)**, Bcl-x_L **(59)**, and A1-a **(48)**]. The precise requirement for pro-survival proteins in the macrophage lineage is therefore unclear. It seems likely that they depend on multiple family members, but it is conceivable that their steady-state survival is independent of the intrinsic apoptosis pathway.

The absence of monocyte and macrophage accumulation in mice lacking both Bak and Bax supports the latter idea. In contrast to neutrophils, no expansion of the macrophage lineage was apparent in either *Bak*$^{-/-}$ *Bax*$^{-/-}$ mice **(26)** or myeloablated mice reconstituted with *Bak*$^{-/-}$ *Bax*$^{-/-}$ bone marrow cells **(60)**. But the analysis has not been extensive, and evidence from Bim-deficient mice indicates that the intrinsic apoptosis pathway does play a role. A fourfold increase in peripheral blood monocytes was observed in *Bim*$^{-/-}$ animals **(27)**, and similar increases were seen in mice additionally lacking Bak or Bax **(61)**. Splenic and peritoneal macrophage numbers, however, appeared normal. This suggests that only monocyte life span in the circulation is regulated by the intrinsic pathway. Do tissue macrophages undergo programmed cell death? Recent evidence indicates that they do, as a result of phagocytosing pyogenic bacteria **(62)**. In primary

bone marrow-derived macrophages, the engulfment of *E. coli* induced apoptotic death postuptake and destruction of the bacteria **(63)**. This mechanism has been proposed to facilitate safe clearance of activated phagocytes and to potentially stimulate a T-cell response to microbial antigen contained within the apoptotic cell. The overexpression of Bcl-2 could protect mouse macrophages from phagocytosis-induced death **(63)**. In macrophages lacking Bim, which were as efficient as wild-type cells at phagocytosing *E. coli* bacteria, the induction of apoptosis measured as both nuclear fragmentation and the binding of annexin V/uptake of propidium iodide was strongly reduced. It remains to be seen whether $Bak^{-/-}$ $Bax^{-/-}$ macrophages are similarly resistant to phagocytosis-induced apoptosis. Taken together, these data suggest that pro-survival proteins mediate the survival of tissue macrophages and that Bim-mediated death represents the final step for these cells once activated. They support the idea that death is the default state, at least for terminally differentiated cells that have fulfilled their *raison d'être*. Interestingly, the deletion of both Bim and Puma resulted in an increase in the number of Mac-1^+ Gr-1^+ macrophages in the spleen **(64)**. Multiple BH3-only proteins may play a role in regulating the survival of the macrophage lineage and the size of the monocyte and macrophage populations.

Megakaryocytes and Platelets

Platelets maintain vascular integrity, prevent spontaneous bleeding, and work in concert with blood coagulation proteins to limit blood leakage following tissue damage. They are small, disk-shaped cells that contain no nucleus but carry abundant mitochondria. Platelets are produced by megakaryocytes: large, polyploid cells that develop in the bone marrow and spleen. Megakaryocytes shed platelets into the bloodstream, where, in humans, they circulate for around 10 days before being cleared by the reticuloendothelial system **(65)**.

We recently demonstrated that the life span of platelets in the circulation is regulated by the interplay between Bcl-2 family proteins **(19)**. Bcl-x_L is the critical platelet survival factor. Mutations in murine *Bcl-x* reduced platelet life span in a dose-dependent fashion, from 5 days in wild type mice to only 24 hours in mice homozygous for a hypomorphic allele. Adoptive transfers demonstrated that these effects were intrinsic to platelets, and not the result of changes in the host. Using a complementary approach, several groups have shown that the pharmacological inhibition of Bcl-x_L with the BH3 mimetic compound ABT-737 causes rapid-onset thrombocytopenia—the clinical term given to a reduction in peripheral blood platelet number—in mice and dogs **(19**, **20**, **66)**. Treating human platelets with ABT-737 *ex vivo* induced cell death associated with the activation of caspase-3 and cleavage of known caspase substrates such as gelsolin. This effect could be ameliorated by the broad-spectrum caspase inhibitor qVD.OPh **(19**, **67)**. Others had previously shown that mitochondrial damage

induced by carbonyl cyanide m-chlorophenylhydrazone (CCCP) resulted in decreased platelet survival posttransfusion, consistent with the idea that mitochondrial disruption is a trigger for clearance **(68)**. The key mediator of platelet death at steady state is Bak. Platelet life span in Bak-deficient mice was extended to eight days, and platelet counts were significantly elevated as a result **(19)**. The deletion of *Bak* rescued the platelet life span defects observed in *Bcl-x* mutants. Interestingly, $Bak^{-/-}$ platelets were only partially protected from the effects of ABT-737, whereas $Bak^{-/-}$ $Bax^{+/-}$ platelets were entirely refractory. This suggests that while Bak is the primary regulator of the platelet cell death program, Bax plays an auxiliary role. Thus, the intrinsic apoptosis pathway regulates circulating platelet life span.

An outstanding question is whether Bcl-2 proteins also contribute to platelet functional responses triggered by activation. In human platelets, physiological agonists cause mitochondrial membrane depolarization, caspase-3 activation, and phosphatidylserine (PS) exposure **(69, 70)**. While the significance of the first two events is unclear, externalized PS serves as a scaffold for assembly of the prothrombinase complex, which catalyzes thrombin generation, thereby potentiating blood coagulation **(71–73)**. In contrast, then, to its well-established role as an "eat-me" signal on apoptotic cells, PS on the surface of activated platelets plays a functional role. Does the intrinsic apoptosis pathway mediate its exposure? Full platelet activation is an irreversible process that eventually leads to platelet death, but evidence suggests it may be calpain- rather than caspase-dependent **(74, 75)**. Thus, even though they likely mediate apoptosis-induced PS **(19, 20)**, Bak and Bax might not be required for activation-induced PS externalization.

In contrast to platelets, it is unclear whether their precursor cell, the megakaryocyte, depends on Bcl-2 family pro-survival proteins. Bcl-x_L and Mcl-1 are both expressed in megakaryocytes **(59, 76, 77)**, but neither has been specifically deleted in the megakaryocyte lineage. Wagner and colleagues used an *MMTV-Cre* line to delete Bcl-x_L in various secretory tissues and the hematopoietic system, and reported severe thrombocytopenia and megakaryocyte hyperplasia, suggesting that Bcl-x_L is not absolutely required for megakaryocyte survival **(59)**. Our own experience with mice homozygous for a hypomorphic allele of *Bcl-x*, which showed no sign of a platelet production defect, supports this idea **(19)**. This may indicate functional redundancy among pro-survival proteins, but their definitive roles remain to be determined.

The function of pro-death Bcl-2 family members in regulating megakaryocyte apoptosis is equally mysterious. A modest increase in bone marrow megakaryocyte/erythroid progenitors was reported in $Bak^{-/-}$ and $Bak^{-/-}$ $Bax^{-/-}$ mice, but the analysis was not extended to megakaryocytes themselves. Thus, there is much to be understood about the intrinsic pathway in the megakaryocyte lineage. This is neatly underscored by intriguing evidence that megakaryocytes require the apoptotic machinery to generate platelets. Once polyploidization and cytoplasmic maturation have occurred, megakaryocytes undergo a complex and dynamic rearrangement, extending large pseudopodia that elongate

and form thin proplatelets from which mature platelets bud off into the circulation—a phenomenon reminiscent of the cytoplasmic blebbing observed in dying cells **(78**, **79)**. Two initial observations implicated the intrinsic apoptosis pathway in this process. First, the overexpression of Bcl-2 in the hematopoietic compartment of mice caused mild thrombocytopenia **(58)**. Second, the deletion of Bim had the same effect **(27)**. Further evidence came from studies of transgenic mice expressing Bcl-x_L under the control of the megakaryocyte lineage-specific *platelet factor 4* promoter. These animals had normal platelet counts, but megakaryocytes exhibited abnormal ultrastructure and a reduced ability to produce proplatelet extensions *in vitro* **(80)**. This led others to specifically examine apoptosis in megakaryocytes. De Botton *et al*. found active forms of caspases-3 and -9 in cultured megakaryocytes and observed cleavage of substrates such as gelsolin and poly (ADP-ribose) polymerase (PARP) in cells that had begun to shed platelets **(81)**. Active caspases exhibited a discrete punctate distribution. Interestingly, while cytochrome c appeared to be present in the cytosol, there was no evidence of DNA fragmentation. Incubation with caspase inhibitors blocked proplatelet extension. The authors suggested that the process of platelet shedding requires a specialized form of apoptosis, with "regional" rather than "whole-cell" death facilitating the necessary cytoskeletal rearrangements. This idea was supported by Clarke *et al*., who utilized the human megakaryocytic cell line MEG-01, which spontaneously produces functional platelets under normal culture conditions **(82)**. They found active caspases in the cell body of proplatelet-bearing megakaryocytes, but not the proplatelets themselves. Both proplatelet formation and the production of functional platelets could be abrogated by caspase inhibition. More recently, a human variant of cytochrome *c* with enhanced pro-apoptotic activity but normal redox function was reported in a family whose only phenotypic indication was mild thrombocytopenia **(83)**. Platelet production appeared abnormal, indicating that megakaryocytes are particularly sensitive to perturbations in the apoptotic machinery. Thus, it would appear that the intrinsic apoptosis pathway, in addition to regulating platelet life span, potentially plays a number of other key roles in the survival of the megakaryocyte lineage, platelet production, and platelet function.

Erythrocytes

Erythrocytes—commonly referred to as red blood cells—deliver oxygen to the tissues. They are biconcave disks that carry no nucleus and no mitochondria. Red blood cell production (erythropoiesis) proceeds from committed erythroid progenitors [burst-forming unit-erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E)] through to the erythroblast, which then matures, becoming progressively smaller and undergoing chromatin condensation, hemoglobinization, cell cycle exit, and enucleation. The resultant reticulocyte, which carries a

small quantity of RNA, is released into the circulation. RNA loss follows as the final mature erythrocyte is realized.

There is good evidence that the erythroid lineage depends on Bcl-2 family proteins for its normal development. The constitutive deletion of *Bcl-x* resulted in a failure of definitive hematopoiesis in the fetal liver **(18)**, and *Bcl-x*$^{-/-}$ ES cells did not contribute to erythropoiesis in chimeras generated by injecting them into wild-type blastocysts **(84)**. The conditional deletion of *Bcl-x* from the hematopoietic system of mice resulted in a severe anemia, accompanied by a dramatic expansion of erythroblasts **(59)**. This was initially proposed to be the result of, and response to, erythrocyte hemolysis, but a second study using the same mice instead suggested that Bcl-x_L plays a key role in the survival of the late-stage erythroblasts **(85)**. *Bcl-x*$^{-/-}$ erythroblasts cultured with erythropoietin underwent apoptosis during the later, hemoglobin-synthesizing stages of differentiation and failed to produce normal numbers of reticulocytes *in vitro*. This was reflected in significantly lower reticulocyte counts in the peripheral blood, suggesting that Bcl-x_L promotes the survival of maturing erythroblasts. The deletion of Bax alone was unable to rescue the erythroid defects in these animals **(59)**, but a third study has recently demonstrated that the deletion of both Bak and Bax can restore red cell counts to near-normal levels and prevent splenomegaly **(86)**. Thus, Bcl-x_L plays a key role in promoting the latter stages of erythroid development by restraining apoptosis mediated by Bak and Bax.

It is unclear whether the intrinsic pathway actively constrains the size of the erythroid lineage at steady state. No increases in red cell counts have been observed in Bak- or Bax-deficient mice; in fact, two studies have suggested they may be slightly reduced **(26, 86)**. This might explain the mild increase in bone marrow megakaryocyte/erythroid progenitors reported in *Bak*$^{-/-}$ and *Bak*$^{-/-}$ *Bax*$^{-/-}$ animals **(26)**, potentially the result of feedback to the erythroid compartment. There may also be other cell death pathways operating in developing red cells, an intriguing possibility in the light of recent data showing that Nix, a protein with homology to the Bcl-2 family, mediates erythrocyte maturation. Autophagy has long been suggested to mediate the elimination of organelles during maturation **(87–91)**, but the molecular mechanism underpinning the process has remained unclear. Several groups have now demonstrated that Nix is required for the autophagic clearance of mitochondria from developing red cells **(86, 92, 93)**. Mice lacking Nix exhibited a decrease in red cell number, but an increase in reticulocytes. Upon closer inspection, it was apparent that *Nix*$^{-/-}$ erythrocytes contained an abnormal subpopulation of cells that had cleared their ribosomes but retained mitochondria. The inhibition of autophagy with 3-methyladenine, wortmannin, or chloroquine suppressed the removal of mitochondria—but not ribosomes—in reticulocytes **(93)**. While autophagosome formation appeared normal, mitochondria failed to localize correctly to these structures, suggesting that Nix has a specific role in targeting the mitochondria to autophagosomes for clearance **(86, 93)**. The nature of the signal that mediates targeting is unclear. Since Nix has been classified as a Bcl-2 family protein, the key events that trigger the signal might be expected to lie downstream

of Bak and Bax. However, *Bax*$^{-/-}$ *Bak*$^{-/-}$ reticulocytes cleared their mitochondria normally, and this was also true of reticulocytes lacking Bim and Puma **(86)**. Thus, in erythrocyte maturation at least, Nix appears to operate independently of the intrinsic apoptosis pathway. Intriguingly, the BH3 mimetic ABT-737 restored the entry of mitochondria into autophagosomes and promoted mitochondrial removal in *Nix*$^{-/-}$ reticulocytes **(93)**. Since both Nix and ABT-737 were shown separately to induce loss of the inner mitochondrial membrane potential, it may be that this event triggers subsequent recognition by the autophagosome **(86, 93)**.

Conclusions

It is becoming increasingly clear that the intrinsic apoptosis pathway plays many essential roles in the development and function of hematopoietic cells of most, if not all, the various lineages. It promotes survival, regulates key aspects of maturation and function, and ultimately triggers the timely demise of functionally expended cells. While much has been learned, many pieces of the puzzle have yet to be found: the exact mechanisms by which Bak and Bax function remain controversial, and several members of the family such as Boo/Diva and Bok have yet to be characterized. Thus, elucidating the complex interplay between pro- and anti-apoptotic regulators, and the specific processes they mediate in hematopoietic cells, presents an ongoing challenge.

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Chapter 20
Cell Death in Acute Neuronal Injury

R. Anne Stetler, Armando P. Signore, and Jun Chen

Abstract Acute neuronal injury models provide an interesting platform for an investigation into the range and diversity of cell death mechanisms within a single insult. Acute toxicity models range from excitotoxic and inflammatory states to activation-delayed programmed cell death. Furthermore, the diversity of cell types and subtypes present within the neuronal system leads to differential sensitivity to cell death stimuli. Within this chapter, we review the major morphological hallmarks and possible molecular mechanisms contributing to pathogenesis following acute neuronal injury models, including cerebral ischemia, traumatic brain injury, seizure, and spinal cord injury.

Keywords Acute neuronal injury · Cerebral ischemia · Traumatic brain injury · Spinal cord injury · Seizure · Apoptosis · Necrosis · Autophagy · Caspase · Calpain · Beclin-1 · c-Jun N-terminal kinase (JNK)

Introduction to Cell Death in Acute Neuronal Injury

Neuronal cell death encompasses a wide range of pathological disease states, leading to various morphologies and cell death mechanisms unique to the nervous system. While many of these mechanisms share similarity with other cellular systems, the complexities evident in neuronal systems provide a fertile ground for the observation of the range and diversity in cell death mechanisms. Within this chapter, we focus on acute neuronal injury, which presents an interesting progression from rapid excitotoxicity to delayed regulated cell death.

The archetypical and perhaps most in-depth studied model of acute neuronal injury is cerebral ischemia. Acute injury and excitotoxic conditions, such as

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traumatic brain injury, spinal cord injury, or seizures, present related toxicity mechanisms and similar cell death paradigms. This chapter discusses acute neuronal injury and its dichotomies in the contexts of morphology and biochemistry, the latter including cell death signaling at the execution as well as the upstream signaling phases. These signaling pathways are altered following many neurological insults, and under different environmental contexts, these pathways may converge and diverge to produce the myriad of features currently being explored in acute neuronal injury models.

Morphology of Acute Neuronal Injury

Necrosis, Apoptosis, Autophagy, or All of the Above?

Due to the complexity of the factors involved in acute neuronal injury, the morphological features following acute neuronal injury, such as ischemia, traumatic brain injury (TBI), spinal cord injury, or seizures, range from the classical necrotic bursting of the cellular membrane to programmed cell death to autophagic-like vacuole formation. The mixture of cell death morphology likely reflects the multiphasic nature of the insult (Fig. 20.1 and Color Plate 9).

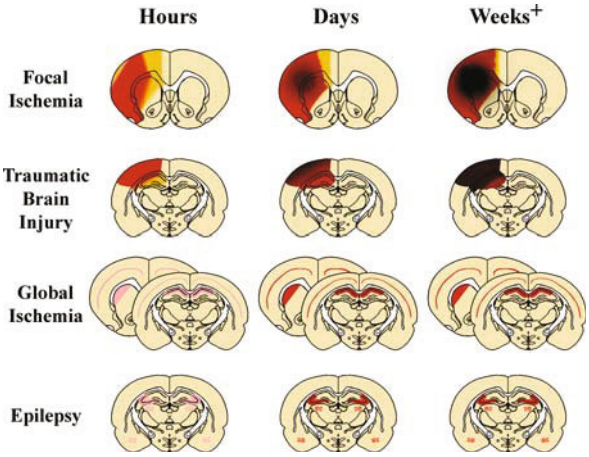

Fig. 20.1 Progression of cell death in multiple models of acute neuronal injury. The progression of cell death varies across cell death models. Focal ischemia and traumatic brain injury display acute injury within hours of the insult, marked by the presence of necrotic morphology within the core of the injury (*black regions*), and progressing rapidly to inflammation (*dark red regions*). Delayed cell death, with morphological features of apoptosis or mixed morphologies, is found in regions proximal to the core of the injury and occurs over days to weeks (*red regions*). Global ischemia and kainic acid-induced epilepsy affect similar overall regions in both hemispheres of the brain, but in differing subregions. Cell death is more delayed compared to focal ischemia or traumatic brain injury and presents hallmarks of programmed cell death, with limited necrotic phenotypes. The figure represents moderate injury models; the range and severity of cell death are highly dependent on the degree of toxicity and the species or method used to induce injury. (*see* Color Plate 9)

Within minutes to hours of acute neuronal injury, excitotoxicity occurs via the release of excitatory amino acids and leads to the rapid appearance of necrotic morphology **(1)**. The second phase of acute neuronal injury involves the activation of inflammatory processes. Inflammation extends into days following the injury and overlaps substantially with the initiation of active cell death processes, leading to morphology characteristic of apoptosis, autophagy, or alternative cell death pathways **(1–3)**. The appearance of these cell death morphologies continues over the course of the ensuing days and weeks following the injury. Sustained and prolonged cell death processes have been observed one year following traumatic brain or spinal injury, leading to progressive atrophy of distantly related structures **(4, 5)**. It is with particular interest that research has focused on the delayed mechanisms of cell death, as therapeutic intervention is feasible at these time points and may provide neurofunctional recovery following acute injury.

The morphological result of the multiphasic nature of acute neurological injury is at best characterized by overlapping cell death phenotypes that can vary greatly from one cell to another within the injured population. Ischemic neuronal death was classically considered to give rise to primarily necrotic morphology, due to hallmarks such as energy depletion, loss of calcium homeostasis, and cellular and organelle swelling in the absence of chromatin condensation. However, at both the light and electron microscopic levels, populations of severely ischemic or damaged neurons manifest morphological changes characteristic of both apoptosis and necrosis, and more recently investigated, autophagy **(1, 6, 7)**. Over the past 20 years, further lines of evidence have solidified the model that cerebral ischemia and many other models of acute neuronal injury produce cell death phenotypes ranging from a predominantly necrotic core zone to a more programmed-like cell death in penumbral or pericontusional regions. Numerous studies, both *in vivo* as well as *in vitro*, have described cell shrinkage, membrane blebbing, chromatin condensation, internucleosomal DNA fragmentation, and the formation of apoptotic bodies following cerebral ischemia **(1, 8–10)**. In addition to apoptotic-like morphologies, evidence of alternative cell death morphologies, including partial chromatin condensation, atypical organelle morphology, and granule formation, occurs in many cerebral ischemic models, indicating that cross-talk may exist between cell death mechanisms **(7, 11)**, potentially leading to a unique cell death process **(12)**.

The morphology of many cells in ischemic vulnerable regions includes autophagic vacuoles and biochemical markers of autophagic processes **(10, 13–15)**, indicating that autophagic processes may contribute to the postischemic morphologies. Following adult hypoxia/ischemia (H/I), microscopic evidence of cytoplasmic vacuolization and degradation of intracellular contents was found **(14)**. Neonatal H/I exhibited electron microscopic evidence of autophagasome formation **(13)**. Seizure models using kainic acid are similar to global ischemia insults, in that overactivation of glutamate receptors occurs and results in a restricted pattern of cell death in the hippocampus and ventral striatum

(Fig. 20.1). The cell death morphology of these cells also has features of both apoptosis as well as autophagy **(16, 17)**. Taken together, these data indicate that ischemic or epileptic cell death presents cells with morphological features of multiple modes of cell death.

In addition to ischemia and epilepsy models, traumatic brain injury has recently been demonstrated to produce a mixed morphology of cell death. Similar to the ischemic core, traumatic brain injury produces a severely damaged cyst at the site of impact and extends into delayed cell death in pericontusional regions, occurring over weeks (Fig. 20.1) **(18, 19)**. Cells exhibiting chromatin condensation, cell shrinkage, and cytoplasmic vacuolization were observed alongside cells bearing more typical necrotic morphology **(18, 20)**. Besides apoptotic and necrotic morphology, recent studies in both rat and human traumatic brain injury tissue have demonstrated the presence of autophagosomes and autolysosomes **(21–25)**. Thus, similar to cerebral ischemic models, traumatic brain injury likely leads to a progressive injury, beginning with necrotic injury in the core region, apoptotic mechanisms in pericontusional areas, and mixed with possible activation of autophagic processes.

Acute spinal injury also results in rapid necrosis and inflammation around the lesion, which gradually is replaced by morphological markers of programmed cell death, including the prolonged degeneration of oligodendrocytes **(26, 27)**. However, morphological and biochemical markers of multiple modes of cell death beyond apoptosis are evident following spinal cord injury **(27, 28)**. Similar to many models of acute neuronal injury, the prevalence of cell death phenotype depends heavily on the proximity and severity of the insult. The degeneration in the spinal cord is not limited to motoneurons but includes apoptosis and delayed cell death of oligodendrocytes **(3, 29, 30)**. Inflammatory processes emanating from microglial activation appear to activate extrinsic apoptotic signaling in surrounding cells, including neurons and oligodendrocytes, and contribute to the acute cell death progression following spinal cord injury **(27, 31, 32)**. Delayed cell death following spinal cord injury may be regulated by alternative mechanisms, as Wallerian degenerative processes are less likely to involve inflammatory responses **(32, 33)**. More recently, morphological evidence of autophagy has surfaced, indicating that autophagy mechanisms may also be activated following spinal cord injury **(34, 35)**. Given that these four models all present varied cell death morphology, a general conclusion can be reached that cell death following acute neuronal injury involves a wide range of cell death mechanisms.

The current debate lies in the functional significance of these particular morphologies; while neuronal "death" may not be desired, apoptosis has long been considered preferable to the excitotoxic conditions that may result from unregulated necrosis. This is particularly relevant in excitable neural areas, where the release of excitatory amino acids may trigger excitotoxicity in neighboring cells. For example, the inhibition of apoptosis by Bcl-xL increased nonapoptotic death following spinal cord injury **(36)**. However, neuronal apoptosis can also be initiated by the atrophy of connecting cells supplying

trophic factors or neural networks, resulting in Wallerian degeneration **(33)**. While the activation of apoptosis may be a damage-control mechanism to contain an excitotoxic injury, it may be responsible for the progression of the injury in more distal areas to the acute injury.

The implication for autophagy in acute neuronal diseases is still a contentious issue. On the one hand, autophagy may be involved in cellular survival, where damaged organelles are recycled by intracellular engulfment. In this sense, autophagy may be a cellular attempt to shut off apoptotic signaling and recover cellular survival **(11, 37)**. Autophagy of damaged mitochondria occurs following recovery from an injury stimulus and is associated with control of functional mitochondrion **(38, 39)**. Controlled engulfment of damaged or leaky mitochondria may then prevent initiation of downstream intrinsic cell death signaling **(39)**. Alternatively, overactivation of autophagy may be an alternative strategy to apoptosis and contribute to cell death **(11)**. Much more work needs to be accomplished in order to define the role of autophagy in acute neuronal injury.

Not All Neurons Are Created Equal

The particular prevalence of morphological phenotypes depends heavily on the severity of the injury induced and the specific sensitivity of neuronal subtypes to different forms of acute injury. For example, while focal ischemia induced by direct occlusion of the middle cerebral artery typically produces a large necrotic core in the striatum followed by a spreading-delayed programmed cell death in cortical penumbral areas, global ischemia (induced via four-vessel occlusion, two-vessel occlusion with hypotension, or cardiac arrest) leads to more localized delayed cell death in specific hippocampal regions (i.e., CA1), thalamus, and, to a more limited extent, striatum and cortex **(1, 40)**. Thus, while all the ischemic models prohibit blood flow to brain regions, the sensitivity and cell death response of neurons differ substantially **(1, 40)**. Even when the mechanism of toxicity may be similar—for example, the excitotoxic activation of glutamate receptors in hippocampal regions in seizure and global ischemia models—and similar morphology of delayed cell death with morphological features of apoptosis and autophagy are present in these models, the specific cellular sensitivity may differ. Global ischemia results in highly localized cell death in hippocampal CA1 populations, whereas kainic acid-induced cell death is restricted to hippocampal CA3 and CA4 neurons. Thus, morphology following acute neuronal injury appears to be quite diverse within a given population of cells. The contribution of the differential expression of receptor subtypes, trophic factors, and antioxidant molecules to cell death phenotypes and sensitivity is still not well understood.

The utility of studying varying degrees of severity and the resulting morphological possibilities has high clinical significance, as the human occurrence of

acute neuronal injury includes a range from severe stroke to transient ischemic attacks to systemic loss of blood flow or mild concussion to severe head trauma. Traumatic brain injury cell death and consequential behavioral deficits are also highly sensitive to subtle differences in insult severity **(41)**. Global ischemia results in distinctly different morphological outcomes than focal ischemia, and both models present different cell death morphologies based on ischemic duration **(1)**, reflecting clinically distinct presentations between stroke and brain injury following cardiac arrest/asphyxiation. Similarly, chronic versus acute glutamate stimulation of spinal motoneurons leads to vastly different cell death phenotypes. Chronic glutamate receptor activation in motoneurons produced delayed morphology with hallmarks of autophagy and limited apoptosis and substantially decreased necrotic cell death compared to acute glutamate receptor activation **(34, 35)**. These phenotypes in motoneuronal toxicity parallel clinical differentials between disease models such as excitotoxic spinal injury and amyotrophic lateral sclerosis **(34, 35)**. Thus, while the range of morphology observed in various acute neuronal injury models is wide, understanding the dynamic range of cell death phenotypes and mechanisms is critical for neuroprotective strategies.

Cell Death Execution in Acute Neuronal Injury

The evidence for the activation and execution of apoptotic and other forms of programmed cell death mechanisms following acute neuronal injury states is plentiful and has been reviewed extensively over the past 15 years. However, over recent years, it has become increasingly clear that the above-described mixed morphological phenotypes are reflective of an amazingly diverse and flexible range of cell death execution. Acute neurological injury has brought to the forefront the concept that executioner molecules typically associated with one pathological process leading to a specific morphology (such as caspase-3 leading to apoptotic nuclear morphology) can, in fact, contribute to different morphological phenotypes **(42)**. In this sense, proteolytic enzymes involved in end-stage cell death are activated throughout acute neuronal injury in distinct but overlapping phases **(11)**. Much of the focus in the literature has been on two major classes of executioner proteolytic enzymes, caspases and calpains.

Caspase-3 was one of the first molecules implicated in both the necrotic as well as programmed phases of cell death following acute neuronal injury and represents an archetypical cell death executioner. A biphasic wave of caspase-3 activity occurs following both global and focal ischemia models, consistent with the rapid death observed in the most vulnerable regions and the delayed death in the penumbra following focal ischemia **(43, 44)**. The inhibition of caspase-3 decreased necrotic phenotypes following traumatic brain injury **(42)**. Caspase-3 activation is also consequential to activation of both the intrinsic and extrinsic caspase activation pathways following acute neuronal injuries such as ischemia, traumatic brain

injury, spinal cord injury, and kainic acid **(27, 43, 45–48)**, and multiple caspase inhibitors are protective to varying degrees in all of these models **(42, 46, 48–53)**. In damaged neuronal tissue, the intrinsic pathway is initiated by cytochrome c release from the mitochondria, and Smac inhibits XIAP and subsequent caspase-3 activation **(54, 55)**. Caspase-8 is also expressed and activated in injured neuronal subpopulations (lamina V) following ischemia **(56)** and is activated in neurons and oligodendrocytes, but not astrocytes, following spinal cord injury **(47)**.

Despite initial results that the inhibition of caspase activity and apoptosis could protect neurons against acute injury such as ischemia, the protection appears to simply delay rather than prevent cell death **(50)**. Infusion of the antiapoptotic protein Bcl-xL linked to a protein transduction domain decreased apoptotic cell death but increased nonapoptotic cell death, increased microglial activation and impaired functional recovery following spinal cord injury **(36)**. Caspase inhibitors may only protect specific populations of neurons. In a seizure model, caspase inhibitors were found to be protective of hippocampal and cortical neuronal populations **(51)** but were ineffective against cerebellar granule cells **(57)**. Furthermore, the activation of specific caspase pathways may be cell-type-dependent. For example, the activation of caspase-8 following focal ischemia was limited to lamina V pyramidal neurons **(56)**. Another limitation of caspase-targeted neuroprotection lies in the therapeutic time window. In traumatic brain injury models, postinjury caspase inhibition appeared to improve histological and neurological outcomes, but this effect was significant only when treatment occurred within one hour postinjury **(42)**, making it limited for clinical use in patient populations.

In addition to the caspase cascade, calpains have widely been implicated in the pathogenesis of neuronal cell death due to the dysregulation of intracellular calcium loads observed in many acute neuronal injury models **(1, 18, 58, 59)**. Calpains were historically ascribed function in excitotoxic conditions due to an acute sensitivity to intracellular calcium levels. Indeed, calpain activity has been observed and implicated in the pathogenesis of cerebral ischemia **(52, 60–64)**, traumatic brain injury **(64)**, spinal cord injury **(28, 65)**, and seizure models **(66)**. Calpains are differentially activated by intracellular calcium concentrations and have a large number of proteolytic substrates, including many common targets shared with caspases. Like caspase inhibition, postinjury calpain inhibition has provided limited neuroprotection following acute neuronal injury, with moderate effects on improved neurological function **(45)**.

Despite the as-yet-unknown role of autophagy in cell death progression following acute neuronal injury, biochemical markers of autophagic signaling have been demonstrated by several groups in multiple models of acute neuronal toxicity. The presence of Beclin-1 as well as LC3-II has been noted in the cerebral ischemic penumbra **(11)**, TBI models, and human traumatic brain injured tissue **(11, 21–25, 67)**, and following kainic acid-induced seizures **(17)**. Beclin-1 and LC3-II have become markers for autophagy, although their roles and the overall mechanism of autophagy in acute neuronal injury are unknown. Beclin-1 is a member of the phosphatidylinositol-3-kinase family. LC3-II is the

microtubule-associated protein 1A/1B-light chain 3 (LC3) modified with a phosphatidylethanolamine during autophagy, which then translocates and incorporates into the membrane of autophagosomes **(68)**.

Simultaneous evidence of multiple classes of proteolytic activation has been found in multiple models of ischemia **(69, 70)**. For example, following cerebral ischemia, cross-talk between calpain and caspase-3 has been noted **(70)**, and cathepsin B activity or spillage from lysosomes was found co-localized in the same cell with active caspases **(7, 62)**. Apoptosis-inducing factor (AIF) nuclear translocation has also been demonstrated to be a caspase-independent cell death mechanism present following cerebral ischemic injury **(60, 71, 72)**. The activation of AIF-dependent cell death under ischemic conditions may also involve cross-talk between cell death pathways via calpain activation. Calpain cleaves and induces AIF translocation from the mitochondria following ischemic insults or isolated mitochondrial stress **(60, 73)**; the inhibition of calpain activity both suppressed AIF translocation and was neuroprotective following global ischemia **(60)**. Furthermore, combinations of caspase and calpain inhibitors are synergistic in neuroprotection **(52, 74)**. A portion of Beclin-1 immunopositive cells also exhibited active caspase-3 following cerebral ischemia **(75)**, indicating that at least some populations of cells with autophagic markers were also apparently committed to cell death. Consistent with the observation of multiple cell death signaling present in neuronal populations undergoing acute stress, the redistribution of LC3-II to punctate cytoplasmic structures resembling autophagosomes in the global ischemia model also exhibited markers of aborted apoptosis, as the initial phases of cell death in these neurons included activated caspases and limited chromatin condensation, but did not fully evolve into the apoptotic phenotype **(14)**. The co-existence and dynamic interaction between multiple classes of cell death executioners and morphology may underlie the presence of various cell death morphologies over the course of acute neuronal injury **(70)**.

These and similar observations in other acute toxicity models lead us to two interesting conclusions. First, the inhibition of one cell death program, such as a caspase-dependent mechanism, may be insufficient to block cell death and trigger cross-talk and the "unmasking" of an alternative cell death pathway. Second, the inhibition of executioner proteases may not be sufficient to block cell death signaling, necessitating targeting much further upstream signaling pathways.

Cell Death Signaling in Acute Neuronal Injury

Given that the execution of cell death following acute neuronal injury appears to involve the co-activation of multiple enzyme systems, a second focus on upstream signaling pathways has attempted to determine if cell fate can be manipulated by the activation of survival pathways or the inactivation of stress

pathways. Kinase cascades have been well described in multiple model systems, but their intricate balance is still being explored following acute neuronal injury. The possible involvement of these cascades in acute neuronal injury models encompasses an extremely large literature; thus, we focus on cascades that have been found to directly impact downstream cell death execution, with particular emphasis on mitochondrial signaling pathways.

One of the major links between cell death execution and upstream signaling cascades rests with the regulation of a cytosolic tethering protein, 14-3-3. Under normal circumstances, 14-3-3 sequesters certain proteins, mostly notably the proapoptotic molecules Bax and Bad, and controls the interaction and thus functions of these proteins **(76)**. The interaction with these molecules is dependent upon phosphorylation states of both the ligands and 14-3-3 itself. In the context of cerebral ischemia, phosphorylation of 14-3-3 has been demonstrated to occur via the c-Jun N-terminal kinase (JNK), leading to the dissociation of Bax from 14-3-3 and translocation of Bax to the mitochondria **(77)**. In cells presumed to survive ischemic injury, Bad is phosphorylated by Akt at serine 136, which increases its affinity and sequestration by 14-3-3 **(78–80)**. However, under stress signaling, JNK targets Bad by phosphorylating serine 128 and 14-3-3, both of which lead to the dissociation of Bad and 14-3-3 and increased affinity between Bad and Bcl-2 or Bcl-xL **(78, 81)**. Bax translocation to the mitochondria and Bad dimerization with Bcl-2 or Bcl-xL have been well documented to be precipitating factors in triggering mitochondrial cell death signals, ultimately leading to caspase activation and cell death **(82)**. Thus, 14-3-3 represents one pivotal intersection between kinase signaling cascades, such as Akt or JNK, and regulation of the Bcl-2 family of proteins and subsequent mitochondrial cell death signaling in acute neuronal cell death.

Beyond 14-3-3, kinase signaling cascades influence acute neuronal toxicity via several other mechanisms. The predominant hypothesis in acute neuronal injury models has been that the MKK4/7/JNK cascade typically leads to or is reflective of cell stress, while the PI3K/Akt cascade promotes cell survival. Although this view is likely to be overly simplistic as multiple levels intersect and cross-talk, substantial literature has implicated JNK in the pathogenesis of acute neuronal injury, including stroke, traumatic brain injury, and seizures **(83)**. JNK can exist as three different isoforms, including the brain-specific JNK3. JNK activity increases following cerebral ischemia in injured regions that later commit to death **(84)**. The pharmacological inhibition or genetic deletion of JNK3 resulted in significant protection against cerebral ischemia **(85–87)** and kainic acid-induced seizures **(83)**. Furthermore, the activation of upstream signaling molecules, such as the apoptosis signal-regulating kinase 1 (ASK1), occurs following ischemia **(88)** and has been demonstrated to be activated by oxidative stress **(89)**, one of the major hallmarks of acute neuronal injury.

Although JNK activity has been closely associated with cell death, several neuroprotective scenarios appear to function via the increased activation of PI3K and Akt **(90–92)**, leading to indirect suppression of JNK activity. Ischemic preconditioning and several other pretreatment strategies increase

the activation of Akt and the modification of downstream targets **(91**, **92)**. The activation of Akt has been found to be negatively correlated with the activation of JNK **(93**, **94)**. However, the interaction between these two kinase signaling cascades is not well understood in the context of neuronal injury. Upstream regulatory molecules, such as PTEN, have been proposed to mediate the inactivation of JNK while stimulating Akt activity in neuroprotective scenarios against cerebral ischemia **(95–97)**.

Major downstream targets for the majority of kinase signaling cascades include phosphorylation and regulation of transcription factors. For example, following cerebral ischemia, JNK activation leads to increased phosphorylation of the transcription factor, c-Jun, and possible subsequent upregulation of Fas ligand **(87**, **98**, **99)**. Consistently, both focal and global cerebral ischemia increases expression of Fas and Fas ligand, leading to the activation of caspase-8 in vulnerable areas **(44**, **72**, **100)**. JNK inhibition led to a suppression of both intrinsic and extrinsic pathways **(44**, **77**, **101)**. On the other hand, the activation of the forkhead transcription factor following cerebral ischemia leads to promotion of cell death gene expression, such as Fas ligand and Bim **(102)**. The activation of the forkhead transcription factor can be inhibited by Akt **(102)**. Due to the opposing effects of Akt and JNK on the same target (e.g., Fas ligand), these two kinase signaling pathways are often thought to be in balance with each other and inherently intertwined. Despite these findings, others have indicated that JNK and Akt may function independently of each other in acute neuronal injury **(79)**. Thus, more investigation is required to firmly grasp the potential interplay between kinase signaling cascades, particularly in nonischemic acute neuronal injury models.

In addition to kinase cascades converging on mitochondrial cell death pathways, recent literature has proposed autophagic mechanisms as potential players in mediating cell death following acute neuronal injury. While the upstream mechanisms leading to autophagy following neuronal injury such as ischemia are poorly understood, critical autophagic activators described in other model systems have been found to be present following acute neuronal injuries, particularly Beclin-1. Beclin-1 is required for autophagy and exhibits several known domains within its protein structure. First, Beclin-1 is capable of binding Class III phosphatidylinositol-3-kinase and promoting autophagy. Second, Beclin-1 contains a BH3-domain-containing protein that can interact with Bcl-2 in nonneuronal models **(103**, **104)**. Conversely, the overexpression of Bcl-2 in apoptosis-incompetent cells can increase autophagy mediated by Beclin-1 **(105)**. The interaction of BH3 domain proteins with Bcl-2 results in mitochondrial cell death signaling or may release Beclin-1 and induce autophagy **(103)**. These seemingly conflicting roles of Beclin-1 in cell death are echoed in the controversy surrounding the role of autophagy in cell death versus survival following acute neuronal injury. The role of Beclin-1 in acute neuronal injury, particularly in the context of interference with upstream mitochondrial cell death signaling, is virtually unexplored and should yield exciting results in the near future.

Cell death signaling and execution in spinal cord injury have not been well defined. However, observations in the progressive role of tumor necrosis factor-α (TNF-α) and Fas receptor signaling in acutely injured motoneurons have been postulated to be critical in mediating the differential cellular responses over the course of the injury state **(29**, **31**, **106**, **107)**. Fas is rapidly induced following acute spinal injury and translocates to lipid rafts, leading to the assembly of signaling complexes, which leads to caspase recruitment **(108)**. Neutralizing antibodies, the infusion of soluble Fas receptor, or the genetic deletion of Fas improved histology and functional recovery following spinal cord injury of motoneurons **(31**, **109**, **110)**. In addition to Fas signaling, TNF-α is quickly upregulated in neurons and glia following traumatic spinal cord injury **(111**, **112)**. The injection of neutralizing antibodies to TNF-α, the genetic deletion of TNF receptor 1, or the pharmacological inhibition of TNF-α decreased the number of apoptotic cells and improved histological outcomes **(111**, **113)**, consistent with a role for TNF-α in the signaling of cell death via nitric oxide. However, as mentioned earlier, recent studies have found diametrically opposed responses of motoneurons to acute versus chronic injury models. In transgenic TNF-α mice, acute injury appeared to favor the overexpression of TNF-α as a cell death signal **(111**, **114)**, whereas chronic phases of spinal injury associated TNF-α overexpression with improved tissue healing **(114)**. Both Fas and TNF-α have also been suggested to influence cell death and survival following cerebral ischemia and traumatic brain injury **(72**, **77**, **100)**, and TNF-α appears to possess similar paradoxical roles described in spinal cord injury **(106)**.

The differences in the perceived role of TNF-α receptors following neuronal injury have been suggested to be due to receptor localization, association in lipid rafts, and expression in different cell types (e.g., neurons versus glia). The last in particular is relevant to different neuronal and glial subclasses, given that the expression of the TNF receptor superfamily members varies greatly **(106)**. The presence of alternative TNF receptor superfamily members can lead to cross-talk between signaling pathways and seemingly paradoxical outcomes in different environmental contexts **(32**, **107)**. The precise mechanisms of these signaling pathways following acute neuronal injury have yet to be well defined, but should provide critical clues into cell type sensitivity to cell death stimuli, as well as more targeted neuroprotectants for clinical use.

Cell Death and Clinical Tools

Currently, targeting cell death pathways following acute neuronal injury is a major focus of translational research. However, as mentioned earlier, acute injuries such as cerebral ischemia or brain trauma result in highly complex and multifaceted cell death signals. Thus, targeting one pathway has had only limited success in neuroprotection or the restoration of neurological function.

Furthermore, the majority of agents assessed thus far have only limited post-injury therapeutic benefit. Because the timing of acute neuronal injury such as ischemia or brain injury is unpredictable, an ideal therapeutic agent would need to have a time window extending beyond several hours. Combination therapies sequentially treating multiple pathways and continued research focusing on upstream modulators of cell death signaling should yield better treatment possibilities in the years to come.

Beyond the potential for direct therapeutics based on the inhibition of cell death signaling, cell death molecules may also be useful as biomarkers and potential diagnostic tools for the progression of brain injury. Currently, the major prognostic tool available for determining therapeutic treatments following acute neuronal injury lies in imaging technology. Unfortunately, this technology is both costly and fairly static. Thus, the potential use of biomarkers could greatly improve therapeutic intervention by allowing a dynamic assessment of injury—and, someday, treatment—progression. The use of radiolabeled cell death markers in imaging could aid in more sensitive determination of the extent and progression of the injury **(115)**. Interestingly, plasma levels of caspase-3 increased following human stroke (in tPA-treated subjects); the levels of plasma caspase-3 positively correlated with infarct growth and poorer neurological outcomes **(116)**. Other markers of neuronal and glial injury have also been detected in plasma or cerebrospinal fluid samples in humans following stroke or traumatic brain injury **(117)**. In addition, serum derived from the jugular bulb of brain-injured patients was capable of inducing cell death in cultured PC12 cells **(118)**. The rate at which PC12 death occurred was predictive of death rate at six months, suggesting that this assay could be used for prognostic purposes. Thus, understanding cell death mechanisms will eventually translate into improved diagnostic and therapeutic tools in the treatment of acute neuronal injury.

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Chapter 21
Apoptosis in Neurodegenerative Diseases

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Abstract Neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and Huntington's disease, are a group of age-dependent, progressive disorders that exhibit prominent neuronal death. Alzheimer's disease and Parkinson's disease are mainly sporadic, whereas Huntington's disease is entirely genetic. Studies on human postmortem brains highlighted the possible involvement of apoptosis and autophagy in neuron death in the diseases. Studies using genetically engineered mouse models confirmed contributions of key apoptosis genes in disease progression in these experimental systems. In addition, mouse models confirmed that neurotoxins may accelerate and exacerbate disease progression. A better understanding of neuron death mechanisms in these diseases will help design better treatment strategies.

Keywords Apoptosis · Autophagy · Neurodegeneration · Alzheimer's disease · Parkinson's disease · Huntington's disease · Postmortem human brains · Mouse models · Genetics · Neurotoxins

Introduction

Neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD), affect more than 5 million people in the United States and more than 20 million people worldwide. These diseases are age-dependent, progressive, and manifested with apoptotic, autophagic, and excitotoxic cell death. This chapter describes existing knowledge regarding cell death mechanisms and regulation in these diseases, specifically studies on AD, PD, and HD postmortem patients and neurotoxin-based and genetically engineered mouse models for these diseases. Neuroprotective strategies derived

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and designed against these neurodegenerative diseases based on cell death studies in human postmortem brains and animal models are also discussed.

Apoptosis in Alzheimer's Disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by impaired memory and cognitive functions. Although several genetic defects have been identified in patients with a family history of this disease, the majority of AD cases are sporadic and late-onset (over the age of 65) and involve individuals with no known genetic predisposition. AD is characterized by the progressive impairment of cognition and behavioral disturbances that strongly correlate with degeneration and death of neurons in the cerebral cortex and limbic brain areas, such as the hippocampus and amygdala. The most acknowledged feature of AD is an accumulation of extracellular amyloid plaques formed by amyloid-β peptide (Aβ), a 40- to 42-amino acid fragment generated by proteolytic processing of amyloid precursor protein (APP), and intracellular neurofibrillary tangles (NFT), whose major constituent is a hyperphosphorylated form of the microtubule-associated protein tau. These deposits constitute the histopathological hallmarks and most widely accepted markers of postmortem AD diagnosis currently available **(1, 2)**. Mutations in APP have been linked to the development of familial AD. Mutations of presenilins (PS), which are part of the gamma-secretase complex that processes APP, also cause a small subset of familial AD. The ApoE E4 allele increases susceptibility to late-onset AD, whereas the E2 allele confers protection against AD. Mutations of tau have been linked to AD-related dementia.

Apoptotic cell death has been studied in AD, based on TUNEL staining, levels of Bcl-2 family proteins, as well as levels and activities of caspases. Compared to age-matched controls, the human postmortem AD brain exhibited a 50-fold increase of DNA fragmentation in neurons in the cortex and hippocampus **(3)**, increased expression of both pro- and antiapoptotic Bcl-2 family members in neurons and in microglia **(4–12)**, increased caspase activities, increased cleavage of caspase substrates in plaque and tangle-associated neurons and microglia **(13–22)**, and increased Fas-associated death domain (FADD) immunoreactivity in cholinergic neurons **(23)**.

From the numerous mouse models overexpressing murine and human APP or Aβ, only a limited neuronal loss was found in some of the models that overexpress human APP or Aβ alone **(24)**. Transgenic mice overexpressing murine $A\beta_{1\text{-}42}$ intracellularly under a strong neurofilament-light (NF-L) promoter exhibited increased TUNEL-positive cells and extensive cell death in the cortex and hippocampus **(25)**. Mice expressing the human Swedish APP double mutation under the Thy-1 promoter exhibited neuronal loss **(26–28)**, a reduced ratio of Bcl-xL/Bax, and mitochondrial dysfunction **(29)**. Mice expressing the APPswe under NSE-1 promoter exhibited increased positive activated

caspase-3 immunostaining and TUNEL staining in the cortex and hippocampus **(30)**. The transgenic expression of wild-type and AD-associated mutant tau has been shown to induce neuronal loss **(31–36)**, in some cases with demonstrated positive capase-3 activation and TUNEL staining **(37)**, while in other cases, apoptosis appeared not to be the primary cell death mechanism. Mice expressing mutant APP together with mutant presenilin 1 (PS1) or tau exhibited accelerated neurodegeneration **(38–40)**. The expression of APP, PSAPP, and tau mutations in transgenic mice frequently causes synaptic dysfunction and axonal degeneration **(24)**.

Stimuli for apoptosis in AD likely include many factors, including increased levels of oxidative stress and dysfunction of mitochondria. The high oxygen consumption, high content of oxidative damage-prone macromolecules such as unsaturated fatty acid, and postmitotic nature of the majority of neurons make the brain susceptible to oxidative damage. A decrease in the endogenous levels of neurotrophic factors, misfolding, and an accumulation of insoluble fibrillated proteins can also induce cell death in AD. Protein accumulation and aggregation may be due to deficiencies of protein sorting or degradation pathways, mutations of the aggregation-prone proteins, oxidative damage, or abnormal posttranslational modification of the aggregation-prone proteins, and they in turn may contribute to the induction of cell death in AD.

Increased levels of cellular oxidative stress have been observed in AD brains. The accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) as well as reduced detoxification enzymes, such as catalase, Cu/Zn-superoxide dismutase, and Mn-superoxide dismutase, have been reported in vulnerable regions of the AD brain in comparison with tissue from aged-matched neurologically normal control patients **(41–47)**. Immunohistology indicated increased protein oxidation and lipid peroxidation in brain areas with neurofibrillary tangles and neuritic plaques **(44, 45)**. Oxidatively damaged membranes cause Aβ to misform and form fibrils *in vitro* **(48)**. Mn-SOD-deficient mice are more prone to amyloid plaque formation *in vivo* **(49)**.

Mitochondrial dysfunction is observed in AD brain and has been proposed as an underlying mechanism of disease pathogenesis since defective energy metabolism is a fundamental component of AD. Decreased energy metabolism and altered cytochrome c oxidase activity are among the earliest detectable defects in AD; such defects can increase ROS, reduce energy stores, and disturb energy metabolism **(42, 44, 50–52)**. Alpha-ketoglutarate dehydrogenase and pyruvate dehydrogenase activities are reduced in AD brains **(50, 52–58)**.

Rat brain mitochondria incubated with Aβ showed decreased mitochondrial respiratory chain complexes III and IV **(59)**. Biochemical studies from transgenic mutant APP mice and AD patients' brains demonstrated that Aβ progressively accumulates in mitochondria and is associated with diminished enzymatic activity of respiratory chain complexes III and IV as well as a reduction in the rate of oxygen consumption **(29, 59–61)**. Aβ at higher

concentrations can induce nonspecific mechanisms that result in mitochondrial dysfunction, by interacting with lipid membranes, generating pores in the inner mitochondrial membrane that result in disruption of ATP production, and assisting the mitochondrial release of ROS **(62, 63)**. Thus, amyloid plaques can be induced by oxidative stress, and conversely, the presence of Aβ can in turn induce ROS production and mitochondrial dysfunction.

Experimental evidence suggests that a blockade of apoptosis may attenuate Aβ-induced cell death. $A\beta_{1\text{-}40}$ microinjected into hippocampal regions of the brains of wild-type mice triggered significant cellular loss, whereas such cellular loss was significantly reduced in caspase-3-deficient mice microinjected with $A\beta_{1\text{-}40}$ **(64)**. Embryonic telencephalic-derived cells from *Apaf1*$^{-/-}$ mice exhibited resistance to common apoptotic stimuli and neurodegenerative inducers such as Aβ peptide **(65)**. Neurons from caspase-2-null mice were resistant to $A\beta_{1\text{-}42}$ toxicity, supporting the importance of this caspase in Aβ-induced death **(66)**. A triple transgenic AD mouse model that carried $PS1_{M146V}$, APPswe, and tau_{P301L} mutations progressively developed plaques and tangles and recapitulated the disease process in the human AD brain. Overexpression of the antiapoptotic protein Bcl-2 in postmitotic neurons protects against disease pathology and improves place recognition memory. These improvements are accompanied by a reduction in caspase activities, which may not be dependent on the antiapoptosis function of Bcl-2 **(67)**. TNFR1 knockout reduces neuronal loss due to APP23 transgenic-expression AD mutation, suggesting that the extrinsic cell death pathway may also contribute to neuronal loss in AD mouse models **(68)**.

Depending on the cell type, timing, dose, and nature of the death stimuli, inducing ROS, ion concentration, and intracellular energy levels, neurodegeneration can exhibit a mixed apoptosis and necrosis phenotype **(69)**. Non-apoptotic neuron death morphologies have been observed in AD brains, including those of necrotic and autophagic cell death **(69–71)**. Excitotoxicity induced by calcium overload has long been suspected to contribute to AD **(72–76)**.

Autophagy clearance of proteins is considered very efficient despite the generation of endosomes and autophagosomes at the distal ends of axons and dendrites and the shuttling of protein substrates to the lysosomes near the cell body. The induction of autophagy by cell stress can be a neuroprotective response. Autophagic stress due to a blockade in the autophagolysosomal pathway accompanies neurodegeneration **(77)**. In AD, endosomal-lysosomal disturbances are early events **(78)**. Autophagic vesicles are highly enriched in components of the gamma-secretase complex and high presenilin-dependent gamma-secretase activity **(79)**. Because APP and tau knockout mice do not cause overt developmental or behavioral deficits **(80, 81)**, reducing $A\beta_{1\text{-}42}$ by boosting Aβ-degrading enzymes such as neprilysin **(82, 83)**, endothelin-converting enzyme **(84)**, or lysosomal cathepsin B **(85)**, inhibiting β- and γ-secretases **(86–89)**, and/or immunizing **(2, 90, 91)** have been investigated.

Apoptosis in Parkinson's Disease

Parkinson's disease (PD) is the second-most common neurodegenerative disorder and the most common movement disorder in the world. The pathogenesis of PD has been linked to a variety of cellular, biochemical, and molecular alterations, with the most characteristic features being the death of 60–90% of substantia nigra neurons and the appearance of Lewy bodies and Lewy neurites in the affected areas **(92–95)**.

The main component of the Lewy bodies and Lewy neurites is α-synuclein (α-syn) aggregates. The development of highly specific antibodies has found that α-syn aggregates appear not only in the substantia nigra, but also in cortical areas in advanced PD. Ubiquitin and ubiquitinated proteins often associate with α-syn aggregates **(96–99)**. The overexpression or mutation of α-syn is the cause of a small subset of PD patients, although sporadic PD patients also invariably exhibit characteristic α-syn aggregates. α-syn overexpression leads to its aggregation and/or neuronal toxicity in various animal models **(100–108)**, while α-syn knockout mice are resistant to MPTP-induced dopaminergic neuron death **(109–111)**. Furthermore, the reduction of α-syn is neuroprotective in multiple animal models **(112, 113)**.

These observations clearly established a critical role of α-syn in PD pathogenesis. Besides the α-syn gene (PARK1 and PARK4), up to 13 genetic loci have been proposed to link to PD **(94, 114, 115)**. Autosomal dominant mutations in *LRRK2* may be responsible for about 5% of familial PD and occur in approximately 1% of sporadic PD; its disease-causing mechanisms may involve kinase activity, GTPase activity, and/or interactions with Parkin, mitochondria, or endosomes **(116–122)**. Parkin and RNF11 mutations may affect protein degradation because of dysfunctional ubiquitin ligase and hydrolase activities **(123, 124)**. PARK9 is a lysosomal-associated ATPase **(125)**. Mutations to PINK1 and DJ-1 may affect mitochondrial function **(126, 127)**. Furthermore, Omi/HtrA2 may be involved in inhibiting IAPs, thereby freeing up caspase activities and apoptosis **(128)**.

One of the first ultrastructural studies of postmortem PD patients used three patients' brains **(129)**. Electron microscopy identified both apoptosis and autophagic degeneration in 6% of 169 melanized dopaminergic neurons in the substantia nigra **(129)**. Mitochondrial structure of apoptotic and autophagic neurons was preserved in these studies **(129)**. In comparison, apoptosis occurs in 2% of the dopaminergic neurons during aging, with dying cells exhibiting shrunken mitochondria **(130)**. These ultrastructural studies were followed by a debate over whether dopaminergic neurons die of apoptosis or undergo a nonapoptotic "withering" death, with arguments favoring either point of view and conclusions dependent on sampling, sensitivities, and interpretation of TUNEL and related techniques **(131–133)**. Later studies found a significant increase in caspase-3 activation in dopaminergic neurons of four PD patients compared to four aged-matched controls **(134)**. Alterations of caspase-8, FADD, Bax, Bcl-2, and

Bcl-xL have also been observed in various studies, although whether these alterations impact the course of the disease pathogenesis is still unclear **(135–139)**.

In contrast to AD, clear cause and consequence have been observed in terms of neurotoxins that induce PD. When drug users in northern California in the early 1980 s developed Parkinsonism, the main neurotoxic product of the synthesized narcotic was found to be 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) **(140)**. MPTP inhibits mitochondrial complex I, increases oxidative stress, and decreases energy production, resulting in neuronal damage and death **(140)**. From these initial observations, the neurotoxin induction of dopaminergic neurodegeneration has been investigated as a possible etiology of PD. Animal models based on neurotoxin-induced dopaminergic neurodegeneration have been used in testing potential therapeutics **(141**, **142)**.

The most frequently used neurotoxin in animal models to aid the study of PD include MPTP, 6-OHDA, rotenone, and paraquat, with mechanisms most likely involving mitochondrial dysfunction and oxidative stress **(143)**. MPTP can easily cross the blood-brain barrier and has a high affinity for dopamine transporter (DAT) to enter dopaminergic neurons. It is converted to MPP^+, which inhibits mitochondrial complex I. 6-OHDA was first used as a neurotoxin because of its structural analogy to catecholamines **(144–146)**. Subsequent studies found 6-OHDA to be a prototypical oxidative stress-generating toxin because it produces hydrogen peroxide in the cell; it is thus frequently used in models for studying the effects of oxidative stress on neurodegeneration **(147–150)**. Since 6-OHDA does not cross the blood-brain barrier well and has severe toxicity to peripheral organs, the unilateral stereotaxic injection of 6-OHDA into the substantia nigra area was performed to produce oxidative stress, neurodegeneration, and associated rotational behaviors **(144**, **146**, **149–159)**. The insecticide rotenone and the herbicide paraquat have been extensively studied because they and related compounds may be potential environmental contaminants that cause sporadic PD. Although only 50% of later epidemiology studies indicated a role of these and related compounds as risk factors in PD, their inhibitory effects on mitochondrial complex I and resulting energy depletion and oxidative stress have been investigated to aid studies of neurodegeneration in PD **(160–164)**.

Oxidative stress damages proteins, lipids, and DNA **(165–168)**. The inhibition of mitochondrial complex I reduces ATP and mitochondrial membrane potential and increases reactive oxygen species (ROS) production and oxidative stress **(169)**. Somatic mitochondrial DNA mutations found in PD can also suppress mitochondrial energy-producing and ROS-reducing functions **(170–176)**, further supporting the possibility of mitochondrial defects in PD development. An energy crisis, a reduction of the mitochondrial membrane potential, and protein, lipid, or DNA damage can potentially induce apoptosis. Indeed, the upregulation of Bax mRNA and protein, the activation of caspases-3 and -8, and subsequent DNA fragmentation have been observed in the SNc in MPTP-treated mice **(139**, **177–179)**. Caspase activation and

positive TUNEL staining in the median forebrain bundle, and consequent apoptotic death of dopaminergic neurons, have been observed in 6-OHDA-treated mice **(151**, **152**, **180)**.

Mutant mice lacking TNFR1 and R2, Bax, p53, or Bim have been shown to be resistant to MPTP toxicity compared to wild-type mice **(181–184)**. AAV-delivered Apaf-1 dominant-negative mutant, Tat-driven expression of Bcl-xL, or transgenic expression of Bcl-2 overexpression attenuates MPTP-induced neurodegeneration **(185**, **186)**. The electron transport chain component coenzyme Q10 (CoQ10) may alleviate the mitochondrial complex I dysfunction observed in PD patients **(187)**. Glial cell-derived neurotrophic factor (GDNF) and its homologue, neurturin (NTN), have been shown to confer neuroprotection in animal models **(188**, **189)**. NTN is currently being evaluated in Phase I trials.

Because of conflicting reports on apoptosis and the observation that autophagic neurons have also been found in substantia nigra neurons in postmortem PD **(129–133)**, and because of the finding that autophagy is reduced during aging **(190–192)**, autophagic neurodegeneration in PD pathogenesis is gathering attention. Defects in both lysosomal and autophagy function have been observed in human patients with Parkinsonism and in mouse models with α-syn inclusion bodies **(193–198)**. Mice with autophagy gene disruption exhibit neurodegeneration phenotypes with accumulation of ubiquitinated proteins, although it is unknown whether polymorphisms of ATG genes are risk factors for PD **(199**, **200)** and whether the enhancement of functions of these genes is therapeutic for PD. Although short on evidence for necrosis playing an active role in PD pathogenesis, delivery of the antagonist of NMDA or the gene encoding GAD, the rate-limiting enzyme for synthesis of inhibitory neurotransmitter GABA, attenuates dopaminergic neuron death **(201)**.

Apoptosis in HD

Compared to AD and PD, HD has a clear genetic basis. George Huntington first described an inherited movement disorder that later, in 1872, became known as Huntington's disease (HD). The disease is characterized by a loss of cognitive ability and motor skills, eventually leading to dementia, changes in personality, severe movement dysfunction known as chorea, and death **(202)**.

HD has been determined as an autosomal dominant disease affecting the central nervous system (CNS) that leads to the dysfunction and death of neuronal cells in the brain **(203)**. The HD-causing gene is *IT15*, whose unstable expansion in the number of CAG repeats in exon 1 results in expansion of a polyglutamine stretch of a large protein Huntingtin (Htt) **(204)**. The number of CAG repeats in the normal population is from 6 to 35. Its expansion to 36 to 41 is associated with an increased risk of HD, and greater than 41 invariably causes HD **(205–208)**. The age of disease onset is inversely proportional to the number

of CAG repeats in the huntingtin gene, and lengths of 80–100 CAG repeats are found in cases of juvenile HD **(206–208)**.

Htt is widely expressed in the whole body, but medium-sized spiny neurons in the caudate are selectively impaired **(203**, **209**, **210)**. Surviving striatal neurons also exhibit dendritic spine loss and synaptic abnormalities **(211)**. A number of findings suggested a role for apoptosis in HD, first by TUNEL staining and later by caspase activation. Portera-Cailliau et al. observed TUNEL-positive striatal neurons in five out of the 10 cases of human HD postmortem brains compared to negative staining in eight control brains **(212)**. Staining was also found in HD striatal tissue by others and was noted to correlate with the length of the CAG repeats **(213–215)**. Because TUNEL-positive staining can also be due to DNA strand breaks during necrosis, other apoptosis characteristics have been investigated in human HD brains. The activation of caspases-1, -2, -3, -8, and -9 and the release of cytochrome c have been demonstrated in human HD postmortem brains **(216–220)**. Reduced IAP levels have also been reported in postmortem HD brain tissues **(221)**.

Transgenic or knockin mice of mutant huntingtin have been generated under HD, CMV, mouse prion, or rat neuron-specific enolase promoters, with exon 1, N-terminal fragment, or full-length human huntingtin **(222)**. These mouse models recapitulate many motor, pathological, brain atrophy, and cell loss phenotypes of human HD **(222)**. Recent work also indicated that expression of mutant Htt in connecting neurons is necessary for the cortical or striatal dark neuron degeneration phenotype **(223)**. Progressive neurological phenotypes were found in the R6/2 mice that contain a segment of exon 1 of the human HD gene carrying 144 CAG repeats **(224)**. Caspases-1 and -3 are transcriptionally upregulated and activated in a sequential fashion in the R6/2 mouse brains **(217**, **225)**. Dark, shrunken cells with pyknotic nuclei consistent with apoptotic morphology and positive TUNEL staining were observed in transgenic mice expressing a full-length huntingtin gene with 8 or 89 CAG repeats under a CMV promoter **(226)**. The proapoptotic BH3-only proteins Bid and Bim_{EL} were increased in the striatum of HD mouse models at different stages of the disease in two different mouse models of HD with exon 1 mutant huntingtin, including the R6/1 mice with 116 CAG repeats, and the R6/1:BDNF+/- mice with BDNF heterozygosity **(227)**.

Although it is a single-gene disease, mutant huntingtin can cause pleiotropic changes in the brain, including impairment of the proteasomes, impairment of mitochondrial functions, alteration of transcription, and blockade of axonal transport **(228)**. Htt interacts with many proteins that are important for HD pathogenesis **(229**, **230)**. Thus, apoptosis itself certainly cannot explain all HD pathologies. Limited studies in rodent models demonstrated a neuroprotective role of the downregulation of proapoptotic genes or the upregulation of anti-apoptotic genes. Overexpressing Bcl-2 in neurons in the R6/2 mice by generating NSE-Bcl-2:R6/2 double transgenic mice delayed the onset of R6/2-induced motor deficits and extended survival **(231)**.

Expression of a caspase-1 dominant-negative transgene (M17Z) under the control of the neuron-specific enolase promoter, administration of a broad

caspase inhibitor (zVAD-fmk), or administration of a tetracycline derivative, minocycline, in R6/2 mice improved motor function and extended life span **(217**, **225)**. Minocycline can inhibit cytochrome c release and decrease the expression of caspases-1 and -3 and the downstream caspase-dependent and -independent cascade that leads to cell death in R6/2 mice **(232)**. Combined pharmacological inhibition of both caspases-1 and -3 was required to increase viability in R6/2 mice, suggesting that several caspases may simultaneously contribute to the progression of HD **(217**, **225)**. A two-year study of minocycline showed improvement in both neuropsychological function and motor skills in patients **(233)**. Treatement with ucf101, an HtrA2-specific inhibitor, counteracts IAPs degradation in HD cells and increases cell survival **(221)**.

Neuron death sometimes occurs many weeks after the onset of neurological symptoms, and TUNEL staining occurs at very late stages of mice morbidity and, in certain cases, is completely absent **(234)**. One likely explanation is that different huntingtin fragments are generated from cleavage by calpains and caspases, and they cause apoptotic, nonapoptotic, or axonal neurodegeneration **(235–239)**. A reduction in NMDA receptors was found in HD postmortem brains, suggesting a special vulnerability of these neurons to mutant huntingtin-induced neuron loss **(240)**. Consistent with the human studies, medium-sized spiny neurons of the striatum are especially susceptible to excitotoxicity, and their susceptibility to excitotoxicity is increased by mutant huntingtin in mouse models **(241–243)**. Mutant huntingtin is also expressed and aggregates in the glia in human HD patients, and its expression in R6/2 transgenic mice sensitizes Htt-induced neurotoxicity in glia-neuron co-cultures through an excitotoxicity mechanism **(244)**. In line with the excitotoxicity theory of HD pathogenesis, neurochemical models of HD have been investigated, using kainic acid, quinolinic acid, 3-nitropropionic acid, and malonate **(237**, **245–249)**. The treatment of M826, a new inhibitor of caspase-3, induced a significant reduction in the number of neurons expressing active caspase-3 and cell death in the rat malonate model of HD **(250)**. A variety of NMDA receptor antagonists have been developed and tested in mouse models and clinical trials **(251–256)**.

In addition to various strategies to directly tackle the apoptotic and excitotoxic cell death machinery, strategies to attenuate the transcriptional deficit generated by huntingtin, provide neurotrophic support **(257**, **258)**, reduce production **(259)**, facilitate folding **(260–262)**, and enhance clearance **(263–272)** of Huntington have been continually investigated.

Conclusion

AD, PD, and HD share common characteristics as age-dependent neurodegenerative diseases with pronounced protein aggregation (Table 21.1). Neurodegeneration is the most pronounced, although not exclusively, in selective populations of neurons in each disease, often accompanied by synaptic dysfunction, axonal

Table 21.1 The commonalities and differences among AD, PD, and HD

The commonalities and differences among AD, PD, and HD					
	Most affected brain areas	Aggregated proteins	Genetics	Common features	Disease-specific contributing factors
AD	Cortex, hippocampus, cholinergic, catecholamine, and glutamate neurons	Aβ and tau aggregate, knockout of APP or tau are well-tolerated	Mostly sporadic. APP and tau mutations, mutations in presenilin 1 (a key component of γ-secretase) that increase amyloid-β 42 deposition, and presenilin 2 mutations give rise to AD. ApoE ε4 allele is a risk factor	Apoptosis, synaptic, and axonal degeneration, mitochondrial dysfunction, oxidative stress	Endosomal and protein sorting and proteolytic pathways are important for generation of Aβ, neuron membrane properties are modulated by Aβ and cholesterol
PD	Substantia nigra, dopaminergic neurons	α-synuclein aggregates, knockout of a-synuclein is well-tolerated	Mostly sporadic, α-synuclein mutation and gene amplification contribute to a small set of familial PD, 14 familial PARK genes have been studied		Substantia nigra neurons are especially susceptible to a-synuclein and neurotoxins that are uptaken by dopamine transporters and inhibitory of mitochondrial complex I
HD	Caudate, medium spiny neurons	Mutant huntingtin (mHtt) aggregates, knockout Htt is lethal	100% genetic with polyQ expansion in huntingtin protein		Striatal medium spiny neruons are especially susceptible to mHtt-induced excitotoxicity, mHtt disrupt many cellular functions

Table 21.2 The unique features of neurodegenerative cell death

Apoptosis, necrosis, and neurodegeneration			
	Regulation	Effects on cell shape and organelles	Effects on surrounding cells
Apoptosis	A cascade of events involving Bcl-2 and caspase family members, fast	Cell shrinkage, relatively intact organelles, membrane blebbing	Inflict minimum damage to surrounding cells
Necrosis	Acute insult-or intense stress-induced, fast, Bax- and caspase-independent	Swollen mitochondria, membrane ruptures	Surrounding cells often die together, cause inflammation
Neurodegeneration (AD, PD, and HD)	Age-dependent, slow, with aggregated proteins	Mixed features, often with synaptic dysfunction, axon degeneration, and accumulation of autophagic vesicles	Often associated with inflammation

degeneration, gliosis, and microglia activation. Neurodegeneration in these diseases appears to involve apoptosis, necrosis, and autophagic features (Table 21.2). The diverse clinical manifestation of these neurodegenerative diseases, the mostly sporadic nature of AD and PD, and the lack of reliable diagnosis biomarkers for AD and PD present special challenges for the treatment of these neurodegenerative diseases. In all three diseases, the levels and activities of proteins involved in apoptosis are altered, due to protein aggregation, oxidative stress, mitochondria dysfunction, and/or deficiencies in proteolytic pathways. Besides apoptosis, necrosis, excitotoxic cell death, and autophagic cell death have also been observed in these diseases. Figure 21.1 highlights possible contributing factors to cell death in AD, PD, and HD.

Alterations in the level, location, and activity of these cell death factors in all three diseases may not represent causality or greatly impact the disease course. Animal models have assisted in proving whether a gain or loss of function of cell death regulators and mediators is necessary and sufficient for aspects of AD, PD, and HD. Understanding the mechanisms, regulation, and contributions of apoptosis, autophagic cell death, and necrosis is important for studying AD, PD, and HD. Although neuron death appears to be a late-stage phenomenon, many factors contributing to cell death start to be dysfunctional before the eventual occurrence of cell death.Also, although apoptosis, autophagy, and/or necrosis alone may not be able to explain every aspect of the neurodegenerative diseases, regulators and mediators of these cell death pathways may be suitable as intervention targets against AD, PD, and HD. Conversely, any strategy improving neuronal functions without improving neuron survival may not be enough to halt disease progression. Thus, improving cell survival still warrants critical consideration for therapeutic purposes. Figure 21.1 also highlights some of the possible

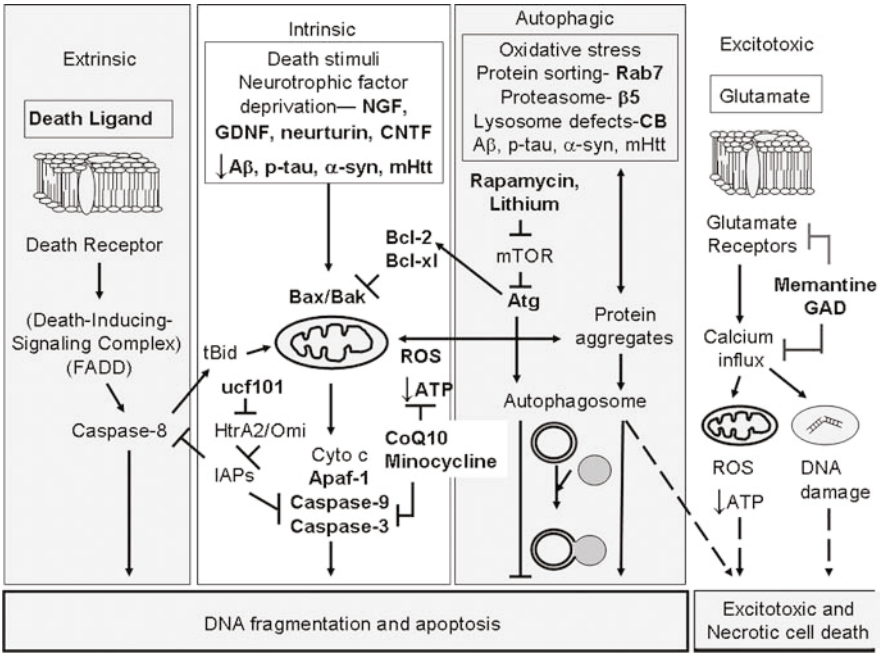

Fig. 21.1 How cells die in neurodegenerative diseases and the therapeutic strategies that target the pathways. In AD, PD, and HD, the levels and activities of molecules that regulate or mediate cell death are altered. These include, but are not limited to, death ligand and receptors, caspases, Bcl2 family anti- and proapoptotic proteins, ROS, autophagy genes (atg), and glutamate receptor-mediated mechanisms. Insults that induce these changes have been proposed to involve ROS and genetic mutations that induce or facilitate protein aggregation. Strategies that intervene with cell death pathways have been investigated in AD, PD, and HD. Highlighted in bold are some of the potential neuroprotective targets examined currently or historically. Neurotrophic factors that are potentially neuroprotective include NGF, GDNF, NTN, and CNTF. The enzymatic or immunological reduction of aggregation-prone proteins has been investigated. Efforts have been made to enhance anti-apoptotic Bcl-2, improve mitochondrial functions, reduce oxidative stress, and decrease caspase activities. Strategies that facilitate protein sorting, proteasome activities, and lysosome activities have been examined. Enhancing the autophagic clearance of aggregated proteins has been achieved at least in cell cultures by rapamycin, lithium, trehalose, and small chemicals. Strategies that reduce excitotoxicity, including the use of memantine and the GABA-producing enzyme GAD, are also included in clinical trials

intervention strategies against neurodegeneration in AD, PD, and HD. Enhancing macroautophagy by rapamycin, lithium, and other small molecules has recently been added to the list of potential therapeutic strategies against PD and HD by clearing α-syn and Htt, at least with promises in cultured cells **(263–272)**. More high-throughput screening of chemical libraries, genes, and networks in more tractable organisms such as yeast, flies, worms, and cultured cells in yeast, *Drosophila*, worm, or cultures in combination with studies in mammalian systems may provide new ideas for understanding and treating these diseases.

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Chapter 22
Apoptosis in Cardiovascular Pathogenesis

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Abstract The loss of cardiomyocytes in the human myocardium results in overloading of the heart, causing structural remodeling of the heart and deterioration of the cardiac function, eventually leading to heart failure. Cellular suicide or apoptosis of cardiac muscle cells has been identified as an essential process in the progression to heart failure. This process entails a highly structured series of events that gradually shuts down cellular functions, leading to removal of the cardiac muscle cell, with minimal consequences to the surrounding tissue. Because of the impact of apoptosis on cardiac function, cardiomyocyte resuscitation by preventing programmed cell death holds a highly interesting potential as a therapeutic target. For future rational drug design aimed at limiting cardiac cellular loss, it is necessary to have a full understanding of the apoptotic pathways that are functional in the cardiac muscle. This chapter summarizes the apoptotic pathways operative in cardiac muscle and discusses therapeutic options related to apoptosis for the future treatment of human heart failure.

Keywords Cardiac cell death · Heart failure · Myocardial infarction

Introduction

Despite considerable advances in the treatment of cardiovascular disease, heart failure is still the leading cause of death in developed countries **(1)**. A powerful predictor for the development of heart failure is the presence of left ventricular hypertrophy **(2)**. It is widely believed that progressive deterioration of the hypertrophied left ventricle (LV) is related to the progressive loss of cardiac myocytes **(3, 4)**. There is increasing evidence from human and animal models suggesting

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that apoptosis or programmed cell death could be a key modulator especially in the transition from "compensatory" hypertrophy to heart failure. Although endothelial cell apoptosis is also frequently found in heart failure biopsies, and could contribute to the ensuing decompensation, here we focus on programmed cell death of cardiac muscle cells, the main functional cellular motors of the cardiac pump. An example of a TUNEL-labeled adult heart muscle cell is provided in Fig. 22.1 and Color Plate 10, with a confocal triple stain for a heart muscle sarcomeric marker, nuclei, and TUNEL-positive nuclei.

Reduced blood supply to the heart muscle due to obstruction of coronary arteries secondary to atherosclerotic heart disease leads to acute regional cardiac ischemic areas, with the widespread death of cardiomyocytes. Several reports have indicated apoptotic rates of 2–12% in the border zone of human myocardial infarcts **(5**, **6)**. Taking these values into account, it is not hard to imagine that such a dramatic loss of viable tissue can have a disastrous effect on the geometry and function of the left ventricle.

These high levels of programmed cell death contrast with the much lower incidence of this form of cell death in postmortem animal and human biopsies with end-stage failure. Human failing hearts in NYHA classes III-IV typically display apoptotic rates ranging anywhere between 0.12% to 0.70% (see Table 22.1) **(7–14)**. Slight variances in technical approaches to detect PCD account for the differing apoptotic incidences found between the studies. Yet a conceptual question is how a loss of 0.1% of contracting muscle could have such a profound influence on the overall geometry and function of the complete organ. To begin to understand this, one should take into account that the documented apoptotic rates were obtained in explanted human tissue, i.e., at one single point of time in the disease. The occurrence of cardiomyocyte apoptosis of less than 0.1% would implicate a minor role in the deterioration of the left ventricular function. Assuming that the period for cardiomyocyte

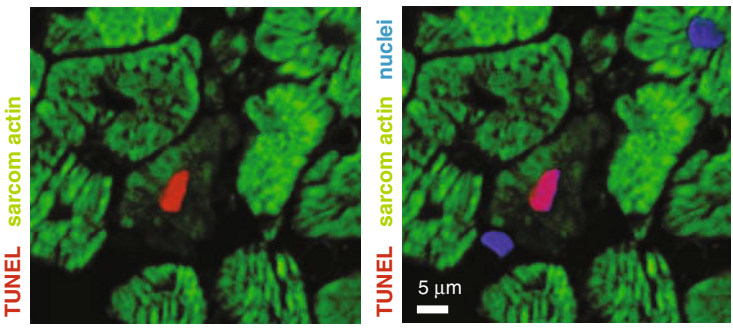

Fig. 22.1 Imaging cardiac muscle apoptosis in vivo. Confocal image of TUNEL-positive cardiomyocyte labeling in the mouse heart after aortic banding, with TUNEL-positive nucleus (*red*), sarcomeric actin (*green*), and nuclei counterstained with DAPI (*blue*) (*see* Color Plate 10)

Table 22.1 Incidence of apoptosis in human heart failure biopsies

Reference	Primary disease		Apoptotic cells (per 10^5cells) HF	Control
Olivetti et al. **(6, 9)**	CHF		232	1
	Ischemic		243	
	Idiopathic		236	
Saraste et al. **(7)**	CHF	Overall	119	11
	Progression to allograft	Rapid	192	
		Intermediate	93	
		Slow	26	
Guerra et al. **(10)**	CHF	Female	80	2
	CHF	Male	180	
Narula et al. **(11)**	Ischemic		576	n/a
	CHF		1837	
Rayment et al. **(12)**	Ischemic		102	n/a
	CHF		553	
Knaapen et al. **(13)**	Ischemic		1.4	n/a
	CHF		1.8	
Latif et al. **(14)**	Ischemic		49	7.1
	DCM		30	

CHF, chronic heart failure; DCM, dilated cardiomyopathy; HF, heart failure; n/a, not applicable.

death to occur due to apoptosis is 24 hours and that the human left ventricle contains up to 6×10^9 cardiomyocytes **(9)**, we may speculate an almost 4% loss of left ventricular contractile mass from apoptosis per year, with even higher percentages under pathological circumstances **(4, 15)**.

In addition, a scattered loss of myocytes due to the apoptosis of cardiomyocytes has been shown to lead to impaired ventricular function **(9)** and provokes the heart into compensatory mechanisms such as left ventricular hypertrophy, left ventricular dilation, and enhanced and sustained activity of the sympathetic nervous system. Studies using genetically modified mice have clearly indicated a direct causal relationship between levels of apoptosis and the progression to advanced heart failure. For example, mice that express a conditionally active caspase exclusively in the myocardium demonstrated that very low levels of myocyte apoptosis (23 myocytes per 10^5 nuclei) caused a lethal, dilated cardiomyopathy in otherwise normal, healthy hearts, indicating that low levels of apoptosis can suffice to cause profound changes in cardiac geometry and function **(4)**. Importantly, the apoptotic rates in the latter study were much lower than those observed in failing human hearts (see Table 22.1). Furthermore, even in studies where apoptosis was not the primary focus, apoptosis is often found to accompany the development of heart disease **(16)**, while conversely, the attenuation of the development of heart failure is often accompanied by the inhibition of apoptosis **(17)**. Thus, limiting cardiac muscle loss by inhibiting apoptosis could have essential implications for the treatment of heart failure.

The Cardiac Apoptotic Pathways

Apoptosis is a highly regulated biological process that regulates the balance between prodeath and prosurvival cell signals, the outcome of which is essential for cell fate. Most apoptotic signaling pathways discovered thus far in extracardiac cell types have also been found to play a crucial role in the induction of apoptosis in the heart; therefore, we give only a brief overview of the mechanisms here, as countless excellent reviews cover these basic mechanisms in detail **(18, 19)**.

The activation and function of caspases, a group of cysteinyl-aspartate-directed proteases, have been shown to play key roles in cardiac apoptosis. In healthy cells, caspases reside in the cytosol as inactive proforms that, in most cases, are activated by proteolytic cleavage **(20)**. Active caspases cleave vital substrates in the cell, leading to cellular demise. In the heart, confirmed caspase substrates include α-actin, α-actinin, α*β*-myosin heavy chain, myosin light chain 1/2, tropomyosin, and cardiac troponins, all of which are crucial molecules for cellular homeostasis **(21)**. The cleavage of key molecules for cellular function (execution) is performed by caspases-3, -6, and -7, which, accordingly, are referred to as executioner caspases. Two major apoptotic pathways, the "extrinsic" and "intrinsic" cascades, eventually lead to activity of executioner caspases.

The intrinsic pathway is characterized by the utilization of mitochondria to propel cell death through opening of the mitochondrial permeability transition pore (MPTP) or rupture of the outer mitochondrial membrane. This triggers the sudden and complete release of cytochrome c and other proteins from the intermembrane space of mitochondria into all other compartments of the cell. The intrinsic pathway is primarily activated in myocytes by cellular stimuli such as hypoxia, ischemia-reperfusion, and oxidative stress, which provoke the mitochondrial permeability transition, an increased permeability of the outer and inner mitochondrial membranes **(22–24)**. The mitochondrial permeability transition pore, a protein complex that spans both membranes, is considered the mediator of this event and consists of at least the voltage-dependent anion channel (VDAC) in the outer membrane, the adenine nucleotide translocase (ANT) in the inner membrane, and cyclophilin-D in the matrix **(22, 24)**. Upon permeabilization of the mitochondrion, several intermembrane proteins are released into the cytosol, including cytochrome c, Smac/DIABLO, endonuclease G (Endo G), Omim/Htr, and apoptosis-inducing factor (AIF) (Fig. 22.2 and Color Plate 11). In the cytosol, cytochrome c binds to the cytosolic protein Apaf-1, facilitating the formation of the "apoptosome" complex, which results in caspase-9 activation, which, in turn, induces caspase-3 activation **(25)**. Smac/DIABLO indirectly activates caspases by sequestering caspase-inhibitory proteins **(26)**, while the release of Endo G and AIF from mitochondria results in their translocation to the nucleus, where they either directly or indirectly facilitate DNA fragmentation **(27)**.

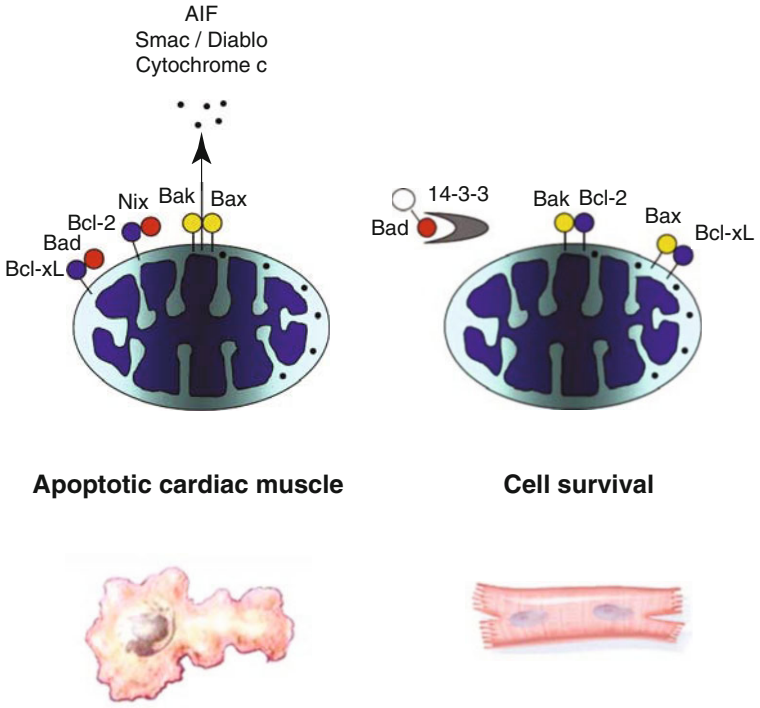

Fig. 22.2 Function of Bcl-2 family proteins. Named after the founding member of the family, which was isolated as a gene involved in B-cell lymphoma (hence the name *bcl*), the Bcl-2 family is comprised of well over a dozen proteins, which have been classified into three functional groups. Members of the first group, such as Bcl-2 and Bcl-xL, are characterized by four short, conserved Bcl-2 homology (BH) domains (BH1–BH4). They also possess a C-terminal hydrophobic tail, which localizes the proteins to the outer surface of mitochondria, with the bulk of the protein facing the cytosol. The key feature of group I members is that they all possess antiapoptotic activity and protect cells from death. In contrast, group II consists of Bcl-2 family members with proapoptotic activity. Members of this group, which includes Bax and Bak, have a similar overall structure to group I proteins, containing the hydrophobic tail and all but the most N-terminal, BH4 domain. Group III consists of a large and diverse collection of proteins whose only common feature is the presence of the ~12- to 16-amino-acid BH3 domain. The Bcl-2 family of proteins function primarily to protect or disrupt the integrity of the mitochondrial membrane and control the mitochondrial release of proapoptotic proteins like cytochrome c, AIF, and Smac/DIABLO. Antiapoptotic Bcl-2 members (Bcl-2, Bcl-xL) protect the mitochondrial membrane. In response to environmental cues, these antiapoptotic proteins engage another set of proapoptotic proteins of the Bax subfamily (which includes Bax, Bak), normally loosely residing on the mitochondrial outer membranes or the cytosol. The interaction between Bak and Bax proteins results in oligomerization and insertion into the mitochondrial membrane of the complete complex (*see* Color Plate 11)

Whereas the intrinsic apoptotic pathway depends on mitochondria, the extrinsic apoptotic pathway is triggered by members of the death-receptor superfamily, such as the Fas receptor or the tumor necrosis factor α receptor (TNFR). Binding of the transmembrane protein Fas ligand to its cognate receptor induces receptor clustering and the formation of a death-inducing signaling complex (DISC) **(28)**. This complex recruits multiple procaspase-8 molecules via the adaptor molecule FADD (Fas-associated death domain protein), resulting in proximity-induced caspase-8 activation **(29)**. Fas ligand is abundantly present in adult cardiomyocytes, and its expression increases in response to pathologic stimuli **(30)**. The overexpression of Fas ligand results in accentuated apoptosis *in vitro*, while Lpr mice, which lack Fas, display less apoptosis and a reduced infarct size in ischemia-reperfusion (I/R) studies **(31)**. Circulating Fas ligand levels are elevated in human heart failure, which may reflect activation of the Fas-Fas ligand system **(32)**.

As apoptosis is still present in mice defective in both caspase-8 (crucial for the death receptor pathway) and caspase-9 (crucial for the mitochondrial pathway), additional genetic programs, apart from the mitochondrial and death receptor pathways, must exist **(33)**. A candidate for this is a pathway initiated by caspase-12. Caspase-12 is localized to the endoplasmic reticulum (ER) and is specifically activated by ER stress **(34)**. The ER is an organelle that ensures correct protein folding; however, under various conditions, such as glucose deprivation or disturbance of intracellular calcium homeostasis, unfolded proteins accumulate in the ER lumen and provoke ER stress **(35)**.

Regulation of Apoptosis

A key regulatory component of the cell death process is the Bcl-2 family of proteins **(36, 37)**. The Bcl-2 family consists of death antagonists (Bcl-2, Bcl-xL) and death agonists (Bax, Bak), which function primarily to protect or disrupt the integrity of the mitochondrial membrane and control the release of (pro)apoptotic intermembrane proteins (Fig. 22.2) **(19)**. BH3-only proteins, another class of death effectors, serve as ligands to activate proapoptotic Bcl-2 family members or inactivate antiapoptotic Bcl-2 members. Gene-targeting studies of the Bcl-2 family have shown that some of these molecules have unique functions in cell death. The overexpression of Bcl-2 is cytoprotective, attenuating apoptosis induced by p53, a transcription factor activated in response to DNA damage **(38)**. In addition, Bcl-2 overexpression in cardiomyocytes also prevents the loss of the electropotential of the mitochondrial membrane as well as the release of mitochondrial intermembrane proteins. Other studies have shown that Bcl-2 overexpression protects against hypoxia-/reoxygenation-induced apoptosis in cardiomyocytes *in vitro* and cardiac ischemia-reperfusion injury *in vivo* **(39, 40)**.

Another nearly ubiquitous BH3-only protein is called *Nix*, a homologue of the E1B 19 K/Bcl-2 binding and the proapoptotic *Bcl2 and 19-kDa interacting protein-3*(*Bnip3*), first described in 1999 by Arnold Greenberg and his group **(41)** and later rediscovered to be specifically upregulated by pressure-overload and Gq-mediated signals in the heart muscle **(42)**. *Nix* gene function in the heart was extensively studied using gene (in)activating strategies in mice to provide valuable new insights into the fundamentals among dying myocytes, hypertrophy signals, and heart failure. First, modest transgenic *Nix*-overexpressing mice with mild cardiac abnormalities **(43)** were crossbred with Gq transgenic mice, which also exhibit a fairly modest cardiomyopathic phenotype. The combination proved lethal, with high rates of dying myocytes, demonstrating the synergy between specific cardiac growth and death pathways in a downward spiral to heart failure. Next, a novel mouse model was created encompassing α-myosin heavy-chain-directed Gq transgenic mice with systemic *Nix* ablation, resulting in reduced Gq-associated peripartum cardiomyopathy and decreased myocardial apoptosis. Finally, the *Nix* gene was specifically deleted from cardiac myocytes by crossbreeding mice harboring a floxed *Nix* allele with a Cre deleter strain driven by the Nkx2.5 promoter. The resultant cardiac-specific *Nix*-deficient mice proved to have a remarkable level of protection against a loss of ejection performance, ventricular remodeling, and diminished myocyte dropout in response to surgical pressure overloading (an animal model for chronic hypertension), without consequences on myocyte hypertrophic growth.

Taken together, the combined studies mechanistically link the induction of *Nix* gene expression, cardiomyocyte dropout, ventricular remodeling, and functional deterioration at the transition point from compensated pressure-overload hypertrophy to decompensated heart failure. The related protein *Bnip3* was also studied in the context of pathological hypertrophy and revealed that this related BH3-only protein responds more selectively to ischemic signals rather than the Gq signals that induce *Nix* expression. Indeed, a *Bnip3*-null allele affords considerable (but not complete) protection against postinfarct remodeling **(44**, **45)**. It will become of interest to determine whether *Nix* and *Bnip3* physically or functionally synergize to propel mitochondrial apoptosis.

Another set of proteins, the IAPs (inhibitor-of-apoptosis proteins), has been shown to alter the functions of the executioner caspases. The inhibitors-of-apoptosis proteins (IAPs) include XIAP, survivin, HIAP1, HIAP2, NAIP, Livin, Ts-IAP, and Apollon. XIAP (X chromosome-linked IAP) is the best-characterized and most potent caspase inhibitor, as it has been demonstrated to target initiator caspase-9 and effector caspase-3 and caspase-7 **(46)**. In failing human hearts, IAPs, such as XIAP, are downregulated, theoretically rendering the cardiomyocytes more sensitive to apoptosis **(47)**.

Smac/DIABLO, which is released from the mitochondria along with cytochrome c during apoptosis, has been shown to be involved in the regulation of IAPs **(48**, **49)**. The binding of Smac/DIABLO to IAPs results in the disruption of the binding of IAPs to caspases and relieves the inhibition of apoptosis **(50)**. Recent studies demonstrate that Smac/DIABLO translocates from the

mitochondria to the cytosol during cardiac ischemia-reperfusion **(51)**, suggesting that Smac/DIABLO and its prodeath function are operative in the cardiac muscle.

Omi/HtrA2 has been identified as another novel protein to have similar characteristics in antagonizing IAP function **(52, 53)**. Studies have now shown that mice treated with ucf-101, a compound that shows high selectivity against the serine protease activity of Omi/HtrA2, resulted in marked protection against ischemia-reperfusion injury of the murine heart **(53, 54)**. Whether Omi/HtrA2 antagonism is similarly efficacious in the setting of heart failure remains to be determined. Nevertheless, this novel pharmacological agent may harbor therapeutic opportunities for testing in animal models of severe heart disease.

Interestingly, XIAP-interacting protein-1 (XAF1) is abundantly expressed in the heart and seems able to interact with and inhibit specifically one IAP, XIAP **(55)**. It has been indicated that the actual inhibition occurs in the nucleus, since activated caspase-9 can disrupt the nuclear membrane enabling XIAP/caspase-3 complex translocation into the nucleus, where XAF1 would relieve the XIAP inhibition of the caspases **(56)**. The abundant presence of XAF1 in the heart indicates a possible important role for XAF1 in regulating cardiac apoptosis, providing a rationale why this organ is relatively susceptible to cell death **(55)**.

ARC (apoptosis regulator with caspase recruitment domain), an apoptosis repressor with a caspase recruitment domain (CARD) that has been shown to protect against oxidative stress-induced apoptosis, was initially found to be highly expressed in terminally differentiated tissues like heart and skeletal muscle. ARC interacts with caspases-2 and -8, but not with caspase-1, -3, or -9. The overexpression of ARC inhibits apoptosis induced by Fas, TNFα, and caspase-8, but not by caspase-9, and inhibited all apoptotic features, including cytochrome c release **(57, 58)**. Furthermore, hypoxia in cell culture induces the loss of endogenous ARC in the cytosol and was associated with translocation of ARC from the cytosol to intracellular membranes, release of cytochrome c from the mitochondria, activation of caspase-3, and cleavage of PARP **(58)**. Because caspase inhibitors fail to inhibit cytochrome c release, these studies predict that ARC acts upstream of caspase activation in this model of apoptosis, perhaps at the mitochondrial level.

Accumulating evidence suggests that oxidative stress triggers cardiac cell death and the pathogenesis of a number of cardiovascular diseases, such as ischemic heart disease, heart failure, and atherosclerosis **(19, 59, 60)**. The production of reactive oxygen species (ROS), such as superoxide anions ($\cdot O_2$-) and hydrogen peroxide (H_2O_2), from mitochondrial sources is enhanced under various pathologic stimuli **(60, 61)**. To counteract oxidative stress, mammalian cells are equipped with elaborate antioxidant mechanisms, including superoxide dismutases (SODs) in mitochondria (MnSOD), the cytosol (CuZn-SOD), plasma membrane, and extracellular spaces (Extracellular SOD), catalase and glutathione peroxidases and other nonenzymatic antioxidants. Antioxidants exert their cellular protective effects by directly scavenging ROS or their precursors and by attenuating the catalysis of ROS generation via binding to metal ions.

AIF is a highly conserved flavoprotein with pyridine nucleotide-disulphide oxidoreductase and DNA binding domains. The AIF precursor is synthesized in the cytosol and imported into mitochondria, where AIF localizes in the mitochondrial intermembrane space. Changes in mitochondrial permeability, secondary to loss of the mitochondrial membrane potential ($\Delta\Psi_m$), induce translocation of AIF into the cytosol and nucleus, where it may participate in chromatinolysis **(62, 63)**. We and others have recently unveiled an additional role for apoptosis-inducing factor (AIF) or programmed cell death 8 (Pdcd8) as a neuronal and cardiac antioxidant, using the harlequin (*Hq*) mutant mouse, harboring a proviral insertion in the first intron of the *aif* gene, resulting in more than a 90% decrease in AIF expression in the brain and heart **(64, 65)**. *Hq* mice have a decreased ability to clear oxygen radicals and display increased sensitivity to necrotic and apoptotic cell death, resulting in progressive degeneration of cerebellar and retinal neurons **(64)**], increased sensitivity of the myocardium to ischemic insults, and accelerated progression to heart failure upon pressure overload **(65)**.

The variety of phenotypes observed in *Hq* mice resembles those in experimental models with premature-aging syndromes: increased oxidative stress, accelerated progression to heart failure in response to stress, neurodegenerative changes, kyphosis, age-related skin changes, growth retardation, and short life span **(66)**. In fact, intrinsic oxidative stress was demonstrated to increase with age in the heart, due to the age-related impairment of transcriptional responses to oxidant stress **(67)**, the diminished expression of antioxidant defense enzymes such as catalase, glutathione peroxidase, and MnSOD **(68)**, the increased myocardial expression of NADPH oxidases **(69)**, and age-dependent cardiac mitochondrial dysfunction **(70)**. Moreover, endogenous cardiac AIF protein levels were found to decrease upon pressure overload and in a genetic model of severe heart failure **(65)**, mimicking the decrease of other antioxidant enzymes in heart failure **(71)**. These age-related effects may contribute to the higher incidence of cardiovascular disorders, such as heart failure, in the elderly. As such, the *Hq* mouse may serve as a relevant model to study premature-aging-related cardiovascular disorders due to a decreased antioxidant ability **(65)**.

Recently, we were able to establish the causality of antioxidant defects in the *Hq* mouse model by chronic treatment of pressure-overloaded *Hq* mice with EUK-8, an antioxidant with powerful superoxide dismutase (SOD), catalase, and oxyradical scavenging properties **(72)**. By administering pressure-overloaded wild-type counterparts with the same compound, the preclinical efficacy of this synthetic antioxidant to protect the myocardium against the noxious effects of pressure overload was demonstrated. EUK-8 ameliorated survival in *Hq* and wild-type mice subjected to pressure overload. Further, EUK-8 improved left ventricular end-systolic dimensions and fractional shortening, prevented myocardial oxidant stress, attenuated necrotic and apoptotic cell death, and attenuated cardiac hypertrophy and fibrosis in both mutant and wild-type mice. The protection against pressure-overload-induced heart failure in *Hq* mice by EUK-8 substantiates the notion that AIF functions as an important mitochondrial

antioxidant in the heart. Further, since antioxidant treatment protected both the oxidative stress-prone *Hq* mouse model and wild-type mice against pressure-overload-induced maladaptive left ventricular remodeling and cardiac decompensation, it may be useful as a novel therapeutic tool in the treatment of human heart failure.

Therapeutic Implications

Many of the illnesses in developed countries can be classified as disorders in which defective regulation of apoptosis contributes to disease pathogenesis or progression involves either cell accumulation, in which cell eradication or cell turnover is impaired (e.g., cancer), or cell loss, in which the cell suicide program is inappropriately triggered (e.g., heart failure, neurodegenerative diseases, inflammation, stroke, type I diabetes, CNS injury). The common pathological finding in clinical heart failure is a loss of cardiac myocytes and functional myocardium. The loss of cardiac myocytes also produces secondary structural changes in the ventricles that result in regional and global hemodynamic overloading that contributes to cardiomyocyte apoptosis. Clearly, heart failure would be classified as one of the clinical disorders where apoptosis should be actively antagonized to limit cell loss. Recent approaches also consider stem cell or progenitor cell therapies, which are aimed at altogether replacing functional myocytes rather than inhibiting the cell death of preexisting muscle cells. The therapeutic options discussed in this section exclusively deal with the salvage of existing cardiac muscle. Table 22.2 presents a number of apoptosis-based therapeutic approaches being tested in clinical trials for a variety of disorders.

Table 22.2 Examples of (pre)clinical status of apoptosis-based therapies

Drug	Company	Target	Type	Effect	Disease	Status
IDN6556	IDUN Pharmaceuticals	Caspases	Small molecule	Anti	Acute liver injury	Phase II
VX-799	Vertex Pharmaceuticals	Caspase-1	Small molecule	Anti	Arthritis	Preclinical
CGP3466B	Novartis	GAPDH	Small molecule	Anti	Parkinson's disease	Phase II
Genasense	Genta	Bcl-2	Antisense technology	Pro	Cancer	Phase II
Vitaxin	Applied Molecular Evolution	$\alpha v\beta 3$-integrin	Humanized antibody	Pro	Angiogenesis	Phase III
INGN201	Introgen Pharmaceuticals	P53	Adenovirus	Pro	Cancer	Phase III
E1A gene therapy	Targeted genetics	E1A	Adenovirus	Pro	Cancer	Phase III
MS-275	National Cancer Institute	HDAC	Small molecule	Anti	Cancer	Phase I

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; E1A, adenovirus E1A protein; HDAC, histone deacetylase.

Here we discuss a number of additional apoptosis targets that may be considered for the development of antiapoptosis clinical strategies for heart failure.

Many studies have posited that activation of "compensatory" neurohormonal pathways under conditions of hemodynamic stress and myocardial injury plays an important role in the natural progression to cardiomyopathy. This indicates that neurohormonal stimulation is closely intertwined with cardiac muscle apoptosis, and a number of its antagonists can modify apoptosis. Interestingly, the neurohormonal axis (renin-angiotensin system, or RAS, and sympathetic system) is (over)active in the failing heart, and neurohormonal antagonism is an important therapeutic tool in protecting the failing myocardium. For example, angiotensin II has potent apoptotic effects in cardiomyocytes that can be blocked with ATII blockers **(73)**. Similarly, norepinephrine causes apoptosis, while apoptosis can be attenuated with beta blockers **(74)**. Indeed, the chronic use of both ACE inhibitors and beta blockers can prevent apoptosis in animal models **(75)**. Taken together, these arguments suggest that clinical strategies aimed at protecting the failing heart from the noxious effects of RAS and catecholamine stimulation already indirectly prevent cardiac muscle apoptosis. In fact, it is likely that the potent antiapoptotic effects of carvedilol may have something to do with its unparalleled efficacy in treating heart failure.

Next in line as a logical target to modulate cardiomyocyte apoptosis more directly in the failing heart would be the executioner caspases. Clinical trials using small molecule caspase inhibitors have been shown to prevent the progression of hepatitis to cirrhosis and other conditions that may destroy the liver. A broad-spectrum and irreversible caspase inhibitor, ZVADfmk, has been shown to reduce apoptosis and infarct size in an ischemia-reperfusion rat model **(76)**, coupled with a 72% reduction in the number of apoptotic cells in the treatment group. Indeed, much evidence points to the beneficial aspects of caspase inhibitors in acute ischemia-reperfusion-induced cardiac injury.

Caspase inhibition may also be of benefit in the slower LV remodeling processes that provoke heart failure. Studying cardiac remodeling and LV function using a pig model with transient coronary occlusion, Yarbrough and colleagues demonstrated that delivery of a pan-caspase inhibitor significantly altered ventricular apoptosis and remodeling in these animals **(77)**. Similar benefits were reported from anticaspase therapy in rats with artery occlusion, demonstrating reduced cardiomyocyte apoptosis in the remote myocardium and attenuated ventricular remodeling **(78)**. In addition, Kitsis's group recently demonstrated that antiapoptotic therapy is also beneficial in transgenic mice with nonischemic cardiomyopathy **(79)**. Collectively, these recent preclinical studies show that caspase inhibitors can have a remarkably positive effect on infarct size, cardiac muscle death in the remote myocardium, and LV remodeling in heart failure animal models.

Besides caspases, targeting other mediators in the apoptotic pathway also holds promise as future therapeutic devices in heart failure. Aurintricarboxylic acid (ATA) is an inhibitor that targets endonucleases, which are situated relatively downstream in the apoptotic pathways and provoke DNA strand

breaks. ATA was recently shown to significantly reduce the number of apoptotic cells in the perinecrotic myocardium of an ischemia-reperfusion dog model. ATA treatment also resulted in a significant increase in Bcl-2, while Bax and activated caspase-3 were significantly reduced, eventually leading to a substantial enhancement in segmental shortening and segmental work in the area-at-risk myocardium, and improved endothelial function and myocardial perfusion **(80)**.

Insulin-like growth factor-1 (IGF-1) is an important survival growth factor in the myocardium. Numerous studies have shown that several reactive pathways implicated in hypertrophic growth modulation of the cardiac muscle also impinge upon specific targets in the intrinsic and extrinsic apoptotic pathways **(81**, **82)**. Animal studies have reported potent antiapoptotic actions of IGF-1 in the heart and improvement of cardiac function in animal models of cardiomyopathy **(81)**. IGF-1 inhibits cardiomyocyte apoptosis mainly by attenuating Bax induction and caspase-3 activation **(83**, **84)**.

Reactive oxygen species (ROS) are formed at an accelerated rate in the failing myocardium, indicating that oxidative stress is common in heart disease. ROS production in the failing myocardium has been shown to trigger the intrinsic apoptotic pathways via multiple mechanisms, including an increase in p53, Bax and Bad translocation to the mitochondria, release of cytochrome c, and caspase activation **(85)**. Due to both the age-related impairment of transcriptional responses to oxidant stress **(67)** and the diminished expression of antioxidant defense enzymes such as glutathione peroxidase and MnSOD, intrinsic oxidative stress was demonstrated to increase with age in the heart **(68)**. Studies using mice deficient in SOD2, a mitochondrial antioxidant enzyme, have shown that these mice spontaneously develop dilated cardiomyopathy, which can be largely overcome by the administration of catalase-SOD-mimetics **(86)**. Similarly, mice with cardiac-specific overexpression of a dominant-negative form of thioredoxin, a cytosolic antioxidant, displayed reduced defense against oxidative stress and accelerated progression to heart failure in response to pressure overload **(87)**. In a rat infarct model, probucol, an antioxidant, prevented internucleosomal DNA fragmentation and upregulation of p53, Bax, and caspase-3 protein expression **(88)**. Interestingly, this inhibition also occurred in the remote nonischemic myocardium, indicating a possible role in the attenuation of LV ventricular remodeling during heart failure development. Given that a large number of commonly used, safe compounds have potent antioxidant as well as antiapoptotic effects, this form of therapy may likely find early introduction in the repertoire of heart failure drugs.

Although inhibiting apoptosis holds a beneficial effect on the development of cardiovascular disease, additional therapeutic targets could enhance this effect, like the muscle-enriched protein ARC and the inhibitor-of-apoptosis protein family (IAPs) such as XIAP and survivin. However, understanding the structure and mode of function of this relatively new group of proteins in more detail is a critical step in developing therapeutic agents.

Despite the great advances that recent research has made in the understanding of the complex processes of the apoptotic signaling pathways in the

myocardium, many challenges must be overcome to counteract cardiomyocyte apoptosis in heart failure. For example, the cell death pathway contains very few conventional drug targets, such as enzymes and small ligand receptors, and the toxicity of the potential pharmacological compounds is still a matter of concern. Therefore, combining strategies such as stem cell and antisense therapy should be considered besides the classical approaches.

Selective modulation of apoptosis in the heart is still a practical limitation. Apart from this practical limitation, theoretical restrictions also exist; e.g., counteracting apoptosis will be beneficial in the treatment of diseases such as heart failure and neurodegenerative disorders; activating apoptosis is, however, essential in treating disorders where there is insufficient cell death, such as cancer. The systemic inhibition of apoptosis may therefore result in increased tumorigenic potential in extra-cardiac and neuronal tissues in patients already affected by serious afflictions such as heart failure.

Although myocardial infarction is typically an acute ischemic event resulting in widespread cardiomyocyte death in the myocardial area, the loss of myocytes exacerbates the imbalance between demand on the heart and its ability to respond, resulting in structural changes and functional deterioration. Because the vast majority of preclinical studies where antiapoptotic strategies were evaluated applied the drugs early in the course of injury, the need for further information about the most appropriate timing of antiapoptotic therapy is important. Additional, noninvasive imaging approaches **(89**, **90)** with high enough resolution to detect apoptotic alterations at the cellular level should be further developed before embarking on clinical trials.

Taken together, a more thorough understanding of the molecular pathways that initiate and execute apoptosis is imperative in designing successful antiapoptotic therapies in humans. However, encouraging efforts toward developing pharmacological inhibitors are currently in progress. Therefore, apoptosis-based therapeutics that modulate the survival of the cardiac muscle cell in human heart failure is definitely within reach.

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Chapter 23
Apoptosis in Lung Injury and Disease

Stefan W. Ryter, Hong Pyo Kim, and Augustine M. K. Choi

Abstract Pulmonary cell death may contribute significantly to acute and chronic lung injuries caused by various adverse environmental agents. Pulmonary cells may die by necrosis, apoptosis, and other forms of regulated cell death. Apoptosis exerts a homeostatic function in lung defense and development, through the removal of dysfunctional cells and by regulating cellular proliferation. Lung cell apoptosis can occur as a response to oxidative stress, mechanical ventilation, ischemia/reperfusion, cigarette smoke exposure, and other forms of acute and chronic lung injuries. The role of apoptosis in the pathogenesis of chronic lung disease remains controversial. Apoptosis may act as an adaptive process, by removing dysfunctional cells, limiting inflammation in damaged tissue, and preventing cell proliferation. On the other hand, excessive apoptosis may contribute to the depletion of critical cell populations, resulting in loss of function, as in emphysema, or in undesirable cell proliferation, as in fibrosis and pulmonary hypertension. An understanding of the cell-type-specific regulation and function of apoptosis in the lung may facilitate the development of therapeutic strategies for the treatment of lung pathologies. This chapter reviews the current evidence for the regulation and function of apoptosis in specific lung diseases, with an emphasis on chronic obstructive lung disease and acute respiratory distress syndrome.

Keywords Apoptosis · Cigarette smoke · COPD · Acute lung injury · Pulmonary medicine

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Introduction

Apoptosis, or programmed cell death, provides essential homeostatic functions in regulating the normal growth and development of organs and in tissue responses to exogenous injurious stimuli, such as exposure to toxins or adverse environments. The occurrence of apoptosis in the context of disease progression can signify an adaptive process through the removal of dysfunctional cells but may also contribute to disease pathogenesis. Apoptosis is defined as a regulated form of cell death that requires the action of proteases (e.g., caspases) and nucleases within an intact plasma membrane **(1)**. The major morphological and biochemical features of apoptosis include DNA fragmentation, cell surface blebbing, cell shrinkage, mitochondrial dysfunction, and cellular decomposition into membrane-bound apoptotic bodies destined for phagocytosis **(1, 2)**.

Cells undergo apoptosis by two distinct pathways: a receptor-dependent or extrinsic apoptotic pathway and an intrinsic apoptosis pathway involving mitochondrial dysfunction **(3–6)** (Fig. 23.1). These pathways are not mutually exclusive and can involve significant cross-regulation. In response to diverse stimuli, proapoptotic Bcl-2 family proteins such as Bax initiate the intrinsic apoptotic pathway by forming channels in the outer mitochondrial membrane, thereby facilitating the release of cytochrome c (Cyt-*c*) and other proapoptotic mediators from the mitochondrial intermembrane space **(3–5)**. Cytosolic Cyt-*c* forms an apoptosome complex with Apaf-1, which activates caspase-9 and, in turn, its downstream caspase-3, resulting in the activation of the apoptotic program. In contrast, the extrinsic apoptotic pathway initiates when a death ligand, such as FasL, interacts with its cell surface receptor (i.e., Fas), forming a death-inducing signal complex (DISC) **(6)**. The activation of Fas triggers its oligomerization and the rapid recruitment of FADD (Fas-associated death domain protein) and caspase-8 to the cytoplasmic death domain of Fas, which activates caspase-8. Active caspase-8 subsequently cleaves Bid into truncated Bid (tBid), which translocates to the mitochondrial membrane, where it triggers Cyt-*c* release and subsequent caspase-9 activation **(6)**.

Apoptosis is distinct from necrosis, a type of cell death resulting from acute, accidental, or nonphysiological injury. Necrosis is characterized by gross membrane damage and leakage of cytosolic components into the extracellular space, which may lead to local inflammation and damage to the surrounding tissues **(2)**. Oncosis is a variant of nonregulated cell death associated with the swelling of intracellular organelles such as nuclei, increased vacuolation, and mitochondrial degradation **(2)**. These morphological definitions are not always distinct, as mixed phenotypes of cell death such as aponecrosis have been reported **(7, 8)**. Other forms of regulated cell death include autophagic or lysosome-dependent cell death (Type II programmed cell death) **(9–11)**.

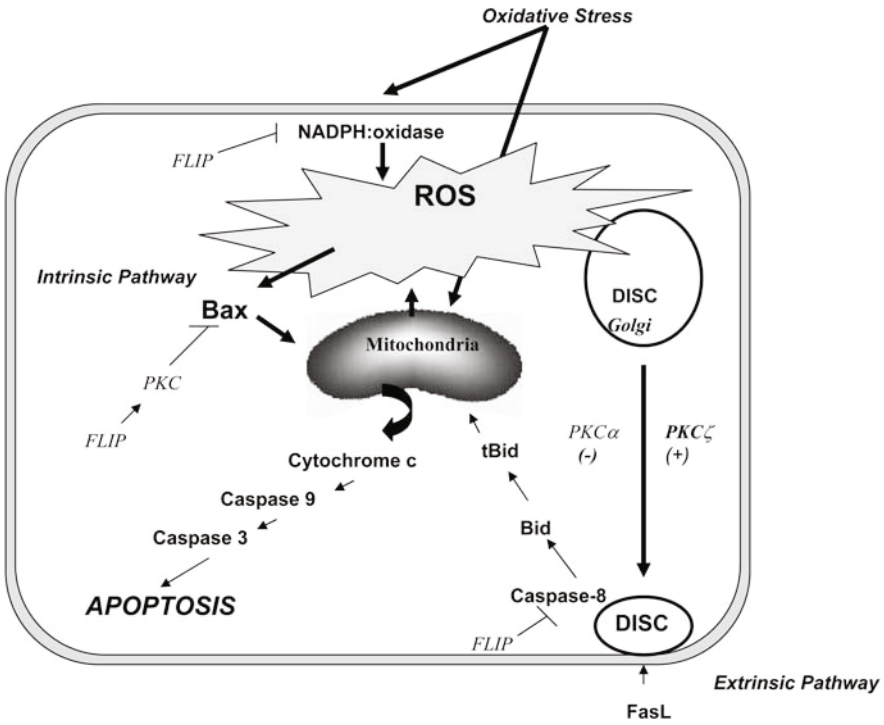

Fig. 23.1 Pathways to apoptosis in lung cells. The schematic diagram displays the features of the extrinsic (receptor-dependent) and intrinsic (mitochondrial) apoptosis pathways that may occur in lung cells exposed to various acute injurious stimuli. In the intrinsic pathway, environmental stress may cause direct mitochondrial damage or promote signaling pathways dependent on reactive oxygen species (ROS) generation, resulting in the activation of Bax. Bax translocates to the mitochondrial outer membrane, where it oligomerizes or forms complexes with other proapoptotic Bcl-2-related proteins such as Bid or Bad. Bax oligomers form pores in the outer mitochondrial membrane, which facilitate the release of proapoptotic molecules such as cytochrome-c. Cytochrome c forms a complex with Apaf-1 and caspase-9, leading to caspase-9 activation. In extrinsic apoptosis, a death-inducing ligand such as Fas ligand (FasL) initiates the death program upon interacting with its corresponding receptor (i.e., Fas). These interactions lead to the recruitment and activation of caspase-8 in a death-inducing signal complex. Caspase-8 activation can lead to caspase-3 activation or to the activation of Bid. Bid assists in the activation and mitochondrial translocation of Bax. Bid activation also results in the release of cytochrome c from the mitochondria. In endothelial cells, we have shown that the DISC preassembly occurs in the Golgi apparatus, followed by its translocation to the plasma membrane, where caspase-8 is activated **(43, 61)**. In the hyperoxia model, the antiapoptotic molecule FLIP, in addition to direct inhibition of caspase-8 activation, can modulate the apoptotic program of endothelial cells by influencing Bax stability, reducing ROS generation, and inhibiting DISC trafficking **(43)**. In the cigarette smoke model, we have shown that specific isoforms of protein kinase c (PKCα, PKCζ) can modulate apoptosis by differentially regulating DISC trafficking **(97)**

Recent studies from this laboratory and others have examined the regulation of apoptosis and other cell death pathways in the lung in response to stress, injury, and pathophysiological conditions **(12)**. The lung is a heterogeneous organ consisting of over 40 distinct cell types, including the epithelial cells of the airways, bronchii, and alveoli, interstitial fibroblasts of the lung parenchyma, endothelial cells of the pulmonary vasculature, and smooth muscle cells that provide the contractility of the airway and pulmonary vasculature. Furthermore, the lung contains resident inflammatory cells such as alveolar macrophages and other specialized cells involved in immune function or secretion **(13)**. Apoptosis may occur in any of these lung cell types, in response to discrete stimuli or in the context of pathological states. Increased apoptosis *in situ* may reflect the increased apoptosis rate of individual cell populations or may reflect the impaired clearance of apoptotic cells (efferocytosis) **(14)**. Thus, the examination of the molecular regulation and function of apoptosis in a cell-type-specific fashion as well as in the whole tissue will be necessary to understand the role of apoptosis in this complex organ.

This chapter summarizes current knowledge with respect to the initiation and propagation of apoptosis in various types of lung cell injury and lung disease. Recent works examining the regulation of lung cell apoptosis in response to oxidative stress, mechanical ventilation, ischemia-reperfusion (I/R), cigarette smoke exposure, and other forms of acute and chronic lung injuries are described. Furthermore, evidence for the regulation and function of apoptosis in specific lung pathologies is also discussed, with an emphasis on chronic obstructive lung disease (COPD) and acute respiratory distress syndrome (ARDS).

Apoptosis in Acute Lung Injury

Apoptosis has been extensively studied in a number of *in vivo* models of acute lung injury (ALI), and in their cell-type-specific *in vitro* correlates, as discussed in the following sections. ALI ranks among the major causes of morbidity and mortality in intensive care units despite advances in therapeutic modalities. ALI can cause or contribute to the development of ARDS, a syndrome characterized by noncardiogenic pulmonary edema, and pulmonary and systemic inflammation resulting in respiratory failure **(15, 16)**. A number of clinical interventions can cause or promote ALI, including mechanical ventilation at high tidal volumes, bacterial infections, or the use of high inspired pO_2 to maintain oxygen delivery to peripheral tissues. Animal models of ALI in rodents include exposure to high physiological oxygen tension (pO_2), mechanical ventilation, and/or bacterial lipopolysaccharide (LPS). Epithelial cell death by apoptosis or necrosis is considered a primary event in the development of ALI (Fig. 23.2).

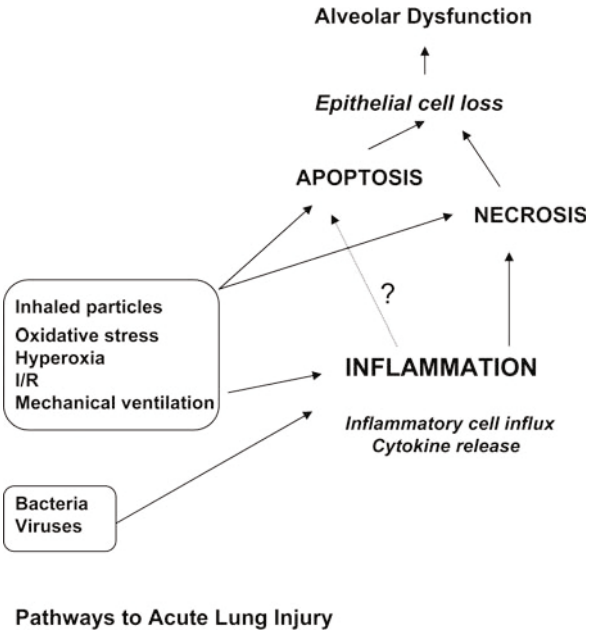

Fig. 23.2 Pathways to acute lung injury. Injurious stimuli such as mechanical ventilation, particle inhalation, or elevated oxygen tension can cause epithelial cell death through apoptosis or necrosis depending on the stimuli and the degree of exposure. Bacterial or viral infections as well as injurious stimuli can activate innate immune responses, leading to inflammation. Necrosis can occur as a secondary response around inflamed tissue. Epithelial cell loss causes alveolar cell dysfunction and increased microvascular permeability, leading to pulmonary edema

Oxidative Lung Injury

Rodents subjected to hyperoxia (>95% pO_2) sustain considerable lung injury associated with inflammatory responses **(17)** and have increased production of intracellular reactive oxygen species (ROS) **(18)**. Mice exposed to high pO_2 show increased indicators of lung injury by 64–72 hours, and death within 90–100 hours of continuous exposure **(19)**. Hyperoxia induces endothelial swelling, interstitial edema, and inflammatory cell influx into the airways, resulting in diffuse alveolar damage. After 72 hours of exposure, type I pulmonary epithelial cells die by necrosis **(20–21)**. The epithelial cell death from prolonged exposure leads to loss of alveolar integrity, airway fluid accumulation, and death. During hyperoxic lung injury in rodents, lung cells can exhibit features of necrosis and/or apoptosis *in situ,* including chromatin condensation, DNA fragmentation, changes in the expression of Bcl-2 family genes, and increases in terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL)-positive cells **(8, 22–26)**. These observations suggest that hyperoxia-induced lung injury involves characteristics of both apoptosis and necrosis, which may occur in distinct lung cell populations or as competing processes in the same cell type. However, it remains unclear how these forms of cell death are distributed among the various lung cell types *in vivo* **(27)**.

A number of apoptosis-related molecules have been implicated in hyperoxic lung injury, including death receptors such as Fas and TNF-R1 **(22, 28)**, Bcl-2 family members (e.g., Bid, Bcl-xL), cell cycle regulatory proteins (e.g., p53 and $p21^{Waf1/Cip1}$) **(22–24, 29–31)**, and mitogen-activated protein kinases (MAPK) **(21, 32)**. Increases in Bcl-xL and Bid protein expression, but not Bax protein expression, have been shown to occur in response to hyperoxia in the mouse lung **(8, 22–24)**.

In vivo studies using genetically altered mouse strains have provided insight into the roles of these and other apoptosis-related proteins in hyperoxia-induced lung injury.

We have shown that mice genetically deleted for c-Jun NH_2 terminal kinase ($jnk^{-/-}$) displayed an increased susceptibility to hyperoxia, suggesting a role for JNK in hyperoxia adaptation *in vivo* **(32)**. We have also recently demonstrated that caveolin-1-deleted mice ($cav\text{-}1^{-/-}$) were resistant to hyperoxic lung injury, which was associated with an increased expression and activation of p38 MAPK in the mouse lung, as well as an upregulation of the antiapoptotic stress protein heme oxygenase-1 (HO-1) **(33)**. Caveolin-1, the principal structural protein of plasma membrane caveolae, regulates many signal transduction processes originating at the membrane, though its specific role in regulating the apoptotic program remains incompletely understood.

Mice genetically deleted for $p21^{Waf1/Cip1}$($p21^{-/-}$) were more sensitive to hyperoxia than wild-type mice, whereas $p53^{-/-}$ mice did not display any modulation of oxygen sensitivity **(22, 24, 31)**. Null mice corresponding to major extrinsic apoptotic pathway proteins such as $fas^{-/-}$ **(22)**, $tnfr1/2^{-/-}$ **(34)**, and $fasl^{-/-}$ **(8)** did not display resistance to hyperoxia relative to the corresponding wild-type mice. In contrast, mice deleted for Bid ($bid^{-/-}$), a downstream effector of the extrinsic apoptosis pathway, were significantly more resistant to hyperoxia than corresponding wild-type mice **(8)**. The activated form of Bid (tBid) increased in the lungs of wild-type mice but not in that of $fas^{-/-}$ or $fasl^{-/-}$ subjected to hyperoxia, whereas an equivalent Bcl-xL expression and caspase-9 activation were observed in all strains **(8)**. The overexpression of FLIP, an endogenous inhibitor of caspase-8, in mouse lungs also resulted in hyperoxia resistance, whereas the overexpression of Bcl-xL did not confer resistance. These results suggested that the caspase-8/Bid pathway represents a major pathway leading to pulmonary cell death during the hyperoxia response *in vivo*. The apparent lack of resistance in $fas^{-/-}$ or $fasl^{-/-}$mice may be explained by compensatory increases in basal and hyperoxia-inducible Bax expression, relative to wild-type mice that did not express Bax protein following hyperoxia. Thus, an alternate pathway to death may occur in $fas^{-/-}$ or $fasl^{-/-}$mice, possibly mediated by a compensatory activation of the Bax-induced pathway or by alternate mechanisms of Bid activation **(8)**.

Pulmonary Cell Death in Hyperoxia

The mechanisms underlying hyperoxic cell death *in vitro* vary in a cell-type-specific fashion and have been reported to involve necrosis, apoptosis, or mixed cell death phenotypes (Table 23.1). Hyperoxia-induced cytotoxicity has been studied most extensively in cultured epithelial cells, which represent the principal cellular targets of oxygen toxicity *in vivo*, although accumulating research has also examined cell death in other pulmonary cell types, such as fibroblasts, endothelial cells, and alveolar macrophages.

Epithelial Cell Responses

Hyperoxia causes cell death in human A549 lung epithelial cells primarily by necrosis, with no indication of apoptotic cell death by fluorescence DNA labeling, *in situ* DNA end-labeling, or ultrastructural analysis **(35)**. Necrotic cell death after hyperoxic exposure was also observed in Type I epithelial and murine lung bronchial cells **(23, 31)**. In contrast, hyperoxia-induced cell death with histological features of oncosis was observed in murine Type II alveolar epithelial cells **(36, 37)**.

Studies from our laboratory have also described necrotic cell death in A549 cells subjected to hyperoxia **(8)**. Despite the final phenotypic outcome of cellular necrosis, however, early events in hyperoxia-induced epithelial cell death involved the activation of apoptogenic factors in both the extrinsic and intrinsic apoptotic pathways. Hyperoxia treatment of A549 cells increased DISC formation, caspase-8 activation, and the expression, activation, and mitochondrial translocation of Bid and Bax, culminating in the release of mitochondrial Cyt-*c* and the cleavage of caspase-9. Compensatory increases in Bcl-xL but not Bcl-2 expression were also observed. While the overexpression of Bcl-xL had no effect, the inhibition of caspase-8 by the overexpression of FLIP protected A549 epithelial cells from hyperoxia-induced cell death, by

Table 23.1 Cell-type-specific responses to hyperoxia in the lung

Cells	Cell death phenotypes	Possible contributors	Ref.
Epithelial cells	*Necrotic* (aponecrosis); type I alveolar, bronchial, A549 *Oncotic;* type II alveolar	DISC formation insensitive to Bcl-xL p21 MAPK (JNK)	**(7, 8, 23, 31, 35–37)**
Macrophages	*Apoptotic*	MAPK (ERK)	**(38, 39)**
Fibroblasts	*Apoptotic*	Bax, Bak, Bid	**(8, 39)**
Endothelial cells	*Apoptotic*	ROS (NADPH: oxidase)-driven intrinsic and extrinsic pathways	**(40, 43)**

reducing the levels of tBid and by inhibiting Bax activation **(8)**. These results suggest that hyperoxic cell death in cultured human A549 epithelial cells involves a cellular death pathway culminating in necrosis, which shares features of both the extrinsic and intrinsic apoptosis and is insensitive to Bcl-xL inhibition **(8)**. Such mixed-death phenotypes have been described in other models and termed "aponecrosis," although their functional significance as a distinct form of cell death remains unclear **(7)**.

Alveolar Macrophages

Despite the predominantly necrotic phenotypes observed in several types of lung epithelial cells exposed to hyperoxia, apoptosis has also been observed in response to hyperoxic stress in a cell-type-specific fashion. Exposure of cultured RAW 267.4 murine macrophages to hyperoxia increased apoptosis, as observed in DNA-laddering, TUNEL, and nucleosomal assays. The induction of apoptosis in these cells by hyperoxia was associated with the selective activation of the ERK MAPK pathway, as evidenced by the time-dependent phosphorylation of ERK1/2, but not of the JNK or p38 MAPK pathways. Chemical or genetic inhibition of the ERK1/2 MAPK pathway inhibited hyperoxia-induced macrophage apoptosis **(38)**.

Fibroblasts

Studies in fibroblasts subjected to hyperoxia also indicate apoptotic cell death. Rodent fibroblasts displayed increased DNA fragmentation, caspase activation, and Cyt-*c* release upon hyperoxia treatment **(39)**. Murine embryonic fibroblasts derived from $bax^{-/-}bak^{-/-}$ mice resisted hyperoxia-induced cytotoxicity, indicating the involvement of Bcl-2 family proteins in the hyperoxic killing of fibroblasts **(39)**. Similarly, lung fibroblasts from $bid^{-/-}$ mice were resistant to hyperoxia-induced cell death in culture **(8)**.

Endothelial Cells

Recently, we have shown that hyperoxia activates the extrinsic and intrinsic apoptotic pathways in mouse lung endothelial cells (MLEC) **(40)**. Hyperoxia induced the plasma membrane assimilation of the DISC and the activation of caspase-8, Bid, and downstream caspases-9 and -3. Hyperoxia has been shown to induce ROS production in various types of endothelial cells, including MLEC **(40–42)**, associated with ERK1/2 and/or p38 MAPK activation, and that that require NADPH: oxidase activation **(40**, **41)**. Inhibitors of NADPH: oxidase diminished the activation and plasma membrane assimilation of the DISC during hyperoxia **(40)**. Hyperoxia also caused the time-dependent downregulation of the antiapoptotic molecule FLIP, an inhibitor of caspase-8, in MLEC. We have shown that the overexpression of FLIP, in cultured MLEC, prevented hyperoxia-induced cell

death, by inhibiting elements of both the intrinsic and extrinsic apoptotic pathways **(43)**. FLIP expression attenuated intracellular ROS generation during hyperoxia exposure, by downregulating ERK1/2 activation and $p47^{Phox}$ expression. FLIP prevented the hyperoxia-induced trafficking of the DISC from the Golgi apparatus to the plasma membrane and also blocked downstream events in both the extrinsic and intrinsic apoptotic pathways. Furthermore, FLIP expression markedly inhibited PKC activation and the expression of distinct PKC isoforms (α, η, and ζ), and stabilized an inhibitory interaction of PKC with Bax. In summary, hyperoxic cell death in endothelial cells involves both the extrinsic and intrinsic apoptosis pathways initiated by elevated intracellular ROS generation **(40, 43)**.

Ventilator-Induced Lung Injury

Mechanical ventilation of rodents with high tidal volumes causes ventilator-induced lung injury (VILI). The injurious consequences of ventilation include alveolar septal thickening, edema, inflammatory cell influx, and protein leakage into the alveoli. Inflammatory responses to mechanical ventilation are associated with an increased production of macrophage-derived cytokines such as macrophage inflammatory protein-2 (MIP-2) and tumor necrosis factor-α (TNFα) **(44–46)**.

The exposure of mice to mechanical ventilation was shown to cause airway epithelial cell apoptosis, neutrophil inflammation, and cytokine release **(45, 46)**. At extreme high tidal volumes, epithelial cell necrosis may supercede apoptosis in the lung tissue, although increased epithelial cell apoptosis was still observed in peripheral tissues such as the kidney **(47)**.

The activation of MAPK pathways has been associated with VILI **(46, 48–50)**, although to date, few studies have addressed the functional role of MAPKs or the specific regulation of apoptosis pathways in VILI. In a model of high tidal volume ventilation, VILI was associated with the activation of apoptotic signaling pathways in the lung, initiated by apoptosis signaling kinase-1 (ASK1) and downstream activation of the JNK/AP-1 pathway **(49),** whereas hyperoxia further promoted VILI by additionally activating ERK1/2 **(50)**. We have recently shown that mice genetically deleted for major MAPK pathway components (i.e., $mkk3^{-/-}$, and $jnk^{-/-}$) were resistant to VILI, as evident by decreased alveolar protein leakage, decreased inflammatory cell index, and reduced epithelial cell death, indicated by TUNEL staining **(46)**. High tidal volume ventilation was also shown to increase in poly(adenosine diphosphate-ribose) polymerase (PARP) activity in the lung **(51)**. Specific inhibitors of PARP activation administered prior to mechanical ventilation reduced markers of epithelial cell apoptosis and protected against VILI, as evidenced by reduced edema and inflammatory cell influx **(51)**.

Lung Ischemia-Reperfusion Injury

Ischemia-reperfusion (I/R) refers to the stoppage and subsequent restitution of blood flow as a component of disease, such as in cardiac arrest and myocardial infarction, shock, transplantation, respiratory failure, or by mechanical intervention. Interruptions in blood flow can lead to diminished O_2 tension (hypoxia) in tissues. The reperfusion of ischemic tissue generates cytotoxic ROS, promotes the recruitment of inflammatory leukocytes, and causes lung injury and cell death involving both necrotic and apoptotic events. Endothelial cells are the primary targets for ROS generated during I/R **(52, 53)**. Cell death may also arise as a secondary consequence of inflammation around dead tissue. The inflammatory response can play a deleterious role in I/R-induced lung injury **(54)**.

In vivo models of I/R injury usually involve temporal clamping of arteries, whereas *in vitro* models of I/R injury, such as in cell culture models lacking a flow component, rely on the manipulation of O_2 tension to generate an artificial hypoxia/reoxygenation (H/R), involving a sustained decrease of O_2, followed by restitution of ambient O_2 tension **(53)**.

Pulmonary I/R caused by temporal clamping of the pulmonary artery induced the biochemical features of apoptosis in rodent lungs, including increased expression of Fas and FasL, activation of caspases-3,-8, and -9, modulation of Bcl-2-related proteins, PARP cleavage, and Cyt-*c* release **(55)**.

Lung Cell Death in Hypoxia/Reoxygenation Injury

The cell death pathways induced by H/R in lung cells and their underlying regulatory mechanisms remain poorly understood and may involve both necrotic and apoptotic forms of cell death. Numerous studies have suggested that H/R can induce apoptosis, depending on the regulation of apoptotic factors. H/R stress can trigger the intrinsic apoptotic cascade in several cell types as the product of mitochondrial damage **(53, 56)**. The potential expression of FasL during H/R may trigger Fas-dependent death pathways characterized by DISC formation and caspase-8 activation **(57)**. Hypoxia also induced a time-dependent mitochondrial translocation of Bax, with the subsequent release of Cyt-*c* and apoptotic cell death upon reoxygenation **(58, 59)**. The antiapoptotic molecules in the Bcl-2 family (i.e., Bcl-2, Bcl-xL) may be downregulated in several cell types during H/R **(53, 58, 59)**.

Recent studies demonstrate that H/R causes cell death in MLEC by stimulating the intrinsic and extrinsic apoptotic pathways **(60–62)**. The cell death was associated with the activation and mitochondrial translocation of Bax and with caspase-9 activation. Additionally, a predominant role for the caspase-8/Bid pathway in H/R-induced cell death was observed in MLEC **(60–62)**. An increased expression of FasL was observed during H/R in MLEC and other cell types **(57, 62)**. H/R induced an early time-dependent cleavage of Bid during

the reoxygenation phase, associated with DISC formation and caspase-8 activation. In addition to the appearance of activated DISC at the plasma membrane, H/R caused an apparent preformation of the DISC in the Golgi apparatus that preceded its translocation to the plasma membrane **(60)**. The mitochondrial Bax translocation following H/R treatment in MLEC occurred without modulating total Bax protein levels **(53, 61)**. In accord with previous studies indicating that PKC can be induced by hypoxia **(63)**, the expression and plasma membrane translocation of PKC were activated by hypoxia in MLEC, which preferentially involved the α and ζ isoforms **(60)**. Under normoxic conditions, PKCα/ζ were found in complex with Bax **(60)**.

Expression of the antiapoptotic factor Bcl-xL in MLEC inhibited both the intrinsic and extrinsic apoptosis pathways induced by H/R. Artificial Bcl-xL expression inhibited Bax and Bid activation and translocation to the mitochondria, as well as downstream Cyt-*c* release and caspase-9 activation **(61)**. Bcl-xL inhibited caspase-8 cleavage and disrupted DISC formation in the plasma membrane of MLEC subjected to H/R stress. Bcl-xL overexpression also antagonized DISC formation in the Golgi complex and inhibited its association with Golgi-associated proteins **(61)**. In contrast, Bcl-xL overexpression increased the appearance of the DISC in the mitochondrial fraction, where caspases-8 cannot undergo efficient processing **(64)**. The effect of Bcl-xL on the redistribution of DISC formation in these cellular compartments protected endothelial cells from the lethal effects of H/R. Bcl-xL overexpression also stimulated the expression of FLIP, which may have contributed to the overall antiapoptotic effect.

In MLEC, the artificial overexpression of FLIP also inhibited H/R-induced cell death by blocking both extrinsic and intrinsic apoptosis **(60)**. FLIP prevented caspase-8 processing, inhibited Bid/Bax activation and Cyt-*c* release, and also upregulated Bcl-xL. FLIP inhibited Bax activation in both wild-type and *bid*$^{-/-}$MLEC, indicating independence from the caspase-8/Bid pathway **(60)**. FLIP expression inhibited the expression and membrane translocation of PKCα, ζ during H/R and promoted an association of these forms of PKC with Bax, resulting in Bax inhibition. The mechanism by which FLIP inhibits PKC translocation remains unclear. Surprisingly, FLIP expression also inhibited DISC formation at the plasma membrane by retaining the DISC in the Golgi. Unlike Bcl-xL overexpression, FLIP overexpression did not prevent DISC formation in the Golgi **(60)**.

Apoptosis in the Pathogenesis of Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is defined as a preventable and treatable disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and is associated with an abnormal inflammatory response of the lungs to noxious particles or gases **(65)**.

The pathogenesis of COPD is primarily attributed to persistent lung inflammation caused by cigarette smoke, environmental pollutants, or bacterial products, associated with an influx of inflammatory cells into the airways and lungs **(66)**. Furthermore, a disruption of the balance between protease and antiprotease activities in COPD patients, resulting in the development of emphysema, also represents a major component of COPD pathogenesis **(67)**. This protease/antiprotease imbalance may arise from the activity of inflammatory cells recruited by cigarette smoke or may result from a genetic deficiency of α-1 antitrypsin. Oxidative stress, which can amplify inflammation, may represent an important mechanism involved in the pathogenesis of COPD. Cigarette smoke itself contains a high concentration of ROS **(68, 69)** and may trigger additional ROS generation by inflammatory cells such as activated macrophages and neutrophils. All of these factors may contribute to the initiation and propagation of apoptosis, which may contribute significantly to lung cell death and tissue injury following exposure to cigarette smoke. The specific role of apoptosis in the pathogenesis of COPD, however, remains unclear **(70)**.

Examination of lung tissue from COPD patients reveals the presence of apoptotic cells in greater numbers than in control lungs or those from smokers without COPD **(71, 72)**. Apoptotic cells include alveolar and bronchial epithelial cells as well as endothelial cells in the parenchyma. Apparent increases in apoptosis may reflect an impaired clearance of apoptotic cells, or efferocytosis **(73)**.

The identification of alveolar cell apoptosis was initially described in emphysematous human lung tissue sections using *in situ* end labeling of fragmented DNA. Among the cells that showed positive signals, most were endothelial cells from capillaries and arterioles. Although less frequent, prominent intranuclear staining was also described in alveolar epithelial cells, interstitial cells, and inflammatory cells, while control lungs were negative **(71)**. Both apoptosis and proliferation of alveolar wall cells were significantly increased in patients with emphysema than in asymptomatic smokers and nonsmokers, as determined by TUNEL and proliferation cell nuclear antigen (PCNA) staining **(72)**. Similarly, lung cell (alveolar epithelial, endothelial, and mesenchymal) apoptosis and cell proliferation were increased in emphysematous lung tissue. Moreover, the activated subunits of caspase-3 and the increased expression of proapoptotic proteins (i.e., Bax and Bad) were detected in emphysematous lungs, while the antiapoptotic protein Bcl-2 was not detected in either normal or emphysematous lung tissue **(74)**. Increased apoptotic epithelial and endothelial alveolar septal cells were observed in emphysematous lungs compared to the lungs from nonsmokers, healthy smokers, and patients with primary pulmonary hypertension **(75)**. In addition, no significant differences were seen in apoptosis indicators between healthy nonsmokers and smokers without emphysema **(75)**. In agreement with this finding, recent data have demonstrated that apoptosis persists despite smoking cessation, suggesting that cigarette smoke itself is not the sole agent causing apoptosis once COPD is established, although COPD individuals are more likely susceptible to smoke-induced cell damage and

apoptosis **(76)**. This finding indicates that factors including protease/antiprotease imbalance, inflammation, and oxidative stress that relate to the pathogenesis of emphysema may also contribute to increased apoptosis **(76)**. Furthermore, genetic alterations in cellular responses to apoptotic stimuli may also play contributory roles.

Cigarette Smoke Exposure

Cigarette smoke, a complex mixture of more than 4,800 chemical compounds, contains high concentrations of free radicals and other oxidants **(69, 77)**. Whereas short-lived radicals in the gas phase of cigarette smoke may be quenched immediately in the epithelial lining fluid, redox reactions in cigarette smoke condensate, which forms in the epithelial lining fluid, may produce reactive oxygen species (ROS) for a considerable time **(78)**.

The chronic exposure of rats to mainstream cigarette smoke produced a significant and time-dependent increase in the proportion of apoptotic cells in the bronchial and bronchiolar epithelium **(79)**. Smoke treatment increased the level of proapoptotic proteins in the terminal bronchiole areas of lung tissue from rats **(80)**. The levels of proapoptotic proteins (caspase-8, caspase-3, Bax, t-Bid, and cytochrome c) were increased in the lungs of cigarette smoke-exposed rats as detected by immunochemical staining, indicating that cigarette smoke sensitizes the lung structural cells toward receptor-dependent as well as mitochondrial-dependent apoptotic pathways **(81)**. Cytokine (e.g., TNF-α, TGF-β1) and Fas levels were increased in the peripheral blood of COPD patients **(82)**. These findings suggest that the Fas-mediated apoptotic pathway may participate in the pathogenesis of COPD.

Aqueous cigarette smoke extracts are now widely used as a model for cigarette smoke exposure *in vitro*. Some limitations exist in the use of CSE to study *in vitro* responses to cigarette smoke, including the exclusion of some volatile components present in CS and variations in the preparation method in between individual laboratories. Because the effect on cells is dependent on the duration of smoke exposure and the concentration of smoke, such variations can lead to differential results even in the same cell lines with regard to cell death. Nevertheless, CSE remains a useful model of *in vitro* smoke exposure that has the potential to uncover important biological pathways **(83)**.

CSE causes cell death by incompletely understood mechanisms. Both oxidative stress and apoptosis exhibited a dose-response relationship with CSE concentrations in human lung fibroblasts **(84)**. Cigarette smoke-induced apoptosis was observed in a variety of human cells, including the monocytic cell line U-937, umbilical vein endothelial cells, alveolar macrophages, bronchial epithelial cells, and the HFL-1 fibroblast strain **(85–89)**.

In vitro studies using alveolar macrophages showed that cell death by CSE occurred in a dose- and time-dependent manner, with 93% of cells showing

apoptosis after 24 hours of exposure to 10% CSE solution **(87)**. CSE induced apoptosis at lower concentrations (10–25%) and necrosis at higher concentrations (50–100%) in human lung fibroblasts **(90)**. CSE activated the caspases-3 and -8, p38 MAPK, and JNK in human aortic endothelial cells (HAEC) **(91)**. On the other hand, some contradiction has arisen in terms of how cigarette smoking affects this apoptotic process, because both the stimulatory and inhibitory potentials of smoking on apoptosis have been described. Cigarette smoke stimulated caspase-3 precursors as well as intact poly (ADP-ribose) polymerase (PARP) production, but did not activate caspase-3 or cleave PARP in human bronchial epithelial cells **(92)**. Furthermore, CSE-induced DNA damage was reversible in human lung fibroblasts, and cells proliferated when CSE was removed after 24 hours of exposure **(93)**. Cell death in response to cigarette smoke by necrosis may be responsible for the loss of alveolar walls and inflammation observed in emphysema **(94)**. We have observed a dose-dependent early induction of apoptosis in Beas-2B cells that peaks at low CSE concentrations or short exposure times. Necrosis, as indicated by membrane leakage, occurs at higher CSE concentrations or longer exposure times **(95)**.

Smoking-induced mitochondrial dysfunction has also been suggested as a prerequisite step toward cell death through the intrinsic apoptotic pathway **(96)**. Our laboratory demonstrated that CSE caused the loss of cellular ATP and the rapid depolarization of mitochondrial membrane potential. Furthermore, we have recently described the functional compartmentalization of heme oxygenase-1 (HO-1) in the mitochondria of lung epithelial cells and its potential role against mitochondria-mediated cell death during cigarette smoke extract exposure **(95)**.

Our laboratory has recently further examined the regulation and role of extrinsic apoptotic pathways in CSE-induced cell death, using human pulmonary fibroblasts **(97)**. We demonstrated that CSE treatment induces DISC formation in human lung fibroblasts (MRC-5) (Fig. 23.3 and Color Plate 12) and promotes DISC trafficking from the Golgi complex to membrane lipid rafts. Using this model, we demonstrated a novel role of protein kinase-C (PKC) in the regulation of DISC formation and trafficking. The PKC isoforms, PKCα, ζ, ε, and η, were activated by CSE exposure. The overexpression of wild-type PKCα inhibited, while PKCζ promoted, CSE-induced cell death. Dominant-negative (dn)PKCζ protected against CSE-induced cell death by suppressing DISC formation and caspase-3 activation, while dnPKCα enhanced cell death by promoting these events. DISC formation was augmented by wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI-3K). CSE-induced Akt phosphorylation was reduced by dnPKCα but increased by dnPKCζ. The potential role of PKC isoforms in modulating apoptosis was also demonstrated *in vivo* in a murine model of chronic cigarette smoke exposure. The expression of PKCα *in vivo* inhibited DISC formation, caspases-3 and -8 activation, lung injury, and cell death after prolonged cigarette smoke exposure, whereas the expression of PKCζ promoted caspase-3 activation. In summary, CSE-induced DISC formation is differentially regulated by

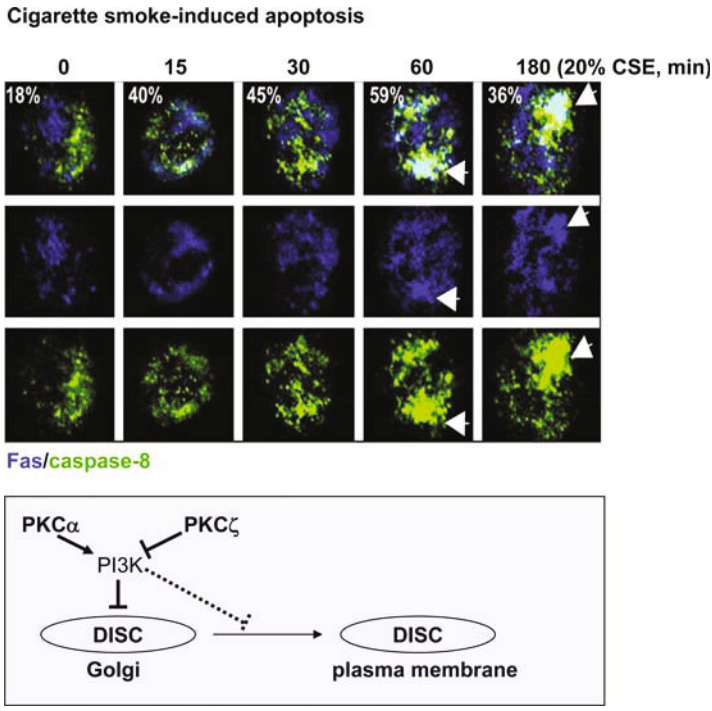

Fig. 23.3 Activation of DISC by cigarette smoke. MRC-5 cells at 70% confluence were exposed to 20% CSE in serum-free media. Immunofluorescence images of MRC-5 double-labeled with indicated antibodies (anti-FAS in *blue* and anticaspase-8 in *green*) are shown. The cyan pseudocolor (*arrow, top panel*) indicates a co-localization of Fas and caspase-8. The same images with either the green or blue color removed are shown for clarity (*middle and bottom panels*). Data in this figure are representative of 20–49 cells analyzed for each time point. All panels are the same scale **(97)**. [Figure reproduced from Park et al. **(97)** with permission from the American Association of Immunologists.]
The cartoon illustrates the potential role of protein kinase-c (PKC) isoforms in regulating DISC formation. PKCα displayed an antiapoptotic effect in CSE-treated cells and in chronic cigarette smoke-exposed mice, whereas PKCζ displayed a proapoptotic effect. PKCα potentially inhbits DISC trafficking by activating the PI3K pathway in fibroblasts. PKCζ promoted DISC trafficking by inhibiting the PI3K pathway **(97)** (*see* Color Plate 12)

PKCα and PKCζ via the PI3-K/Akt pathway. These results suggest that the modulation of PKC may have therapeutic potential in the prevention of smoke-related lung injury **(97)**.

Other Emphysema Models

In rodents, the induction of endothelial or epithelial apoptosis is accompanied by the loss of pulmonary alveoli and pathological evidence of emphysematous changes **(98)**. Blockade of the vascular endothelial growth factor

receptors caused alveolar cell apoptosis, oxidative stress, and emphysema-like disease in both rats and mice **(98**, **99)**. The intratracheal administration of ceramide, a proapoptotic signaling molecule, caused emphysema-like symptoms in mice, whereas the inhibition of ceramide biosynthesis prevented emphysema and alveolar cell apoptosis caused by VEGF receptor blockade **(98)**. This evidence suggests that apoptosis may play a crucial role in emphysema and COPD.

Pulmonary Hypertension

Pulmonary arterial hypertension (PAH) is a terminal disease characterized by a progressive increase in pulmonary vascular resistance leading to right ventricular heart failure.

The disease is primarily a hyperpoliferative disorder in which excessive smooth muscle cell proliferation causes intimal hyperplasia, leading to vascular remodeling. An initial increase in endothelial cell apoptosis may lead to the proliferation and expansion of an apoptosis-resistant endothelial cell population, which also contributes to vessel occlusion **(100)**. The potential counter-regulatory role of apoptosis in the development of PAH has been demonstrated. The expression of dominant-negative mutants of an inhibitor-of-apoptosis protein (*survivin*) resulted in the reversal of established monocrotaline-induced PAH in rats, associated with increased pulmonary artery smooth muscle cell apoptosis both *in vivo* and *in vitro* **(101)**. Similarly, the induction of vascular smooth muscle or endothelial cell apoptosis with pharmacological agents such as simvastatin effectively reversed established pulmonary hypertension in several experimental models including VEGF receptor blockade and monocrotaline-induced hypertension **(102**, **103)**.

Fibrosis

Idiopathic pulmonary fibrosis (IPF) is a terminal disease characterized by scarring or thickening of lung tissues associated with fibroblast hyperproliferation and extracellular matrix remodeling with no known etiology or effective treatment **(104)**. IPF primarily affects the lower respiratory tract, resulting in compromised efficiency of alveolar gas exchange **(104)**. Alveolar epithelial cell apoptosis may be an initiating factor in the progression of fibrosis **(105)**.Epithelial cell loss may permit the migration of fibroblasts, resulting in intra-alveolar fibrosis. Increased epithelial cell apoptosis has been observed in tissues from IPF patients **(106)** and also in experimental models of bleomycin-induced pulmonary fibrosis in mice **(107)**. The critical role of apoptosis in the latter model is supported by observations that caspase inhibitors **(108)** or neutralizing

antibodies against activation of the extrinsic apoptotic (Fas-mediated) pathway prevent bleomycin-induced pulmonary fibrosis **(109)**.

Conclusions and Therapeutic Strategies

Apoptosis appears to play a dual role in the progression and pathogenesis of lung injury and disease. This process may serve as an adaptive process by removing dysfunctional cells, limiting inflammation in damaged tissue, and preventing cell proliferation. On the other hand, excessive apoptosis may contribute to the loss of critical cell populations, resulting in loss of function, as in emphysema, or in undesirable cell proliferation, as in fibrosis (Fig. 23.4). Thus, the complex role of apoptosis in lung diseases warrants a detailed examination of regulatory mechanisms.

Conceivably, strategies aimed at modulating apoptotic outcomes by targeting specific regulatory elements in apoptosis signaling pathways might be developed for potential therapeutic gain in lung diseases. These may include small molecule inhibitors of signal transduction pathway components such as MAPK or PKCs, or antagonists of cytokine or death receptors.

Although therapeutic agents targeting apoptosis pathways are currently starting to emerge in biomedical research and human clinical trials, we have yet to observe the translational application of these targets in specific lung diseases. Among experimental approaches, carbon monoxide at low concentration has been shown in animal models to exert antiapoptotic and antiinflammatory effects in various rodent models of ALI **(110)**.

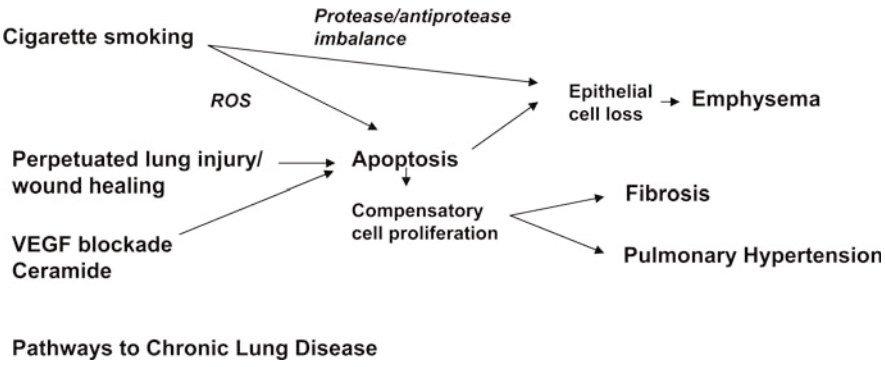

Fig. 23.4 Pathways to chronic lung disease. The role of apoptosis in the development of chronic lung diseases such as chronic obstructive lung disease (COPD) and pulmonary fibrosis remains incompletely understood. Epithelial cell apoptosis caused by cigarette smoke or other stimuli may contribute to loss of alveolar function and the development of emphysema. Epithelial cell apoptosis may permit fibroblast cell proliferation, leading to pulmonary fibrosis, and smooth muscle or secondary endothelial cell proliferation, contributing to pulmonary hypertension

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Chapter 24
Apoptosis in Liver Injury and Liver Diseases

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Abstract The liver is a multifunctional organ that has important roles such as metabolism, synthesis, and detoxification. Enhanced hepatocyte apoptosis and impaired liver regeneration are the most common liver disorders in acute liver failure. Hepatocyte apoptosis also emerges as a fundamental component of chronic liver diseases. Liver tissue fibrosis is triggered by hepatocyte apoptosis, and the excess fibrosis causes liver disease to progress to cirrhosis, which causes chronic liver failure. Although hepatocyte apoptosis is a cardinal feature of liver diseases, it is generally believed that apoptosis deletes hepatitis virus-infected hepatocytes and prevents carcinogenesis and that antiapoptotic factors are activated in the chronic injured liver. This chapter highlights the mechanism of pro- and antiapoptosis in liver diseases' underlying molecular basis.

Keywords Hepatocyte · Apoptosis · Death receptor · Virus · ASH · NASH · AIH · Drug · I/R injury · HCC

Many liver diseases are associated with inflammatory cell infiltration, inflammatory cytokine production, and hepatocyte cell death including apoptosis. Massive hepatocyte apoptosis results in acute liver failure. Chronic hepatocyte apoptosis with impairment of hepatocyte regeneration followed by the excessive deposition of extracellular matrix proteins causes liver fibrosis and subsequent cirrhosis that results in chronic hepatic dysfunction. Although hepatocyte apoptosis is a cardinal feature of liver diseases, the depletion of virus-infected hepatocytes by apoptosis is associated with the prevention of carcinogenesis (Fig. 24.1 and Color Plate 13). In liver injury, death receptors, such as tumor necrosis factor (TNF) receptor (TNFR) or Fas, -mediated signals are critical components for

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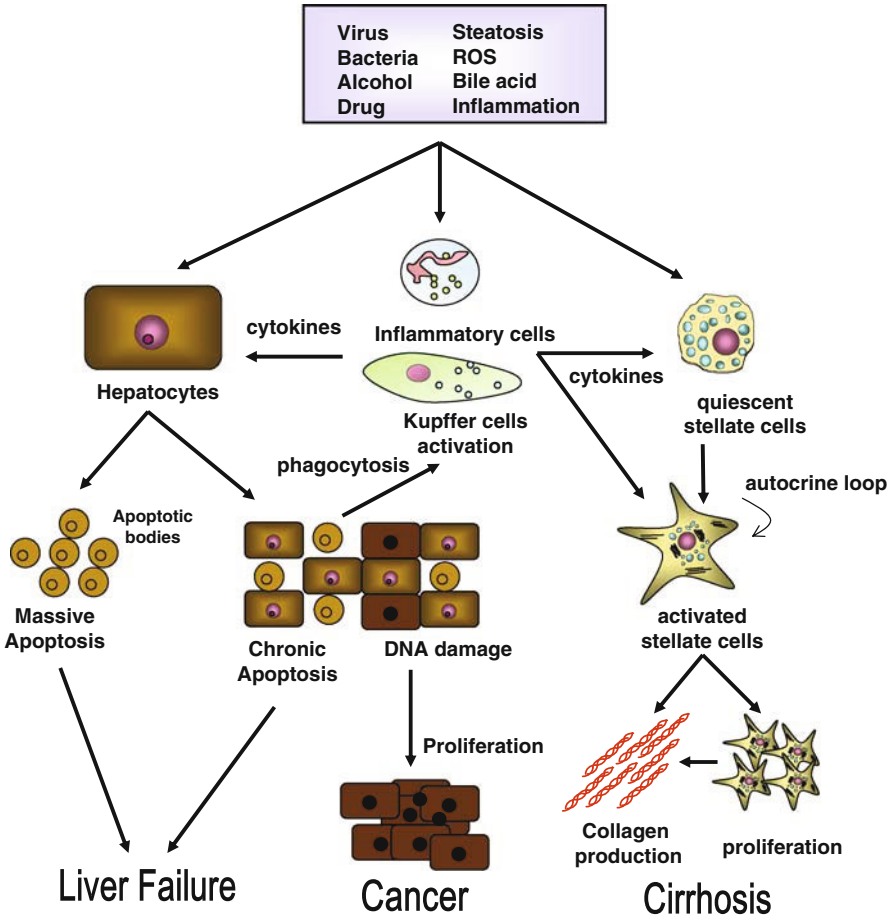

Fig. 24.1 Schematic representation of liver diseases. Hepatocyte apoptosis is initiated by various stimuli via direct effects and/or inflammatory responses. Massive hepatocyte apoptosis with impairment of hepatocyte regeneration results in acute liver failure. Chronic hepatocyte apoptosis leads to liver cirrhosis and liver cancer. Kupffer cells engulf apoptotic bodies of hepatocytes and release fibrogenic cytokines, which trigger collagen production by hepatic stellate cells. Chronic hepatocyte apoptosis also stimulates hepatocyte regeneration, and dysregulation of the balance between hepatocyte proliferation and cell death causes hepatocarcinogenesis (*see* Color Plate 13)

hepatocyte apoptosis. The signals from TNFR or Fas control downstream signals, such as caspase-dependent, caspase-independent, and mitochondria-dependent apoptosis pathways. The receptors also activate nuclear factor (NF)-kappaB, c-Jun N-terminal kinase (JNK)/AP-1, p38, and reactive oxigen speoes (ROS), which regulate the expression of apoptotic and antiapoptotic factors.

Death Receptor-Mediated Liver Injury

The activation of Fas and TNFR induces hepatocyte apoptosis in a wide variety of liver diseases, such as viral hepatitis, alcoholic hepatitis, ischemia-reperfusion liver injury, and fulminant hepatic failure. Fas and TNFR-mediated signals are induced by Fas ligand (FasL) and TNF-alpha, respectively. FasL binds to Fas, a transmembrane protein containing a "death domain" that is necessary for inducing cell death **(1)**. After the activation of Fas, the intracellular domain of Fas interacts with the Fas-associated protein with death domain (FADD) adaptor molecule, which contains a conserved death domain, which in turn binds to procaspase-8. This forms the death-inducing signaling complex (DISC) that activates caspase-8 to initiate the programmed cell death **(2)**. Activated caspase-8 leads to the processing of effector caspases including caspase-3. The effector caspases cleave and inactive proteins whose functional loss induces apoptosis **(3)**. TNF-alpha interacts with two membrane receptors, TNFR1 and TNFR2. Soluble TNF-alpha activates apoptotic signaling via TNFR1 **(4)** in hepatocytes, and membrane-bound TNF-alpha is required for TNFR2-mediated hepatocyte apoptosis **(5)**. TNFR1 contains the death domain in its cytoplasmic portion. Upon the activation of TNFR1, the intracellular domain of TNFR1 interacts with the TNF receptor-associated protein with death domain (TRADD) adaptor molecule and then interacts with FADD followed by caspase-8 activation. The role of TNFR2 in hepatocyte apoptosis is not clearly understood, because it lacks the intracellular death domain.

Although both FasL and TNF-alpha induce hepatocyte apoptosis, they transduce distinct signals. TNFR1/TRADD or TNFR2 interacts with TNF-alpha receptor-associated factor (TRAF)-2, and then the signals transmit to inhibitor-of-apoptosis (IAP) molecules **(6)**. Since IAPs interfere with the activation of caspase-8, TNF-alpha/TNFR1 signaling induces a weak and transient formation of DISC. TNFR1-mediated signaling further induces a mitochondria-dependent signal that mediates hepatocyte apoptosis. Cleavage of the Bcl-2 family member Bid by activated caspase-8 results in oligomerization of the proapoptotic proteins Bax and Bak, which induce the mitochondrial permeability transition (MPT) and the release of cytochrome c **(7)**. In mitochondria-dependent cell death, cytochrome c interacts with Apaf-1 to activate procapase-9 that subsequently activates caspase-3. The inhibition of caspase-9 prevents TNFR-mediated, but not Fas-mediated, liver injury and hepatocyte apoptosis *in vivo* **(8)**. Additionally, overexpression of the antiapoptotic factors Bcl-2 and Bcl-xL, which inhibit Bid and Bax activation, prevents TNF-alpha-induced liver injury **(9, 10)**. Thus, TNFR-mediated hepatocyte apoptosis depends on mitochondria-mediated caspase-9 activation. In animal models, agonistic Fas antibody (Jo2) induces pronounced hepatocyte damage and apoptosis with massive hemorrhage through Fas, but TNF-alpha alone does not induce hepatocyte apoptosis. Unlike Fas-induced apoptosis, TNFR-mediated apoptosis requires hepatocyte sensitization by D-galactosamine *in vivo* **(11)**. D-galactosamine specifically blocks

hepatic transcription by metabolic depletion of uridin nucleotides **(12)**. The transcriptional inhibitor actinomycin D also sensitizes hepatocytes against TNF-alpha. Additionally, NF-kappaB activated by TNF-alpha or AKT activated by bile duct ligation in hepatocytes inhibit TNFR-mediated, but not Fas-mediated, hepatocyte apoptosis *in vivo* **(13, 14)**.

TNF-alpha activates JNK, which phosphorylates c-Jun, ATF-2, and JunD. These transcription factors are members of the AP-1 family and induce the transcription of AP-1-dependent genes, which are involved in the regulation of inflammation, proliferation, and cell death. Upon TNFR activation, the intracellular domain of TNFR binds to TRADD, which recruits TNFR associated factor (TRAF)2 and receptor interacting protein (RIP) to activate JNK through the phosphorylation of JNK kinase kinases such as MEK kinase 1 (MEKK1) and apoptosis signaling kinase 1 (ASK1) and MKK7 **(15, 16)**. Hepatocytes express two JNK genes, *jnk1* and *jnk2* **(17)**. In hepatocytes, JNK2 is essential for TNF-alpha-induced hepatocyte apoptosis **(18)**. Liver injury induced by GalN plus lipopoly saccharide (LPS) (a strong inducer of TNF-alpha) is markedly decreased in JNK2-deficient mice compared with wild-type and JNK1-deficient mice. In addition, JNK2 is required for caspase-8 activation, Bid cleavage, and induction of the mitochondrial death pathway. However, the mechanism by which JNK activation leads to mitochondrial dysfunction is not clear. JNK1 mediates apoptosis in other situations. In cultured hepatocytes, bile acid toxicity depends on JNK1, whereas JNK2 has a cytoprotective function **(19)**. TNF-alpha-induced apoptosis is suppressed in JNK1-inactivated fibroblasts but increased in JNK2-null cells **(20)**. ROS are also involved in TNF-alpha-mediated liver injury **(15)**. The depletion of reduced antioxidant glutathione sensitizes cultured mouse hepatocytes to TNF-alpha to induce cell death without transcription inhibitor, such as actinomycin D **(21)**. The overexpression of antioxidant thioredoxin attenuates GalN plus LPS-induced liver injury **(22)**, indicating the critical role of ROS in TNF-alpha-induced hepatocyte apoptosis. The ROS formation by TNF-alpha depends on RIP, TRAF2, and FADD **(23)**, which regulate the generation of ROS in mitochondria to control the cellular redox status **(24)**. TNF-induced ROS activate ASK1 and JNK kinase kinase and also inactivates MAPK phosphatases (MKP) **(25)**, leading to sustained activation of JNK, which contributes to TNF-induced apoptosis **(26)**. The lysosomal cysteine protease cathepsin B is also involved in TNF-alpha-induced hepatocyte apoptosis. Cathepsin B-deficient hepatocytes are resistant to TNF-alpha-induced apoptosis **(27)**. Cathepsin B release is regulated by caspase-8 and Bid **(28)**, which leads to activation of the mitochondrial apoptotic pathway by cytochrome c release.

As described above, hepatocytes are resistant to TNF-alpha-mediated cytotoxicity. In addition to apoptotic signals, TNF-alpha also activates signals that protect from cell death such as the transcription factor NF-kappaB. TNF-alpha activates NF-kappaB through I-kappaB kinase (IKK) activation, which in turn phosphorylates I-kappaB, which is the inhibitory component of the NF-kappaB/Rel complex. The inhibition of NF-kappaB by the overexpression of

a mutant I-kappaB superrepressor sensitizes hepatocytes to apoptosis induced by TNF-alpha **(29, 30)**. The deletion of the RelA/p65 gene also renders hepatocytes sensitive to TNF-alpha-induced apoptosis *in vitro* and *in vivo* **(31)**. The deletion of conditional NF-kappaB essential modulator (NEMO) (also known as IKK-gamma) in hepatocytes results in blocking NF-kappaB activation and massive hepatocyte apoptosis after TNF-alpha stimulation **(32, 33)**. The hepatocyte-specific deletion of IKK-beta does not lead to impaired activation of NF-kappaB nor increased apoptosis after circulating TNF-alpha administration, whereas the liver is highly susceptible to damage by cell-bound TNF-alpha via attenuation of JNK activity **(30, 32)**. NF-kappaB regulates the expression of antiapoptotic genes, such as caspase-8 inhibitor c-FLICE-like inhibitory protein (c-FLIP)(L), the Bcl-2 family members Bcl-xL and A1/Bfl-1, X-linked inhibitor of apoptosis (XIAP), TRAF2, inducible nitric oxide synthase, and cellular inhibitor of apoptosis (c-IAP)1 and c-IAP2. These genes block the signals of either the death receptor-dependent or the mitochondria-dependent apoptosis pathways **(6, 34–36)**. The inactivation of NF-kappaB results in sustained JNK activation by TNF-alpha **(37, 38)**. Manganese superoxide dismutase, which acts as a scavenger of potentially toxic ROS, is an NF-kappaB-dependent target gene induced by TNF-alpha **(39)**. Thus, NF-kappaB regulates JNK activation and ROS accumulation. The phosphatidylinositol 3 kinase (PI3K)/AKT pathway is also activated by TNF-alpha in hepatocytes and induces potent antiapoptotic functions **(40, 41)**, through its negative modulation of Raf, Bad, and forkhead transcription factors **(42)** and positive modulation of antiapoptotic members of the Bcl-2 family such as Mcl-1 **(43, 44)**.

Ceramides are sphingolipid-derived signals and regulators in the stress response and cell death **(45)**. Ceramides are generated from the major membrane sphingolipid sphingomyelin by acid or neutral sphingomyelinases (SMase) that are activated in response to TNF-alpha. Acid SMase knockout mice are resistant to TNF-alpha-induced liver injury **(46)**. TNF-alpha also activates sphingosine kinase (SphK). Proapoptotic ceramide is deacylated by ceramidase and converted to sphingosine. Sphingosine is phosphorylated to sphingosine 1-phosphate (S1P) by SphK and prevents the cytotoxic action of TNF-alpha **(41, 47)**. A model has been proposed in which the dynamic balance between the intracellular levels of ceramide and S1P (the "ceramide/S1P rheostat") determine cell survival or death. S1P stimulates various signaling pathways, such as cyclic adenosine monophosphate (cAMP)-dependent kinase, focal adhesion kinase, extracellular signal-regulated kinase, AP-1, NF-kappaB, and PI3K/AKT pathway **(47, 48)**. Notably, S1P also functions as an extracellular ligand for Edg receptor(s) in human and rat hepatocytes. S1P may contribute to protect hepatocytes from apoptosis by AKT activation **(40, 41)**.

It has been reported that TNF-related apoptosis-inducing factor (TRAIL) selectively induces apoptosis in various transformed cell lines but not in normal cells **(49)**. TRAIL receptors 1 and 2 contain the death domain in their intracellular portion that signals apoptosis in hepatocytes **(50, 51)**. TRAIL is expressed on the surface of activated immune cells such as natural killer (NK) cells, T lymphocytes,

macrophages, and dendritic cells, where it apparently functions as an immune effector molecule, antitumor cytotoxicity, and immune surveillance **(52, 53)**. TRAIL does not induce apoptosis in the healthy liver. Hepatic TRAIL and TRAIL receptor 2 are upregulated in mice and humans after viral infection. Viral infection makes hepatocytes highly susceptible to TRAIL to induce apoptosis *in vivo* **(54)**.

Transforming growth factor (TGF)-beta is involved in hepatocyte apoptosis via the TGF-beta receptor **(55)**. In the liver, TGF-beta is produced by Kupffer cells and hepatic stellate cells. The produced TGF-beta inhibits proliferation and induces hepatocytes apoptosis by a SMAD-dependent manner and also stimulates hepatic stellate cells to induce fibrogenic responses upon injury **(56–58)**.

Viral Injury

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are major causes of acute and chronic viral hepatitis. Chronic hepatitis results in fibrosis and then cirrhosis, leading to major complications of cirrhosis, hepatocellular carcinoma (HCC), portal hypertension, and hepatic decompensation. HBV is a partially double-stranded circular DNA virus. After infection, the DNA is made fully double-stranded and transformed into closed circular supercoiled DNA (cccDNA), which becomes the template for viral mRNAs. The viral DNA replicates via a reverse transcription of the RNA pregenome and predominantly infects human hepatocytes in the liver. HBV causes an acute hepatitis that is self-limited in 90% of adults. The remaining patients and the patients infected perinatally develop chronic hepatitis. A series of studies using HBV transgenic mice reveals the critical role of HBV-specific $CD8^+$ cytotoxic T cells, $CD4^+$ helper T cells, and IFN-gamma in HBV persistence.Cytotoxic T lymphocytes (CTL) eliminate damaged or infected cells and mediate the host immune response against viral infection. The adaptive immune system generates viral-specific CTL that damage hepatocytes **(59–61)**. CTL produce the cytotoxins perforin, granulysin, and granzyme that damage the target cell's plasma membrane and activate caspases resulting in hepatocyte apoptosis. FasL-Fas and TNF-TNFR systems also contribute to CTL-mediated hepatocyte apoptosis. In patients with chronic hepatitis B, the HBV may increase serum TRAIL, which may induce cell death in infected hepatocytes **(54)**. Although HBV is a noncytopathic virus, cytopathic effects of HBV have been described in several experimental models. The X protein of HBV (HBx), a potent transactivator for viral replication, has oncogenic properties in animal models **(62)**. HBx is also involved in hepatocyte apoptosis in both a p53-dependent **(63)** and a p53-independent manner **(64)**. HBx interacts with c-FLIP to induce apoptosis **(65)**. HBx increases the susceptibility of hepatocytes to cell death through activation of MAPK kinase 1 and n-Myc **(66)**. In parallel, HBx also has anti-apoptotic effects, which cause continuous inflammation and carcinogenesis. HBx activates NF-kappaB **(67)** via ROS formation, resulting in enhancement of the

viral replication **(68)**. HBx also stimulates PI3K/AKT **(69)**. In human hepatoma cell lines, intracellular retention of the HBV large surface protein induces apoptosis with cellular vacuolization **(70)**. HBV core promoter mutations that are associated with fulminant hepatitis induce apoptosis in hepatocytes **(71)**. In addition, the accumulation of cccDNA results in a strong cytopathic effect in hepatocytes **(72)**.

HCV is a positive, single-stranded RNA virus. After the infection, the HCV genome is translated to three structural proteins (Core, E1, E2) and seven nonstructural proteins (NS1, 2, 3, 4A, 4B, 5A, 5B). The NS5B, which is a viral RNA-dependent RNA polymerase, produces the negative-stranded RNA as a template for new positive viral genomes. HCV may cause both immune response and direct cytopathic effects, which induce liver injury **(73)**. CTL is important in liver injury induced by HCV **(74)**. The number of HCV-specific CTL is inversely associated with the reduction of viral titers and directly associated with the elevation of transaminase **(75)**. However, it has been reported that HCV-specific $CD8^+$ cells do not correlate with the level of liver damage **(76)**. Natural killer (NK) cells, which do not express antigen-specific receptors, are important for antiviral immunity, including HCV infection **(77)**. NK cells directly induce apoptosis of virus-infected hepatocytes **(78)** and are also necessary for optimal priming and cytolytic function of virus-specific CTL and nonantigen-specific T lymphocytes by the production of interferon (IFN)-gamma **(74)**. IFN-gamma increases susceptibility to TNF-alpha-mediated hepatic damage **(79)** with the recruitment of natural killer T (NKT) cells **(80)**, which are important cell populations for liver injury during chronic hepatitis C **(81)**. HCV also has a direct cytopathic effect that is involved in both pro- and antiapoptosis. Antiapoptotic effects by HCV on hepatocytes allow the virus to proliferate. The structural HCV core protein, which is the virion nucleocapsid protein of virus particles, has both pro- and antiapoptotic effects **(73, 82)** by associating with apoptosis-related molecules. HCV envelope proteins E1 and E2 are proapoptotic **(83, 84)**. HCV NS3/4A proteases cleave the mitochondrial antiviral signaling protein (MAVS), which induces apoptosis **(85, 86)**. NS3 promotes caspase-8-mediated apoptosis **(87)**, and the NS5A protein has anti-apoptotic effects **(88, 89)**. Although many reports have described the effects of HCV proteins on apoptosis, their role *in vivo* still remains unclear.

Alcoholic Liver Injury

Alcoholic liver disease is toxic injury from excessive ethanol consumption and is a common cause of liver disease. Alcoholic liver disease is characterized by the excessive accumulation of fat in hepatocytes and is associated with the disruption of mitochondrial fatty acid oxidation. This step of alcohol liver injury may be reversible by abstinence, whereas the continuous consumption of alcohol may progress to chronic inflammation with hepatocyte apoptosis producing alcoholic hepatitis and cirrhosis **(90)**. Ethanol is metabolized to acetaldehyde by

alcohol dehydrogenase, cytochrome P450 (CYP) 2E1, and catalase. Since CYP2E1 has strong NADPH: oxidase activity, chronic alcohol abuse leads to the high-level production of ROS such as O_2^- and H_2O_2, which result in hepatocyte apoptosis **(91)**. Alcohol and its metabolites reduce the levels of certain antioxidants, such as glutathione levels in mitochondria **(92)**. The ROS formation and reduced glutathione make mitochondria more susceptible to oxidative damage **(93)**. The oxidative stress induced in the mitochondria is associated with the collapse of mitochondrial membrane potential and the onset of MPT **(94)**, leading to a release of proapoptotic factors such as cytochrome c from mitochondria. Death receptors and their ligands are involved in ethanol-induced hepatocyte apoptosis. FasL and Fas are highly expressed in alcoholic liver injury **(95, 96)**. Alcohol directly or indirectly activates Kupffer cells by the increased levels of bacterial endotoxin, leading to TNF-alpha production. The gut microflora-derived endotoxin LPS triggers both cytokine release and oxidative stress **(97)**. Alcohol also increases the sensitivity of hepatocytes to TNF-alpha **(98)**. TNFR1-deficient mice are resistant to ethanol-induced liver injury **(99)**, indicating that TNF-alpha mediates alcoholic liver injury **(100)**. Acetaldehyde is the most important metabolite of ethanol, leading to hepatocyte damage. Acetaldehyde forms adducts with intracellular proteins. In addition, acetaldehyde-protein adducts trigger an abnormal immune response characterized by the production of antibodies against acetaldehyde epitopes **(97)**.

Nonalcoholic Steatohepatitis

Nonalcoholic fatty liver disease (NAFLD) has been recognized as one of the most common forms of chronic liver disease. Patients with NAFLD modestly increase the risk of death compared with the general population **(101)**. Nonalcoholic steatohepatitis (NASH) consists of steatosis with lobular inflammation and hepatocellular ballooning, or any stage of fibrosis. Patients with simple steatosis have a benign prognosis **(102)**, whereas those with NASH have the risk of disease progression, and 15–50% of patients may develop cirrhosis **(103)** and HCC **(104)**. Hepatocyte apoptosis may be a key component of the "second hit" involved in the progression of simple steatosis to NASH. Apoptotic cells are increased in the liver of patients with NASH compared with the liver of simple steatotic patients. A positive correlation is observed between hepatocyte apoptosis and hepatic fibrosis and inflammatory activity **(105)**. Insulin resistance may increase the release of free fatty acids (FFAs) from adipose tissue. FFAs may activate Toll-like receptor-4. FFAs accumulated in the liver stimulate the synthesis of triglycerides resulting in fatty liver. Further lipid accumulation is limited by oxidation of FFAs in mitochondria, peroxisome, or microsomes. The beta-oxidation of fatty acid promotes oxidative stress, leading to mitochondrial dysfunction **(106)**. Indeed, induction of the prooxidant microsomal CYP2E1 is

increased in NASH patients **(107)**. These ROS formations play a central role in the pathogenesis of NASH **(108)**. In addition, the expression of TNFR1 **(109)** and Fas **(105)** in the liver is increased in the patients with NASH. JNK 1 but not JNK2 promotes the development of steatohepatitis in mice **(110)**.

Autoimmune Hepatitis

Autoimmune hepatitis (AIH) is characterized by the presence of interface hepatitis and portal plasma cell infiltration, hypergamma-globulinemia, and autoantibodies. Complex interactions between triggering factors, autoantigens, genetic predispositions, and immunoregulatory networks are involved in AIH **(111)**. FasL and granzyme levels are increased and associated with hepatocyte apoptosis in AIH **(112)**. Liver lymphocytes isolated from AIH patients polarize to Th1 phenotype **(113)**. The activation of resting T lymphocytes results in polyclonal activation of lymphocytes, which show cross-reactivity to foreign antigens and self-proteins **(114)**. $CD8^+$ T lymphocytes constitute the major cell type in areas of interface hepatitis, and $CD4^+$ T lymphocytes predominate in the central part of the portal tract **(115)**. The $CD8^+$ T lymphocytes have cytotoxic effects to induce hepatocyte apoptosis in AIH. In addition to CTL, plasma cells and NK cells infiltrated in the portal area are involved in relapse after steroid therapy **(116)**. B lymphocytes from peripheral blood have been reported to lyse isolated hepatocytes in an autoantibody-dependent manner **(115)**.

Cholestatic Liver Injury

Bile acids are amphipathic molecules synthesized by hepatocytes and have detergent action required for lipid absorption. When bile acid homeostasis is impaired, hepatocytes are exposed to elevated concentrations of bile acid, leading to hepatocyte cell death by necrosis and apoptosis. A high concentration of hydrophobic bile acids (>100 μM) may induce necrosis, while a low concentration may induce apoptosis **(117)**. In cholestatic patients, bile acid concentrations in the serum are not enough for detergent action but trigger cell death, suggesting that the toxicity and detergent action are independent **(118)**. Bile acid-associated death receptor-mediated apoptosis is one of the common mechanisms for cholestatic hepatocyte injury **(119)**. Toxic hydrophobic bile acids activate Fas signaling in a ligand-independent manner by altering cellular trafficking of Fas. Bile acids initiate ROS generation from mitochondria, leading to apoptosis **(120)**. Steatosis may impair bile secretory function **(121)**, and the hepatocellular retention of toxic bile acids in fat-laden hepatocytes may also provide a "second hit" through ROS generation and signaling cascades that trigger hepatocellular injury **(117)**.

Drug-Induced Liver Injury

Acetaminophen overdose is the most frequent cause of acute drug-induced liver failure. Acetaminophen induces both apoptosis and necrosis in hepatocytes **(122**, **123)**. Apoptosis is induced in primary hepatocytes when necrotic cell death is prevented by fructose and glycine treatment **(124)**. A large portion of acetaminophen is conjugated with glucuronic acid or sulfate and excreted into bile or blood. The remaining unconjugated acetaminophen is metabolized by mainly CYP2E1 to a reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI) with the concomitant formation of ROS **(122)**. Because glutathione is exhausted by NAPQI, NAPQI makes mitochondria more susceptible to oxidative damage, leading to the onset of the MPT that leads to apoptosis. A number of medications, such as corticosteroids, synthetic estrogens, amiodarone, perhexiline, nifedipine, and salicylates, cause NASH. Similarly with ethanol, these drugs damage mitochondria and induce an increased formation of ROS, causing steatohepatitis **(97**, **122)**. Some drugs, such as rifampicin and estrogen, damage hepatobiliary transporter systems, causing cholestasis **(125)**. Taken together, steatohepatitis or cholestasis may cause hepatocyte apoptosis.

Ischemia-Reperfusion Injury

Liver ischemia results from many clinical situations, such as hepatic resections, liver trauma, liver transplantation, and circulatory shock. The reduction of ATP by a decreased oxygen supply causes disturbances in membrane ion translocation that result in cell swelling and death. Upon reperfusion, the liver is subjected to a further injury that is generally more severe than the ischemia. Complex networks of hepatic and extrahepatic mechanisms are involved in the injury **(126)**. In the early phase after reperfusion, Kupffer cells are activated to produce ROS, cytokines, and chemokines **(127)**, which damage hepatocytes or lead to the infiltration and activation of inflammatory cells such as neutrophils and $CD4^+$ lymphocytes **(128**, **129)**. The activated neutrophils release ROS during the late phase of reperfusion injury **(130)**, and the T lymphocytes activate Kupffer cells. Because apoptosis requires ATP, ATP depletion promotes necrosis after ischemia. In an acute phase of liver injury after ischemia-reperfusion, necrosis, but not apoptosis, is induced **(131)**. When ATP levels are restored, caspase-dependent apoptosis is induced **(132)**. The inactivation of caspases-8 and -3 by siRNA or caspase inhibitors shows significant protection against ischemia-reperfusion injury in the liver **(133)**. Thus, both necrosis and apoptosis are involved in ischemia-reperfusion liver injury.

Hepatocellular Carcinoma (HCC)

HCC is characterized by the loss of physiological homeostasis through the activation of proliferation and the inhibition of apoptosis. The imbalance of apoptosis/survival signals **(134)** and the insufficient apoptosis are essential for hepatocarcinogenesis **(135)**. Many genetic changes are related to the insufficient apoptosis-mediated HCC development. The most common genetic alterations in HCC are mutations in the p53 gene. p53 is stabilized and activated by diverse stress stimuli, including DNA damage, oncogene activation, and hypoxia. Activated p53 induces cell cycle arrest, apoptosis, or senescence by transactivating a large panel of target genes involved in these cellular responses **(136)**. Many p53 mutations are missense and lead to the synthesis of structurally altered proteins that have lost their transactivation activity due to single amino acid changes, resulting in impaired tumor-suppressing function **(137)**. The PI3K/AKT pathway is also altered in HCC. PTEN, which blocks AKT activation by the dephosphorylation of phosphatidylinositol-3,4,5-triphosphate, is decreased in patients with HCC, and the decreased PTEN expression correlates with increased tumor grade, advanced disease stage, and poor prognosis **(138)**. Hepatocyte-specific PTEN deficiency in mice leads to liver tumorigenesis, with abnormal activation of AKT **(139)**. The activation of SphK is increased in the HCC cell line, and the inhibition of SphK activity enforces the differentiation **(140)**. The antiapoptotic Bcl-2 family, Bcl-xL, Mcl-1, and a caspase inhibitor, XIAP, are frequently overexpressed in HCCs **(141–143)**. The constitutive activation of NF-kappaB is observed more frequently in tumor tissue compared with nontumor tissue from patients with HCC **(144)**. Additionally, many apoptosis-/survival-related factors, such as hepatocyte growth factor (HGF), epithelial growth factor receptor (EGFR) ligands, THF-converting enzyme (TACE), beta-catenin, Fas, and SPREDs, have been reported to be altered in HCC **(134)**.

Summary

The liver is one of the most important organs, and the organism cannot survive without a healthy liver. Although apoptosis and subsequent regeneration contribute to physiological homeostasis, apoptosis is the essential feature that contributes to liver damage in a wide range of acute and chronic liver diseases. Thus, control of apoptosis might become a rational target of therapy in liver diseases, including hepatic cirrhosis, NASH, ischemia-reperfusion injury, and drug-induced liver failure. The features that induce hepatocyte apoptosis also suppress hepatocyte regeneration and may result in acute or chronic liver dysfunction or hepatic fibrosis. Normalizing the imbalance between cell death and regenerative responses in liver diseases might become a good therapeutic strategy. Selectively enhancing apoptosis might also become a therapy to delete cancer cells or virus-infected cells. Finally, controversies still exist because of the

complexity of proapoptosis and antiapoptosis signals in various forms of liver disease and injury. Resolution of these controversies and further research of the complex signals may modulate apoptosis in the liver and provide new strategies for the treatment of liver diseases.

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Chapter 25
Apoptosis in Acute Kidney Injury

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Abstract Acute kidney injury (AKI), also called acute renal failure, is a major kidney disease associated with high mortality. Under the disease condition, renal tubular cells are reversibly or irreversibly injured, undergoing cell death in both forms of necrosis and apoptosis. Research during the last few years has established an important role for tubular cell apoptosis in ischemic as well as nephrotoxic AKI. The mechanisms underlying tubular cell apoptosis are being elucidated. Research in this area may lead to the development of novel therapeutic strategies for the prevention and treatment of AKI.

Keywords Apoptosis · Acute kidney injury · Ischemia · Cisplatin · Nephrotoxicity

Introduction

Acute kidney injury (AKI) leads to a rapid decrease of renal function, i.e., acute renal failure. AKI can be induced in many clinical settings, including dehydration, hypotension, septic shock, trauma, operative arterial clamping, and toxicity of nephrotoxins. In the kidneys, two major intrinsic causes of AKI are renal ischemia and nephrotoxicity. Under these conditions, renal tubular cells are reversibly or irreversibly injured, leading to cell death. Despite the previous recognition of necrosis, compelling evidence has now been demonstrated for the occurrence of tubular cell apoptosis. Further investigation has suggested an important role for apoptosis in ischemic and nephrotoxic AKI. Mechanistically, tubular cell apoptosis has been shown to be induced by multiple factors and to occur via several pathways, particularly the mitochondrial and death receptor-mediated pathways. Here we discuss the current

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understanding of apoptotic mechanisms of AKI with a focus of ischemic and cisplatin-induced nephrotoxic injury.

Apoptosis in Ischemic AKI and Cisplatin Nephrotoxicity

During ischemia, kidney tissues are deprived of blood supply, leading to the depletion of oxygen and nutrients. This leads to ATP depletion or energetic failure in renal tubular cells, particularly those of the proximal tubules, due to their high metabolic rate and limited glycolytic activity. As a result, cellular homeostasis is disturbed, leading to the activation of various injurious and autolytic processes and ultimately cell death. In addition, the reperfusion of ischemic tissues can activate detrimental factors such as oxidative stress and inflammation, further exacerbating cell injury and death.

For a long time, necrosis was recognized to be the major form of tubular cell death. However, in 1992, Schumer et al. showed the presence of apoptotic bodies within renal tubules by electron microscopy in ischemia-reperfused kidneys **(1)**. In the tissues, they further demonstrated a distinct pattern of DNA cleavage indicative of apoptosis. These initial observations have been confirmed and extended by subsequent studies using a variety of examination techniques. The most commonly used method for *in vivo* apoptosis detection is TUNEL assay, which has been shown to be relatively specific for the examination of renal tissues **(2)**. Biochemically, caspase activation has been shown in ischemia-reperfused renal tissues, especially tubular cells **(3)**. In addition, the regulation of apoptotic genes, including caspases and Bcl-2 proteins, has been detected during renal ischemia-reperfusion **(4–5)**. Importantly, tubular cell apoptosis has also been shown in human kidneys under conditions of ischemia-reperfusion. For example, Castaneda et al. detected apoptosis primarily in tubular cells in cadaveric kidney allografts injured by ischemia-reperfusion **(6)**.

Interestingly, studies in animals models have revealed two phases of apoptosis in ischemia-reperfused kidneys **(7–9)**. The first phase of apoptosis peaks shortly after the ischemic injury and is considered to be important to the acute tissue damage, while the second phase appears at the recovery period, which may help eliminate the damaged cells to restore tissue homeostasis. It is noteworthy that *in vivo* detection at any given time point would underestimate apoptosis, because **(1)** cells in tissues are quite heterogeneous and apoptosis is not synchronized and **(2)** apoptotic cells are rapidly cleared up or phagocytosed by macrophages and neighboring cells. It is also noteworthy that, despite earlier controversy regarding the site of apoptosis, more recent studies have shown apoptosis in both the renal cortex and the outer medulla. Apoptosis is detected in several segments of the renal tubule, including proximal tubules, distal tubules, and Henle's loop **(3, 10–14)**.

Similarly, renal cell apoptosis has been detected during cisplatin nephrotoxicity. Cisplatin is one of the most potent and widely used chemotherapy drugs

for cancer treatment. However, the therapeutic efficacy of cisplatin is limited by its side effects in normal tissues and organs, especially nephrotoxicity in the kidneys. About one third of patients develop renal functional defects during or soon after cisplatin-based chemotherapy. One major pathological damage during cisplatin nephrotoxicity is tubular cell injury and death, including both necrosis and apoptosis **(15–16)**.

In 1998, Megyesi et al. showed one of the first *in vivo* pieces of evidence for tubular cell apoptosis during cisplatin nephrotoxicity in mice **(17)**. Using TUNEL assay, they detected a cisplatin treatment time-dependent increase of apoptotic cells in renal tubules. These observations have been confirmed and extended by other investigators **(18–21)**. Of note, although in their initial study Megyesi et al. indicated that cisplatin-induced apoptosis was restricted to distal tubules, later studies have also suggested the occurrence of apoptosis in proximal tubular cells **(18–21)**. Our recent work has further identified the cell type(s) of apoptosis using proximal and distal tubule-specific lectin markers **(18)**. It was shown that many apoptotic cells (TUNEL-positive) were stained by PHA, a proximal tubule-binding lectin, whereas significantly fewer apoptotic cells were stained by PNA, a distal tubule-binding lectin. Thus, during cisplatin nephrotoxicity, apoptosis occurs in both tubular segments, but mainly in the proximal tubules.

Taken together, these studies have demonstrated convincing evidence for renal cell apoptosis during ischemic and cisplatin nephrotoxic AKI. Depending on the experimental or clinical conditions, apoptosis occurs in different segments of the renal tubule, particularly the proximal and distal tubules.

Apoptotic Pathways Activated During Ischemic AKI and Cisplatin Nephrotoxicity

Depending on the triggering signals, cells can undergo apoptosis by multiple pathways, including the intrinsic and extrinsic pathways. In the intrinsic pathway, the mitochondrial outer membrane is permeabilized, leading to the release of apoptogenic factors such as cytochrome c. Cytochrome c then binds to Apaf-1 in the cytosol and recruits and activates caspase-9. In the extrinsic pathway, ligand engagement of death receptors (e.g., Fas) at the plasma membrane leads to the recruitment and activation of caspase-8. Studies during the last few years have demonstrated the activation of both the intrinsic and extrinsic pathways under the pathological conditions of AKI.

Apoptotic Pathways in Ischemic AKI

In 1998, using *in vitro* models of ischemia-reperfusion, we demonstrated the first evidence of mitochondrial outer membrane permeabilization in renal tubular

cells. Importantly, it was shown that Bax translocated to mitochondria, and the translocation was critical to the outer membrane permeabilization and subsequent release of cytochrome c, caspase activation, and apoptosis **(22)**. Further studies indicate that Bax, after translocation to mitochondria, forms oligomers with Bak, presumably leading to porous defects for mitochondrial leakage. Notably, the upregulation of Bcl-2 either pharmacologically or by gene transfection blocks Bax/Bak activation or oligomerization, resulting in the preservation of mitochondrial integrity and cell viability **(22–25)**. Similar results are shown for Bcl-xL, an antiapoptotic Bcl-2 homologue **(25–26)**. These findings have been confirmed and extended to *in vivo* models of renal ischemia-reperfusion. For example, Kelly et al. detected cytochrome c release in ischemically injured kidneys in rats **(27)**. In humans, the intrinsic pathway of apoptosis seems to be a key to apoptotic cell death under conditions of renal ischemia-reperfusion. Castaneda et al. detected tubular cell apoptosis in ischemically injured cadaveric kidney allografts **(6)**. Notably, in the allografts, Bax and Bak were expressed predominantly in apoptotic cells, associated with cytochrome c release. Together, these *in vitro* and *in vivo* studies have established a role for the intrinsic pathway in tubular cell apoptosis during renal ischemia-reperfusion.

Compared with the intrinsic pathway, evidence for the involvement of the extrinsic pathway of apoptosis in renal ischemic injury is limited. Nevertheless, using a unilateral clamping mouse model, Nogae et al. showed that Fas was upregulated during renal ischemia-reperfusion in distal tubular cells, accompanied by apoptosis in these cells **(28)**. Notably, apoptosis was reduced in Fas mutant (lpr/lpr) mice. The role of Fas was further suggested by RNA interference studies. Hamar et al. showed that a single hydrodynamic injection of Fas interference RNA could downregulate Fas in the kidney, leading to significantly lower apoptosis and better renal function following renal ischemic injury **(29)**. Consistently, Du et al. showed that inferior vena cava delivery of Fas interference RNA protected against renal injury during ischemia-reperfusion **(30)**. These findings suggest that the extrinsic pathway of apoptosis, particularly that mediated by Fas, contributes to ischemic kidney injury. However, in human cadaveric kidney allografts, Fas expression does not show a significant correlation with tubular cell apoptosis following ischemic injury, raising questions about the role of the extrinsic pathway of apoptosis in clinical settings of ischemic renal failure **(6)**.

Apoptotic Pathways Activated During Cisplatin Nephrotoxicity

Similar to ischemic renal injury, cisplatin nephrotoxicity also involves multiple apoptotic pathways, including the extrinsic pathway mediated by death receptors, the intrinsic pathway involving mitochondria, and the endoplasmic reticulum (ER)-stress pathway.

Direct evidence for a role of the extrinsic pathway in cisplatin nephrotoxicity was initially suggested by Ramesh and Reeves **(31)**. They showed that after cisplatin treatment, there was massive induction of TNFα in mice. Importantly, the pharmacological and genetic inhibition of TNFα attenuated the production of several inflammatory cytokines, resulting in amelioration of cisplatin nephrotoxicity. Despite these observations, it has been controversial as to which TNFα receptor (TNFR) is critical to cisplatin nephrotoxicity. Ramesh et al. showed that TNFR2 is important in cisplatin nephrotoxicity, but Tsuruya et al. suggested TNFR1 plays an important role **(21, 32)**. It is noteworthy that TNFα may regulate cisplatin injury by regulating the inflammatory response instead of directly activating the TNFR-mediated apoptosis. The role of Fas, another death receptor, has not been studied in detail during cisplatin nephrotoxicity, although Razzaque et al. showed an upregulation of Fas and Fas ligand after cisplatin treatment of cultured proximal tubular cells **(33)**.

In contrast, the intrinsic pathway has emerged as the major apoptotic pathway in cisplatin nephrotoxicity. Lee et al. and Park et al. demonstrated Bax activation during cisplatin treatment of cultured renal cells, which was followed by cytochrome c release, activation of caspase-9, and apoptosis **(34–35)**. Importantly, the overexpression of Bcl-2 diminished Bax activation and ameliorated mitochondrial injury and cisplatin-induced apoptosis. Using mouse models, we have recently demonstrated compelling evidence for renal tubular apoptosis via the mitochondrial pathway during cisplatin nephrotoxicity *in vivo*. In C57BL/6 mice, Bax was induced, activated, and further accumulated in mitochondria in renal tubular cells **(18)**. Importantly, Bax-deficient mice showed significantly lower apoptosis following cisplatin treatment and were resistant to cisplatin-induced renal failure. In addition, primary cultures of tubular cells isolated from Bax-deficient mice were resistant to cisplatin-induced apoptosis, indicating that Bax plays a very important role during cisplatin nephrotoxicity **(18)**. How Bax is activated under the pathological condition is not entirely clear. Nevertheless, the balance between the pro- and antiapoptotic Bcl-2 family proteins shifts in favor of cell death. Notably, PUMAα, a proapoptotic BH3-domain-only protein, is induced both *in vitro* and *in vivo* during cisplatin treatment **(36)**. PUMAα then translocates to the mitochondria and binds antiapoptotic proteins like Bcl-xL. This leads to the neutralization of antiapoptotic proteins and results in Bax activation and subsequent apoptosis **(36)**. Interestingly, our recent work has further demonstrated a striking morphological change of mitochondria, i.e., mitochondrial fragmentation, during cisplatin-induced apoptosis **(37)**. Importantly, the inhibition of mitochondrial fragmentation can abrogate apoptosis, suggesting that the morphological change may contribute to apoptosis during cisplatin nephrotoxicity **(37)**.

Cisplatin-induced tubular cell apoptosis may also involve the ER-stress pathway. ER stress activates caspase-12, which is localized at the cytosolic face of the ER. Using an *in vitro* model, Liu et al. demonstrated caspase-12 activation during cisplatin treatment of cultured tubular cells **(38)**. Importantly, transfection of an anti-caspase-12 antibody significantly ameliorated cisplatin-induced apoptosis in

this model. *In vivo,* in a rat model of cisplatin nephrotoxicity, caspase-12 cleavage and activation have been reported **(39)**. Also, Cummings et al. showed that the pharmacological inhibition of ER- associated $iPLA_2$ led to an amelioration of cisplatin-induced apoptosis in cultured tubular cells **(40)**. Despite these findings, the role and regulation of ER stress during cisplatin nephrotoxicity remain to be determined.

Upstream Triggers of Apoptosis in Ischemic and Cisplatin Nephrotoxic AKI

Apoptotic Triggers in Ischemic Renal Injury

The exact trigger(s) of apoptosis during ischemic renal injury is not clear, but it may involve several detrimental events.

Energy Depletion

High-energy phosphate in the form of ATP is required for various activities within the cell, from maintenance of ion homeostasis and biochemical regulation to synthesis and proliferation. In mammalian cells, ATP is produced mainly in two ways: oxygen-dependent oxidative phosphorylation and anaerobic glycolysis. During ischemia, cells are deprived of nutrients and oxygen, inactivating both glycolysis and oxidative phosphorylation, resulting in a rapid decline of ATP. Consequently, all the energy-dependent cellular activities are disturbed **(41–43)**. In renal tubular cells, ATP depletion can activate Bax, leading to the activation of the intrinsic pathway of apoptosis **(22, 44)**.

Disturbance in Ion Homeostasis

Ion homeostasis is critical for cell physiology; the loss of ion homeostasis is usually associated with the development of cell injury and death. During ischemia, the deprivation of ATP blocks the activity of various ion pumps, resulting in the loss of cellular ion homeostasis and finally leading to cell death **(45)**. The uncontrolled Ca^{2+} in the cytosol will cause mitochondria permeabilization and consequent cell death **(46)**. In kidney cells, the blockage of calcium influx has been reported to attenuate mitochondrial injury and apoptosis *in vitro* **(47)**.

Reactive Oxygen Species (ROS) Accumulation

Reactive oxygen species such as hydroxyl radical, superoxide, and H_2O_2 are cytotoxic when those radicals are above physiological levels. ROS can induce the oxidation of proteins, lipids, DNA, etc., resulting in apoptosis **(48)**. During ischemia, a lack of oxygen leads to the cessation of mitochondrial respiration

and the reduction of ubiquinone (complexes I and III), which may generate superoxide in the presence of residual oxygen. However, the production of oxidative radicals in the ischemic period is usually limited, due to low availability, and ultimately the lack of oxygen. On the other hand, significant amounts of oxidative free radicals are generated when the blood flow is restored during the reperfusion period, which may account in large part for cell injury caused by reperfusion **(49)**. In support of the role of ROS, SOD-1-deficient mice were shown to be more susceptible to ischemic renal injury, and several oxidant scavengers were reported to ameliorate ischemic AKI with less apoptosis in renal tissues **(50–52)**.

Inflammation

Ischemic AKI involves complement activation, the generation of cytokines and chemokines, and infiltration of leukocytes to the kidney. Some signaling pathways activated by those cytokines and chemokines can mediate both inflammation and apoptosis **(53–54)**. Although the original call for leukocytes is to clean up dead cells and debris, inflammation by itself leads to the secondary damage of tissue. Leukocytes may induce cell injury by producing numerous toxic factors. Also, the accumulation of inflammatory cells may occlude blood vessels and block blood flow to the ischemic region, worsening the degree of ischemia **(55)**.

Induction and Activation of Apoptotic Genes

An important trigger of apoptosis during ischemic AKI may be the induction and activation of apoptotic genes. In a microarray analysis of ischemic renal tissues, Supavekin et al. detected the upregulation of various transcription factors, growth factors, signal transduction molecules, and proapoptotic genes such as FADD, DAXX, BAD, BAK, and p53 **(56)**. Since the balance between pro- and antiapoptotic Bcl-2 family proteins is a critical determinant of cell death and survival, several studies examined the expression of these proteins during renal ischemia-reperfusion. Basile et al. showed that both Bcl-2 and Bax were induced in a rat model of ischemic AKI **(5)**. Gobe et al. further showed that the pattern of Bcl-2 family protein expression is different in distal tubules and proximal tubules **(57–58)**. Bax was shown to be markedly increased in proximal tubules following renal ischemia, with a moderate increase in Bcl-xL **(58)**. However, the expression of Bcl-2 family proteins may be related to the severity of renal injury. In a rat model of moderate ischemic injury, we detected an immediate increase in Bax/Bak and a mild increase in Bcl-2, while Bcl-xL was induced first but decreased after 48 hours of reperfusion. In a mouse model with severe injury, all the Bcl-2 family proteins examined decreased (Wei and Dong, unpublished). In addition to Bcl-2 family proteins, apoptotic factors of the extrinsic pathway may also be subjected to expression regulation **(28, 29, 56, 59)**. For example, Fas expression is greatly induced by renal ischemia/reperfusion **(28)**. Zf9, a transcription factor to regulate TGFβ-1, was shown to be maximally

induced during I/R injury and also was suggested to be critical for the early apoptotic response **(60)**. At a downstream level, caspase expression has also been reported to be altered during renal ischemia-reperfusion **(4)**.

Recent studies have suggested a role for Bid in the activation of tubular cell apoptosis during renal ischemia-reperfusion. Bid activation was detected both in ATP-depletion-induced kidney cell apoptosis *in vitro* and in ischemic kidney injury *in vivo* **(3, 61)**. Although Bid was found to be cleaved mainly by caspase-9 *in vitro* during reoxygenation, the activation of Bid *in vivo* occurred during ischemia and early reperfusion **(61)**. Importantly, Bid-deficient mice were protected from ischemic renal injury, suggesting that Bid may have an important role in the initiation of tubular cell apoptosis during renal ischemia-reperfusion **(3)**.

Upstream Events Leading to Tubular Cell Apoptosis During Cisplatin Nephrotoxicity

In cells, cisplatin is aquated and probably metabolically activated, becoming highly reactive, leading to the activation of multiple injurious events or factors **(16)**.

ROS

Cisplatin can induce oxidative stress via several pathways. First and foremost, cisplatin reacts with thiol-containing compounds, resulting in depletion of important antioxidants like glutathione and ROS accumulation **(16)**. Second, cisplatin can also induce mitochondrial dysfunction, leading to higher ROS production by the disrupted respiratory chain complexes **(62)**. Finally, cisplatin can induce ROS formation in the microsomes via the cytochrome P450 complex **(63–64)**. The inhibition of ROS production during cisplatin treatment results in reduced apoptosis in both *in vitro* and *in vivo* conditions **(15, 16, 65–67)**. Various signaling pathways, including MAPK and p53, have been suggested to be regulated by ROS **(66–68)**. Despite this evidence, the exact mechanism and targets of ROS in this pathological condition are still not clear.

DNA Damage

Genomic DNA is considered one of the major targets of cisplatin. Cross-linking DNA by cisplatin leads to a blockade of replication and transcription **(69)**. If the DNA damage is extensive, the cell undergoes apoptosis. In renal tubular cells, the tumor suppressor protein p53 has emerged as the key signaling molecule that couples the DNA damage response to apoptosis. Cummings and Schnellmann showed that pifithrin-α, a pharmacological inhibitor of p53, could partially suppress cisplatin-induced apoptosis **(70)**. These preliminary

observations were further extended by a series of studies using both *in vitro* and *in vivo* models **(19, 36, 71, 72)**. The emerging picture is that the DNA damage response proteins ATR and Chk2 are activated early during cisplatin nephrotoxicity. ATR-Chk2 then activates and stabilizes p53 by phosphorylation, leading to the transactivation of various proapoptotic proteins, including PUMA-α and PIDD **(36, 72, 73)**. PUMA-α accumulates in the mitochondria, where it neutralizes antiapoptotic proteins like Bcl-xL, resulting in Bax/Bak activation and the mitochondrial release of cytochrome c **(36)**. PIDD, on the other hand, may activate caspase-2, resulting in apoptosis-inducing factor (AIF) release from mitochondria **(73)**.

CDK2-p21 Pathway

Proteins involved in cell cycle regulation modulate renal cell death and survival during cisplatin nephrotoxicity **(74)**. In this regard, the cyclin-dependent kinase-2 (cdk2) and its inhibitor p21 have been suggested to be important regulators of renal injury during cisplatin nephrotoxicity. Price, Safirstein, and colleagues have shown in a series of studies that renal cell injury is dependent on the balance between cdk2 and p21 **(17, 19, 75–77)**. p21 is induced both *in vivo* and *in vitro* during cisplatin nephrotoxicity **(17)**. Importantly, the p21 knockout mice are hypersensitive to cisplatin nephrotoxicity. The overexpression of p21 in cultured cells makes them resistant to cisplatin-induced apoptosis **(76)**. It has also been shown that cdk2 is activated during cisplatin nephrotoxicity and that p21 overexpression inhibits cdk2 activation **(75)**. Also, cdk2 gene knockout or pharmacological inhibition significantly ameliorates cisplatin-induced renal injury. Recent studies have suggested that E2F1 is an important downstream target of cdk2 during cisplatin nephrotoxicity **(77)**. These studies have shown that the cdk2-E2F1 pathway plays a proapoptotic role during cisplatin nephrotoxicity and that p21 inhibits this pathway. However, it remains unclear how CDK2 is activated during cisplatin nephrotoxicity and how it triggers apoptosis.

Inflammation

A robust induction of proinflammatory cytokines is associated with cisplatin nephrotoxicity. Earlier studies by Deng et al. showed that anti-inflammatory cytokines like IL-10 could significantly reduce renal injury during cisplatin treatment **(78)**. Later studies by Ramesh and Reeves identified the upstream regulators of inflammation during cisplatin-induced renal injury **(31, 79)**. TNF-α was shown to be the central regulator of various cytokines during cisplatin treatment **(31)**. TNF-α is induced during cisplatin nephrotoxicity and then upregulates various other inflammatory cytokines. Importantly, TNF-α knockout mice and

the pharmacological inhibition of TNF-α ameliorate cisplatin-induced renal cell death **(31, 67, 80)**. These studies show that TNF-α is the central regulator of inflammation during cisplatin nephrotoxicity. However, whether TNF-α directly induces apoptosis and whether TNFR1 or 2 is involved are currently unclear **(21, 32)**.

Inhibition of Apoptosis for the Prevention and Treatment of AKI

Based on the role played by apoptosis in ischemic and nephrotoxic renal injury, new therapeutic strategies for AKI have been proposed. By blocking apoptotic pathways, various reagents were shown to be renoprotective in experimental models of renal ischemia-reperfusion **(25, 27, 29, 43, 50, 51, 81–94)**. Genetic knockout of apoptotic genes such as Bid protected the animal, while a deficiency of antiapoptotic PPARβ/δ showed worsened renal dysfunction **(3, 95, 96)**. In addition, both the RNA interference of Fas and the overexpression of Bcl-2 significantly reduced renal cell apoptosis in experimental models of ischemia-reperfusion **(29, 97)**. Pharmacologically, a variety of reagents were shown to diminish tubular cell apoptosis and protect against ischemic renal injury, by blocking p53, inhibiting caspases, reducing oxidative stress, or activating antiapoptotic proteins, including HSP70, Akt, and hepatocyte growth factor **(27, 53, 81, 83, 84, 86, 90, 92, 93, 98, 99)**. More recent studies have further identified novel renoprotective reagents that attenuate tubular cell apoptosis during renal ischemia-reperfusion, including erythropoietin, minocycline, and furosemide **(25, 27, 81, 85, 86, 88, 93)**.

In experimental models of cisplatin nephrotoxicity, earlier studies showed that knockout of TNFR1 or TNFR2 led to the preservation of renal histology and function **(21, 32)**. It was also shown that Bcl-2 overexpression could almost completely ameliorate cisplatin-induced renal cell apoptosis **(97)**. Moreover, the knockout of Bax in mice results in a cisplatin injury-resistant phenotype **(18)**. Importantly, the inhibition of p53 and the consequent blockade of apoptotic gene induction provided significant renoprotective effects in mice **(19)**. Caspase-1 and caspase-12 knockdown also conferred resistance to cisplatin-induced apoptosis in renal tubular cells **(38, 100)**. In addition, VAD, the pan-caspase inhibitor, was able to partially decrease cisplatin-induced renal cell apoptosis, although its *in vi*vo effects remain to be demonstrated **(70–71)**.

Collectively, these studies have suggested that blocking apoptosis may offer a promising strategy for renoprotection during ischemic and cisplatin nephrotoxic AKI. Certainly, further research needs to thoroughly evaluate this possibility through additional experimental and clinical investigations. Of note, the studies conducted thus far have shown significant, but partial, renoprotection, indicating that the antiapoptosis strategy must be combined with other therapies for the treatment of AKI.

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Chapter 26
Apoptosis in Cancer Biology and Cancer Therapeutics

Simone Fulda

Abstract Most anticancer therapies commonly used in the treatment of human cancer, e.g., chemotherapy, γ-irradiation, immunotherapy, or suicide gene therapy, kill tumor cells by triggering cell death pathways including apoptosis in cancer cells. Hence, the failure to activate such pathways may lead to the resistance of cancers to current treatment approaches. A better understanding of the molecular events that regulate apoptosis in cancers and upon cancer therapy provides the basis for a more rational approach to developing molecular targeted therapies in the fight against cancer.

Keywords Cancer · Apoptosis · Cancer therapy · Bcl-2 family proteins · BH3 mimetics · Death receptor · TRAIL · IAPs

Introduction

Programmed cell death or apoptosis is the cell's intrinsic death program that plays an important role in various physiological and pathological situations and is highly conserved throughout evolution **(1)**. Tissue homeostasis is maintained by a subtle balance between proliferation on one side and cell death on the other side **(2)**. As a consequence, too little apoptosis can contribute to tumor formation **(3)**. In addition, defects in apoptosis programs may confer resistance to cytotoxic therapies, since the response of cancer cells to current treatment approaches is, to a large extent, due to their ability to undergo cell death in response to cytotoxic stimuli **(4–6)**. Thus, further insight into the regulation of apoptosis pathways upon cancer therapy will provide a molecular basis for the design of new strategies targeting apoptosis in cancer cells and opens new perspectives to overcome apoptosis resistance in a variety of human cancers.

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Apoptosis Pathways

Under most circumstances, the activation of apoptosis pathways by anticancer therapy eventually leads to the activation of caspases **(7)**. The activation of caspases can be initiated at different sites, e.g., at the plasma membrane upon ligation of death receptors or at the level of the mitochondria **(1)**. Stimulation of death receptors of the tumor necrosis factor (TNF) receptor superfamily such as CD95 (APO-1/Fas) or TRAIL receptors results in activation of the initiator caspase-8, which can propagate the apoptosis signal by direct cleavage of downstream effector caspases such as caspase-3 **(8)**. The mitochondrial pathway is initiated by the release of apoptogenic factors such as cytochrome c, apoptosis-inducing factor (AIF), Smac/DIABLO, or Omi/HtrA2 from the mitochondrial intermembrane space **(9)**. The release of cytochrome c into the cytosol results in caspase-3 activation through formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex. Smac/DIABLO or Omi/HtrA2 promotes caspase activation through neutralizing the inhibitory effects of IAPs **(9)**. Most cytotoxic drugs are considered to primarily initiate cell death by triggering a cytochrome c/Apaf-1/caspase-9-dependent pathway linked to mitochondria **(10)**. However, the concept of mitochondria being the central initiator to integrate stress stimuli into an apoptotic response has also been challenged by recent reports showing that a functional apoptosome is dispensable for stress-induced apoptosis. To this end, the activation of caspases, e.g., caspase-2, in response to cellular stress was found to be required for mitochondrial permeabilization **(11)**. Because of the potential detrimental effects on cell survival in case of inappropriate caspase activation, the activation of caspases has to be tightly controlled. The antiapoptotic mechanisms regulating cell death have also been implicated in conferring drug resistance to tumor cells. Although caspases are crucial for cell death execution in many systems, caspase-independent apoptosis as well as nonapoptotic modes of cell death, e.g., necrosis and autophagy, also have to be considered **(12)**. The activation of these nonapoptotic forms of cell death presents an alternative strategy to trigger cell death in apoptosis-resistant types of human cancers **(13)**.

Apoptosis and Cancer Biology

A hallmark of human cancers is that they have developed various strategies to evade apoptosis. In principle, signaling to cell death can be blocked by an increase in antiapoptotic molecules and/or by a decrease or defective function of proapoptotic proteins. The following two sections discuss examples of alterations in the death receptor and mitochondrial pathway that may occur in human cancers (Fig. 26.1).

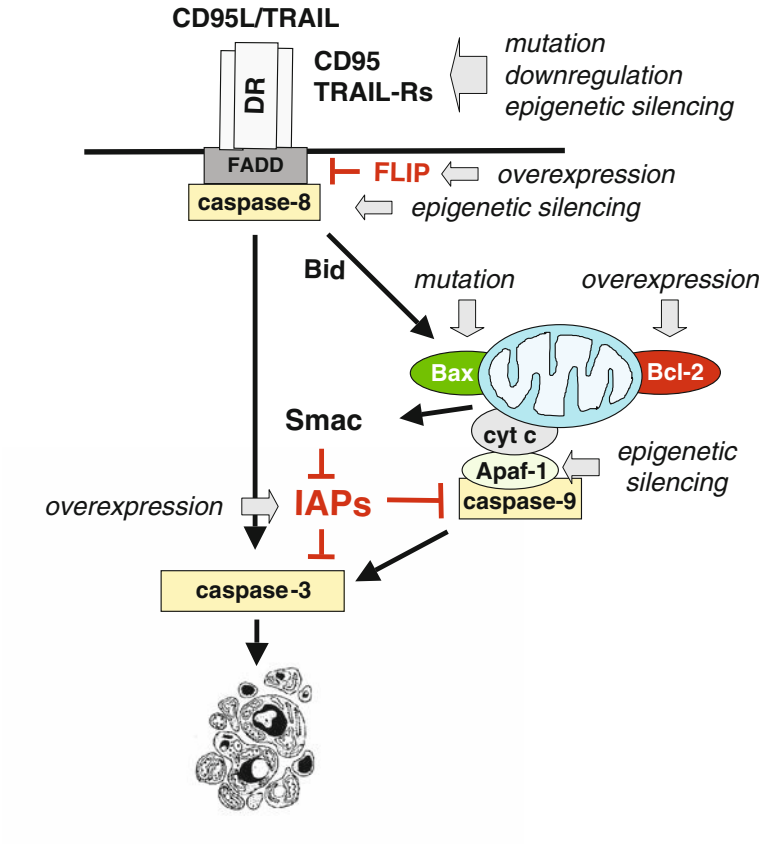

Fig. 26.1 Defects in apoptosis pathways in human cancers. Signaling via the death receptor pathway may be impaired by mutations, downregulation, epigenetic silencing of death receptors (DR) such as CD95 or TRAIL receptors (TRAIL-Rs), overexpression of FLIP, or epigenetic silencing of caspase-8. The mitochondrial pathway may be blocked by the overexpression of antiapoptotic Bcl-2 family proteins, Bax mutations, epigenetic silencing of Apaf-1, or high levels of inhibitor-of-apoptosis proteins (IAPs). See the text for more details

Defects in the Death Receptor Pathway in Human Cancers

At the receptor level, the surface expression of death receptors may be decreased or completely absent in resistant forms of cancers. The downregulation of CD95 expression was found in drug-resistant leukemia or neuroblastoma cells, suggesting that critical levels of CD95 expression may determine drug sensitivity **(14**, **15)**. In addition, defective transport of the apoptosis-inducing TRAIL receptors TRAIL-R1 and TRAIL-R2 from intracellular stores such as the endoplasmatic reticulum to the cell surface was shown to confer resistance

to TRAIL in colon carcinoma **(16)**. Moreover, mutations of the CD95 gene were detected in hematological malignancies as well as in solid tumors **(17)**. In the TRAIL system, loss of expression of the agonistic TRAIL receptors TRAIL-R1 and R2 can account for TRAIL resistance. Both receptors are located on chromosome 8p, which is frequently affected by the loss of heterozygosity (LOH) in human cancers **(18)**. Also, deletions or mutations were detected in a small percentage of cancers, e.g., non-Hodgkin's lymphoma, colorectal, breast, head, and neck cancer, osteosarcoma, or lung carcinoma, leading to the loss of both copies of TRAIL-R1 or R2 **(19**, **20)**. Death receptor signaling can also be impaired by a series of decoy receptors. For example, the genetic amplification or overexpression of decoy receptor 3 (DcR3) was identified in lung or colon carcinoma and glioblastoma as a mechanism to counteract CD95-triggered apoptosis by competitively binding CD95 ligand **(21**, **22)**. TRAIL-R3, a decoy receptor for TRAIL, was reported to be overexpressed in gastric carcinoma **(23)**.

Alternatively, epigenetic changes such as CpG-island hypermethylation of gene promoters may contribute to the impaired surface expression of death receptors **(24**, **25)**. Also, epigenetic changes in CD95 expression may favor immune escape, as the restoration of CD95 expression by histone deacetylase inhibitors in tumors with epigenetically silenced CD95 resulted in the restoration of chemosensitivity in an NK cell-dependent manner **(26)**.

Death receptor signaling may also be dampened by molecules that associate with their cytoplasmatic domains and interfere with the recruitment of procaspase-8 or -10 to the DISC, e.g., FLIP or phosphoprotein enriched in diabetes/phosphoprotein enriched in astrocytes-15 kDa (PED/PEA-15) **(27**, **28)**. High FLIP expression is a characteristic feature of many tumors and has been correlated with a resistance to death receptor- and also to chemotherapy-induced apoptosis **(29**, **30)**. Further, caspase-8 expression may be impaired by epigenetic mechanisms in cancer cells. To this end, caspase-8 expression was found to be inactivated by the hypermethylation of regulatory sequences of the caspase-8 gene in a number of different tumor cells derived from neuroblastoma, malignant brain tumors, Ewing tumor, retinoblastoma, rhabdomyosarcoma, or small lung cell carcinoma both *in vitro* and *in vivo* in primary tumor samples **(31**–**35)**. It is interesting to note that co-methylation for caspase-8 and FLIP and also for the pairs of agonistic TRAIL receptors TRAIL-R1/TRAIL-R2 or antagonistic TRAIL receptors TRAIL-R3/TRAIL-R4 was detected in neuroblastoma, suggesting that these genes are not randomly targeted by methylation **(36)**.

Defects in the Mitochondrial Pathway in Human Cancers

Alterations in genes or proteins involved in the regulation of the mitochondrial pathway frequently occur in human cancers. For example, the overexpression of Bcl-2 is present in approximately 85% of human follicular lymphoma and is

the result of chromosomal translocation of the *bcl*-2 oncogene into the immunglobulin heavy-chain gene locus **(37)**. Experiments with transgenic mice showed that Bcl-2 overexpression promotes the neoplastic transformation of B and T lymphocytes as well as of myeloid cells **(38, 39)**. Moreover, somatic mutations that inactivate the proapoptotic *bax* gene have been found in certain solid tumors and hematological malignancies. To give an example, single-nucleotide substitution or frameshift mutations of the *bax* gene were described in mismatch repair-deficient (MMR) colon cancer or hematopoietic malignancies **(40, 41)**. Furthermore, BH3-only proteins can function as bona fide tumor suppressors and may contribute to the suppression of malignant transformation. To this end, the loss of a single allele of Bim was shown to accelerate B-cell lymphomagenesis induced by the expression of a *c-myc* transgene **(42)**. It is also interesting to note that homozygous deletions in the chromosomal region of the *bim* gene were identified in patients with mantle cell lymphoma **(43)**. Mice lacking the *bid* gene spontaneously develop a myeloproliferative disorder that may eventually progress to a chronic myelomonocytic form of leukemia **(44)**. Besides alterations in Bcl-2 family proteins, the mitochondrial pathway of apoptosis may also be impaired in human cancers at the postmitochondrial level, e.g., because of the decreased, or absent, activity of Apaf-1 as reported in melanoma and leukemia **(45, 46)**.

Moreover, defective signaling via either the death receptor or the mitochondrial pathway in human cancers may be caused by the aberrant expression of IAP proteins, e.g., due to increased mRNA or protein expression or the loss of endogenous antagonists such as XAF1 **(47–49)**. In addition, XIAP stability can be enhanced in response to phosphorylation by Akt **(50)**. The co-expression of survivin or the overexpression of other IAPs may function as a sink for Smac, thereby freeing XIAP to inhibit effector caspases **(51, 52)**. In gene-profiling studies, survivin was found to represent the fourth-most common transcriptome of the human genome, while survivin was not expressed in normal adult tissues, indicating that survivin may contribute to the malignant phenotype of cancer cells **(53)**. The *cIAP2*gene is affected by the t(11;18)(q21;q21) translocation, which occurs in 50% of mucosa-associated lymphoid tissue (MALT) lymphoma **(54)**.

Apoptosis and Cancer Therapy

Based on the concept that the evasion of apoptosis is a hallmark of human cancers that contributes to tumor formation and progression, many efforts have been made over recent years to develop strategies that trigger programmed cell death in cancer cells (Table 26.1). Besides the direct activation of cell death pathways in cancer cells, apoptosis-targeted therapies may also enhance the responsiveness of human cancers toward conventional treatments that are currently used in the clinic, e.g., chemo- or radiotherapy, since these therapies primarily exert their antitumor activity by inducing apoptosis in cancer cells.

Table 26.1 Examples of apoptosis targeting cancer therapeutics

Name	Clinical trial	Cancer type	Single/Combined	References
(1) TRAIL receptor agonists				
• TRAIL	Phase I	Solid tumors, NHL	Single	**(79)**
• TRAIL-R1 mAb	Phase I	Solid tumors	Single, combination with chemotherapeutics	**(77, 81, 82)**
• TRAIL-R2 mAb	Phase I	Solid tumors	Single	**(78)**
(2) Bcl-2/Bcl-xL targeting agents				
• Bcl-2 antisense	Phases I-III	Solid tumors, leukemia/ lymphoma	Combination (chemotherapy)	**(83)**
• Bcl-2/Bcl-xL inhibitor	Phase I	Leukemia	Single	
(3) XIAP targeting agents				
• XIAP antisense	Phase I/II	Solid tumors, AML	Single	**(117)**

Cancer Therapeutics Targeting the Death Receptor Pathway

The concept to selectively trigger death receptors to induce cell death in cancer cells is attractive for cancer therapy, since death receptors are directly linked to the cell's intrinsic death machinery **(55)**. However, the systemic administration of CD95 ligand or TNFα has been hampered by severe toxicities **(8)**. In contrast, TRAIL turned out to be a more promising candidate for clinical development, since TRAIL predominantly kills cancer cells while sparing normal cells **(18)**. The underlying mechanisms for the differential sensitivity of malignant versus nonmalignant cells for TRAIL have not exactly been defined and may be related to intracellular patterns of pro- and antiapoptotic molecules **(18)**. Notably, studies in nonhuman primates such as chimpanzees and cynomolgus monkeys showed no toxicity upon intravenous infusion, even at high doses **(56)**. In addition, TRAIL exhibited no cytotoxic activity in a variety of normal human cells of different lineages, including fibroblasts, endothelial cells, smooth muscle cells, epithelial cells, or astrocytes **(57)**. However, some concerns about potential toxic side effects on human hepatocytes or brain tissue have also been raised **(58, 59)**, which may be related to the TRAIL preparations used in these studies. Thus, TRAIL preparations, which are antibody-cross-linked or not optimized for Zn content, may overpass the threshold of sensitivity of normal cells by forming multimeric aggregates **(18)**. Importantly, recent evidence suggests that TRAIL can induce survival and proliferation under certain circumstances, e.g., in cancer cells resistant to TRAIL-induced apoptosis, which involves activation of the transcription factor NF-κB **(60)**. These findings

suggest that the death-inducing ligand TRAIL might paradoxically promote tumor growth in TRAIL-resistant tumors.

Recombinant soluble TRAIL triggered apoptosis in a wide range of cancer cell lines and also *in vivo* in several xenograft models of human cancers **(18)**. Also, monoclonal antibodies targeting TRAIL receptors TRAIL-R1 or TRAIL-R2 demonstrated potent antitumor activity **(61, 62)**. Interestingly, TRAIL-R2 antibody-based therapy was recently reported as an efficient strategy not only to eliminate TRAIL-sensitive tumor cells, but also to induce tumor-specific T-cell memory, which afforded long-term protection from tumor recurrence **(63)**. In addition to recombinant soluble TRAIL ligand, several gene therapy approaches have been developed to specifically target tumor cells. Using the hTERT promoter, an adenoviral vector-based system resulted in high levels of TRAIL expression and apoptosis specifically in breast cancer cells, whereas only minimal transgenic expression and toxicity were detected in normal human primary mammary epithelial cells **(64)**. The intralesional administration of adenoviral TRAIL effectively suppressed the growth of human breast cancer xenografts, resulting in the long-term, tumor-free survival of mice **(64)**.

Although many tumors express both agonistic TRAIL receptors, they often remain resistant to induction by TRAIL, which has been related to the dominance of antiapoptotic signals. Importantly, a synergistic interaction between TRAIL and chemotherapy or γ-irradiation was found in various cancers, including malignant glioma, melanoma, leukemia, and breast, colon, or prostate carcinoma **(65–70)**. Remarkably, TRAIL and anticancer agents also cooperated to suppress tumor growth in mouse models of human cancers **(71, 72)**. The molecular mechanisms that account for this synergistic interaction may include the transcriptional upregulation of the agonistic TRAIL receptors TRAIL-R1 and TRAIL-R2, which has been reported to occur in a p53-dependent or -independent manner **(73, 74)**. Recent evidence suggests that p53 is crucial for sensitization to TRAIL by chemotherapy through the transcriptional upregulation of TRAIL-R2 in some tumors, e.g., mismatch repair-deficient colorectal cancer cells harboring Bax mutations **(75)**. Intriguingly, preexposure to chemotherapy restored TRAIL sensitivity through the p53-mediated increase of TRAIL-R2 expression even in resistant colorectal carcinoma cells lacking Bax expression, indicating that the sequential combination of anticancer agents with TRAIL may overcome some forms of resistance **(75)**. In addition, chemotherapy has been reported to enhance the receptor assembly of CD95 or TRAIL receptors, e.g., in colon carcinoma cells **(76)**.

Currently, agents directed at agonistic TRAIL receptors, i.e., recombinant soluble TRAIL and fully human monoclonal antibodies for TRAIL-R1 or -R2, are evaluated in early clinical trials (Table 26.1). Results from ongoing trials in patients with advanced solid tumors revealed no major dose-limiting toxicities for recombinant TRAIL or fully human monoclonal antibodies to TRAIL-R1 and defined the maximum tolerated dose for monoclonal antibodies to TRAIL-R2 **(77–79)**. In a Phase II trial with a fully human monoclonal antibodies

against TRAIL-R1 (mapatumumab; Humane Genome Sciences, Rockville, MD) that was conducted in patients with non-Hodgkin's lymphoma, tumor responses were observed in 3 of 40 patients (8%) **(80)**. In that study, the antibody was reported to be well tolerated **(80)**. Based on preclinical studies that demonstrated cooperative interaction of TRAIL receptor agonists with anticancer drugs, clinical trials using HGS-ETR1 in combination with chemotherapeutic agents, e.g., carboplatin and paclitaxel or gemcitabine and cisplatin, were also launched **(81, 82)**.

Cancer Therapeutics Targeting Bcl-2 Family Proteins

Since the overexpression of antiapoptotic Bcl-2-related proteins confers tumor cell resistance by blocking the mitochondrial pathway, several attempts aimed at overcoming the cytoprotective effect of Bcl-2. To this end, nuclease-resistant Bcl-2 antisense oligonucleotides downregulating Bcl-2 mRNA were tested in clinical trials for hematological malignancies or solid tumors, as a single agent or in combination with chemotherapy **(83)**. Moreover, a multidisciplinary effort to target the protein-protein interaction site between antiapoptotic Bcl-2 proteins and Bax or Bak has recently resulted in the generation of small molecule antagonists that bind to the surface groove of Bcl-2, Bcl-xL, and Bcl-w, which normally bind to the BH3 domain of Bax or Bak **(84)**. By preventing the binding of these antiapoptotic Bcl-2 proteins to Bax or Bak, the small molecule inhibitor can directly trigger apoptosis in some susceptible cell lines or sensitize cancer cells for apoptosis **(84)**. In addition, ABT-737 sensitized cancer cells for apoptosis when combined with conventional chemotherapeutics **(84–86)**. Since ABT-737 targets Bcl-2/Bcl-xL but not Mcl-1, a high expression of Mcl-1 may confer resistance to this novel agent. Indeed, recent reports indicate that Mcl-1 represents a key determinant of ABT-737 sensitivity and resistance in cancer cells **(85, 86)**. Consequently, Mcl-1 downregulation by genetic approaches or pharmacological compounds, including CDK inhibitors (e.g., roscovitine, flavopiridol, seliciclib), Raf/Mek inhibitors (e.g., sorafenib), or proteasome inhibitors, has been demonstrated to dramatically increase ABT-737 cytotoxicity in various malignant cell types **(85–89)**. These findings also show that the multidomain proapoptotic proteins Bax and Bak play important functional roles in ABT-737-mediated apoptosis **(86)**. Of note, ABT-737 exerted potent antitumor effects *in vivo* in xenograft mouse models and was also active against primary tumor cells, e.g., from AML samples **(84–86)**. Furthermore, BH3 peptides, which mimic BH3-only proteins in activating proapoptotic Bax and Bak proteins, are under preclinical evaluation **(90)**. Collectively, these findings suggest that small molecule inhibitors of antiapoptotic Bcl-2 family proteins may open new perspectives to reactivate the mitochondrial pathway of apoptosis in cancer cells.

Cancer Therapeutics Targeting Inhibitor-of-Apoptosis Proteins

There is mounting evidence that cancer cells have an intrinsic drive to apoptosis that is held in check by inhibitor-of-apoptosis proteins (IAPs). To this end, high basal levels of caspase-3 and caspase-8 activities and active caspase-3 fragments in the absence of apoptosis were detected in various tumor cell lines and cancer tissues, but not in normal cells **(91)**. Tumor cells, but not normal cells, also expressed high levels of IAPs, suggesting that upregulated IAP expression counteracted the high basal caspase activity selectively in tumor cells **(91)**.

Several strategies have been developed to target the enhanced expression of IAPs in cancers. For the design of potentially therapeutic small molecules to target XIAP, the binding groove of the BIR3 domain of XIAP, to which Smac binds after its release from mitochondria, has attracted the most attention **(92)**. Smac peptides harboring the N-terminal part of Smac that is essential for binding Smac to XIAP were reported to promote caspase activation and to sensitize various tumor cell lines and also primary patients' derived tumor cells for apoptosis induced by death receptor ligation or cytotoxic drugs **(93)**. Importantly, Smac peptides even enhanced the antitumor activity of TRAIL *in vivo* in an intracranial malignant glioma xenograft model **(70)**. Also, Smac peptides potentiated the antitumor activity of TRAIL and epothilone B derivative BMS 247550 in T-cell leukemia cells **(94)**. To facilitate intracellular delivery, Smac peptides were linked to a carrier, e.g., the protein transduction motif of the HIV Tat protein, the *Drosophila* antennapaedia penetratin sequence, or a polyarginine stretch **(93**, **95**, **96)**. Moreover, on the basis of the three-dimensional structure of Smac in complex with XIAP BIR3, Smac peptidomimetics were designed that bind to one or several of the XIAP-BIR3, cIAP1-BIR3, cIAP2-BIR3, or livin-BIR domains and cooperate with TRAIL, TNFα, or anticancer drugs to trigger apoptosis in tumor cells **(97**–**103)**. Recently, IAP antagonists were reported to kill cancer cells by inducing the autoubiquitination of c-IAPs, NF-κB activation, and TNFalpha-dependent apoptosis **(104**–**106)**.

Besides Smac mimetics, an oncolytic adenoviral vector of Smac increased the antitumor activity of TRAIL against hepatocellular carcinoma *in vitro* and also in mice **(107)**. Similarly, the transfection-enforced expression of Smac lowered the threshold for TRAIL-induced killing in different tumors **(108**, **109)**. Also, an expression system using ubiquitin fusions to express mature, biologically active Smac in the cytosol of transfected cells was developed, circumventing the requirement for mitochondrial processing and release of full-length Smac **(110)**. In addition, the natural product embelin from the Japanese Ardisia herb was discovered as a cell-permeable, nonpeptidic, small-molecular-weight inhibitor of XIAP through the structure-based computational screening of a traditional herbal medicine three-dimensional structure database **(111)**. Embelin was shown to effectively overcome the protective effect of XIAP in prostate cancer cells with high endogenous levels of XIAP or in Jurkat cells transfected with

XIAP through binding to the XIAP BIR3 domain **(111)**. Besides the BIR3 domain of XIAP, its BIR2 motif has also served as a target for the development of small molecule compounds. To this end, nonpeptidic XIAP antagonists were identified by screening a polyphenylurea library using a caspase derepression assay **(112, 113)**. These compounds induced the apoptosis of different tumor cell lines in culture without the requirement of an additional cytotoxic stimulus, sensitized cancer cells to chemotherapeutic drugs or TRAIL, and also suppressed the growth of established colorectal or prostate tumors in xenograft models in mice **(112, 114)**.

Also, XIAP antisense oligonucleotide as a single agent exhibited potent antitumor activity compared to control oligonucleotide in several xenograft models representing major cancer types, including colon, breast, lung, ovarian, and prostate carcinoma **(115)**. Of note, XIAP antisense oligonucleotide cooperated with clinically relevant chemotherapeutic drugs, e.g., platinum compounds or taxanes, to suppress tumor growth *in vivo,* suggesting that XIAP antisense oligonucleotide will likely act optimally in combination protocols with chemotherapy **(116)**. The antitumor activity of XIAP antisense oligonucleotide in these models correlated with the downregulation of XIAP levels in tumors **(116)**. Currently, XIAP antisense oligonucleotide is being evaluated in Phase I/II clinical trials either as a single agent or in combination with chemotherapy in advanced tumors (Table 26.1) **(117)**. Thus, Smac agonists, low-molecular-weight XIAP antagonists, or XIAP antisense oligonucleotides are promising approaches to trigger apoptosis or to lower the threshold for apoptosis induction in cancer cells.

Conclusions

Cell death by apoptosis is one of the key mediators of the antitumor effect of many currently used anticancer therapies and, thus, a crucial determinant of treatment response. The elucidation of key components of the apoptotic machinery that are activated in response to cytotoxic therapy has provided substantial insights into mechanisms of anticancer drug action and has identified many molecular targets for therapeutic interventions. Several strategies that either directly trigger apoptosis or target defects in apoptosis programs in cancer cells have already been translated into early clinical trials. Such approaches may provide novel opportunities in the fight against cancer.

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Part IV
Alternative Cell Death Mechanisms and Pathways

Chapter 27
Necrosis: Molecular Mechanisms and Physiological Roles

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Abstract For a long time, necrosis has been considered an accidental and uncontrolled form of cell death, lacking underlying signaling events. However, accumulating evidence supports the existence of a caspase-independent cell death pathway that is also regulated and controlled. This regulated form of necrosis seems to be of relevance in both physiological and pathological conditions. For example, necrosis occurs during excitotoxicity, ischemia-reperfusion injury, neurodegenerative diseases, and infections. Research over the past decade indicates that necrosis results from an extensive interplay between several signaling events and a wide range of mediators. However, it is still not clear whether these many mediators represent various subroutines in different cell lines and what the precise relationship is between the signaling events and the activation of the mediators. The serine/threonine kinase receptor-interacting protein 1 (RIP1) apparently is a central initiator of necrotic cell death. Reactive oxygen species (ROS) and calcium are important mediators of necrosis, but several other mediators have also been described, such as phospholipases, calpains, cathepsins, ceramide, and methylglyoxal, all of which contribute to the disruption of organelles and the plasma membrane. Necrotically dying cells initiate a proinflammatory response by actively releasing immunomodulatory factors and passively releasing their contents when they lyse. Knowledge of the molecular mechanisms involved in necrosis has contributed to the development of therapeutic strategies for the treatment of pathologies associated with necrosis. This chapter focuses on the molecular events during regulated necrotic cell death and the link with several necrosis-associated pathologies.

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Introduction

Cell death is an essential process in the development, homeostasis, and immune regulation of multicellular organisms. Based on the typical morphological features of dying cells, three major types of cell death have been described: apoptotic, autophagic, and necrotic cell death **(1)**. Apoptotic (type I) cell death, as reviewed in **(2)**, is an active, programmed, and regulated cellular suicide mechanism that functions under both homeostatic and pathological conditions. Morphologically, apoptosis is associated with cell membrane blebbing, condensation of the nucleus, and internucleosomal cleavage of DNA. These features are the consequence of the activation of caspases, an evolutionarily conserved family of cysteine proteases. Autophagic (type II) cell death is a cellular survival mechanism initiated in response to cellular stress or nutrient deprivation. It is characterized by the formation of double-membrane autophagosomes and ultimately results in either caspase-dependent or caspase-independent cell death [reviewed in **(3)**]. It is still debated whether autophagic cell death is an autonomous cell death program or whether it uses other cell death pathways as a consequence of continued cellular stress. Necrotic, or type III, cell death is characterized by cytoplasmic and organelle swelling, followed by the loss of cell membrane integrity [reviewed in **(4)** and **(5)**]. This leads to the release of the cellular contents into the surrounding extracellular space, with subsequent inflammation.

For a long time, necrosis has been considered an accidental and uncontrolled form of cell death lacking underlying signaling events. This might be true for cell death resulting from severe physical damage, such as hyperthermia or detergent-induced cytolysis. However, accumulating evidence supports the existence of caspase-independent cell death pathways that can function even in a strictly regulated developmental context, such as interdigital cell death **(6)**. Often, the same type of insult can induce either apoptosis or necrosis, and which mode of cell death predominates usually depends on the type and severity of the insult, the cell type, and the cellular context. For example, TNF induces apoptotic cell death in most cell lines, whereas in the L929 mouse fibrosarcoma cell line, it induces necrotic cell death **(7)**. Also, the treatment of these cells with anti-Fas results in apoptosis, but this response can be shifted to necrosis by the addition of caspase inhibitors **(8)**. As outlined in Fig. 27.1, this chapter focuses on several stimuli and molecular events leading to regulated necrotic cell death and on their relationship to several necrosis-associated pathologies.

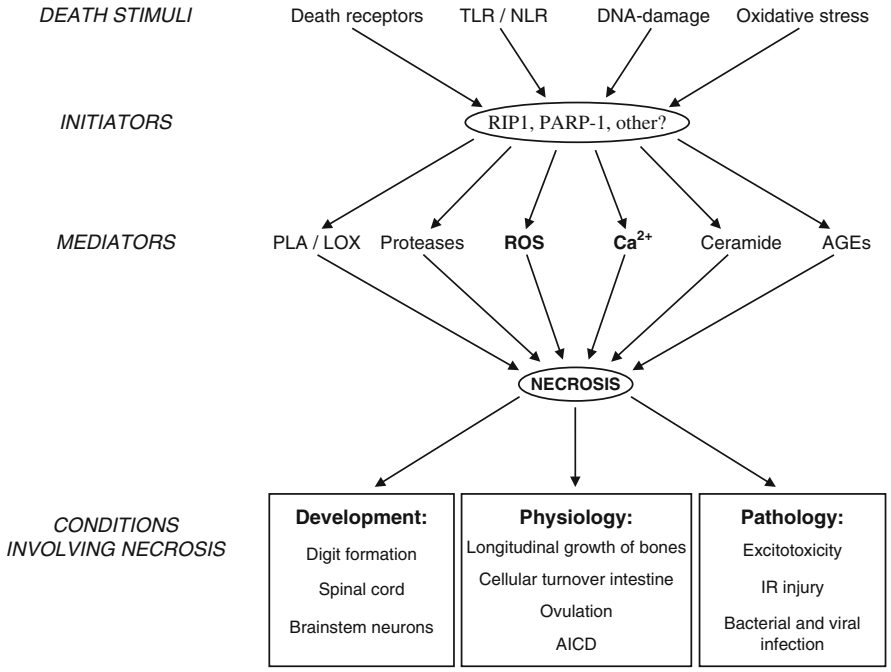

Fig. 27.1 Schematic overview of stimuli, initiators, and mediators contributing to necrotic cell death and associated (patho)physiological conditions. Stimulation of death receptors, TLRs, and NLRs or the occurrence of extensive DNA damage and oxidative stress leads to the activation of necrotic initiators, such as RIP1 and PARP-1. The subsequent action of a wide range of necrotic mediators ultimately results in necrotic cell death. Among these mediators, ROS and calcium are the two main players. Necrosis generally occurs during several pathological conditions, but is also important during the development and homeostasis of a multicellular organism

Physiological and Pathological Significance of Necrotic Cell Death

Although it was generally accepted for a long time that necrosis occurs only under extreme conditions, such as severe hyperthermia and hypoxia, increasing evidence supports the existence of caspase-independent cell death pathways involved in physiological and pathological conditions. Apoptosis is indispensable for tissue remodeling during embryogenesis, but necrosis can, at least in some conditions, substitute for apoptosis to eliminate unwanted cells. For example, the removal of interdigital cells during the development of digits in the presence of the caspase inhibitor zVAD-fmk or in Apaf-$1^{-/-}$ mice occurs by a caspase-independent necrotic-like process **(6)**. Moreover, in wild-type mice, some interdigital cells were found to die with a necrotic morphology **(6)**. In addition, the genetic deletion of caspases-3 and -9 did not impair the development-related loss of spinal cord and brainstem neurons, although the morphology of the

forebrain suffered a marked perturbation **(9)**. Necrosis is also involved in physiologically relevant signaling processes, such as in ovulation **(10)**, in the death of chondrocytes associated with the longitudinal growth of bones **(11)**, and in cellular turnover in the small and large intestines **(12, 13)**. In addition, necrotic cell death participates in the activation-induced cell death (AICD) of T lymphocytes, which is mostly driven by Fas and which constitutes an important mechanism for reducing T-cell numbers after an immune response **(14)**. This could explain why FLIP and CrmA transgenic mice, both overexpressing proteins interfering with caspase-8 activation, are phenotypically different from Fas-deficient mice (*lpr* mice). Fas-deficient mice develop lymphoproliferative or autoimmune diseases, in contrast to the FLIP and CrmA transgenic mice **(15, 16)**. Whereas the overexpression of FLIP or CrmA inhibits only the caspase-8-dependent route of Fas-mediated AICD, Fas deficiency apparently also affects the caspase-independent necrotic cell death pathway. However, it is important to point out that in most of the above-mentioned pathways, necrotic cell death is always observed together with apoptosis or in the presence of caspase inhibitors, suggesting that it functions as a backup mechanism and is never the sole cell death pathway.

Necrotic cell death is also associated with several pathological conditions. Ischemia-reperfusion (IR) injury is caused by an obstruction of blood flow to a tissue that is followed by reperfusion. The concomitant necrotic cell death of endothelial cells and nonproliferating cells of surrounding tissues (e.g., neurons, cardiomyocytes, renal cells) can lead to injury of organs, including the heart, brain, liver, kidney, and intestine. The relative contribution of apoptosis and necrosis to postischemic cell death is controversial and appears to depend on the type of ischemia (e.g., complete or incomplete, global or focal), severity of insult, time point examined, and criteria used to define both cell death types **(17)**. Necrotic cell death is prevalent in the core of ischemic regions, but in peripheral regions, where oxygen supply and energy depletion are less severe and less prolonged, apoptotic cell death is commonly observed **(18)**.

Necrotic cell death is also involved in excitotoxicity, the pathological process by which neuronal cells die due to the overactivation of glutamate receptors by excess neurotransmitters, such as N-methyl-D-aspartate (NMDA), 2-amino-3-propionate (AMPA), and kainate. Excitotoxicity may be involved in stroke, traumatic brain injury, and neurodegenerative disorders, such as multiple sclerosis, Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD). During excitotoxicity, apoptotic cell death, as defined by both morphological and biochemical criteria, also occurs simultaneously with necrosis, or in a temporal sequence, or in a particular spatial distribution **(19, 20)**. Several reports also illustrate the occurrence of necrotic cell death during viral and bacterial infections. HIV-1 was shown to kill $CD4^+$ T lymphocytes by necrosis rather than apoptosis **(21, 22)**, and infection of Vero cells with high doses of West Nile virus induced necrosis associated with the rapid loss of plasma membrane integrity and abundant budding of the progeny virus particles at the cell surface **(23)**. Necrotic-like cell death was also observed in

human Jurkat cells and in pancreatic β cells after infection with Coxsackievirus B **(24, 25)**. Furthermore, the Gram-negative bacterium *Shigella flexneri* can induce necrotic cell death in infected neutrophils and macrophages **(26)**.

Regulated Necrotic Cell Death

Death Receptor-Induced Necrotic Signaling

Death receptors, such as tumor necrosis factor receptor 1 (TNFR1), Fas, and TNF-related apoptosis-inducing ligand (TRAIL) receptor, belong to the TNFR superfamily. Once they bind their extracellular ligands, they can induce a variety of cellular responses, including proliferation, differentiation, and death. The aggregation of death receptors leads to the initiation of a complex signal transduction pathway by the recruitment of Fas-associated death domain (FADD) to the receptor via its C-terminal death domain (DD) **(27)**. FADD also contains an N-terminal death effector domain (DED) that recruits caspase-8 to the receptor complex via a homophilic DED interaction **(28)**. The subsequent activation of caspase-8 initiates a signaling cascade that eventually leads to apoptotic cell death in most cell types. However, if caspase activation in this pathway is hampered, necrotic cell death might ensue instead. In contrast to signaling induced by Fas and TRAIL-R, TNFR1 aggregation leads to the sequential formation of two complexes **(29, 30)**. Complex I consists of TNFR1, TNF receptor-associated factor 2 (TRAF2), and serine/threonine kinase receptor-interacting protein 1 (RIP1) and is formed at the plasma membrane. TRAF2 and RIP1 are important mediators of TNF-induced activation of NF-κB and MAPKs **(31, 32)**. The endocytosis of TNFR1 is followed by the formation of complex II, in which TNF receptor-associated death domain (TRADD) recruits FADD, procaspase-8, and procaspase-10 **(33)**. If complex I fails to induce sufficient antiapoptotic proteins, caspase-8 is activated and apoptosis is the end result.

However, if the apoptotic pathway is blocked during death receptor-mediated signaling, the response can shift to necrosis. The type of cell death mediated by these death receptors depends on the cellular context and on the stimulus. In the L929 mouse fibrosarcoma cell line, TNF induces necrotic cell death, which can be sensitized by the addition of caspase inhibitors, such as the broad-spectrum inhibitor zVAD-fmk **(34)**. In many other cell lines, TNF induces apoptotic cell death, which can be shifted to necrosis if apoptosis is blocked. For example, when caspases are inhibited in mouse embryonic fibroblast (MEF) cells or Jurkat cells, stimulation with TNF/cycloheximide results in necrotic cell death **(14, 35)**. Similarly, FADD-deficient Jurkat cells, in which FADD-mediated caspase-8 activation is abrogated, die by necrosis upon TNF treatment **(14)**. Triggering Fas in L929 cells in the presence of zVAD-fmk also leads to necrotic cell death **(8)**. In addition, upon stimulation with FasL,

necrosis was observed in Jurkat cells under a caspase-inhibited condition and in Jurkat cells deficient in caspase-8 **(14)**. In the presence of cycloheximide and zVAD-fmk, TRAIL induces necrotic cell death in Jurkat cells **(14)**. These findings illustrate that necrotic cell death acts as a backup pathway that is activated when the apoptotic machinery fails to induce cell death. FADD remains a crucial adaptor protein in Fas- and TRAIL-R-induced necrosis **(14)**, but the importance of FADD in TNF-induced necrosis is controversial. In MEF cells, FADD seems necessary for TNF-induced necrotic cell death **(35)**, but in Jurkat cells it is dispensable **(14**, **36)**, indicating the involvement of different adaptor proteins, depending on the cellular context.

RIP1 is another protein recruited to death receptors, where it fulfills a dual role [reviewed in **(37)**]. On the one hand, RIP1 exerts a prosurvival effect by activating NF-κB and MAPKs in a complex that also includes TRAF2 **(38)**. On the other hand, RIP1 is a crucial initiator of death receptor-mediated necrosis. TNF-, FasL-, and TRAIL-induced necrotic cell death is abrogated in RIP1-deficient Jurkat cells under caspase-inhibited conditions **(14**, **36)**. Reintroducing a kinase-active RIP1 restores the necrotic signaling pathway **(14)**. In addition, dimerization of the RIP1 kinase domain induces necrotic cell death, in contrast to dimerization of a kinase-dead mutant **(39)**. The importance of RIP1 in necrotic signaling processes is also demonstrated by studies on heat-shock protein (Hsp) 90, a cytosolic chaperone for RIP1 **(40)**. Treating L929 cells with Hsp90 inhibitors (geldanamycin or radicicol) results in strong reduction of RIP1 levels and protection against Fas- and TNFR1-induced necrosis **(14**, **41)**. Furthermore, RIP1 is cleaved by caspase-8 during TNF-, FasL-, and TRAIL-induced apoptosis, thereby suppressing its pronecrotic and antiapoptotic properties **(36**, **42)**. Moreover, the C-terminal cleavage fragment of RIP1 sensitizes cells to apoptosis by interfering with NF-κB activation **(42**, **43)**.

TLR- and NLR-Induced Necrotic Signaling

Besides death receptor-mediated necrosis, triggering of pathogen recognition receptors (PRRs) can also lead to necrotic cell death. Receptors of this family include the transmembrane Toll-like receptors (TLRs), the cytosolic NOD-like receptors (NLRs), and the RIG-I-like receptors (RLRs). They all recognize molecular patterns found in bacteria or viruses, such as LPS, flagellin, and double-stranded RNA, and stimulation of these receptors leads to the activation of innate immunity and/or cell death [reviewed in **(44)**]. In human Jurkat cells and murine L929 cells, the recognition of synthetic dsRNA by TLR3 induces necrotic cell death **(45)**. TLR4 is expressed on macrophages and monocytes and is critical for the recognition of LPS of Gram-negative bacteria. Although TLR4 ligation induces NF-κB activation, which promotes cell survival through the induction of antiapoptotic genes,

TLR4 activation can also result in macrophage apoptosis **(46–49)**. Impeding caspase-8 activation switches TLR4-induced cell death from apoptosis to RIP1-dependent necrosis **(50)**.

Cryopyrin/NALP3, an NLR member, mediates the necrotic cell death of macrophages infected with *Shigella flexneri* at a high multiplicity of infection (MOI) **(51)**. Moreover, patients carrying a disease-associated mutation in cryopyrin show excessive necrotic-like cell death **(51)**. Both cryopyrin-mediated *S. flexneri* and disease-associated necrotic cell death depend on ASC/PYCARD, a component of the inflammasome, and cathepsin B, suggesting the involvement of lysosomal membrane permeabilization (LMP) in this necrotic cell death process. Whether RIP1 is a crucial initiator in cryopyrin/NALP3-mediated necrosis is still an open question. At a lower MOI, pyroptosis is the primary death mode induced by *S. flexneri* **(52)**, the cell death process that depends on caspase-1 activation and that is accompanied by IL-1β maturation **(53)**.

DNA Damage and Hydrogen Peroxide-Induced Necrotic Signaling

Several pathological conditions, such as IR, diabetes, reactive oxygen species (ROS)-induced injury, and glutamate excitotoxicity, are associated with massive DNA damage [reviewed in **(54)**]. The extensive formation of breaks in the DNA strands causes hyperactivation of poly(ADP-ribose) polymerase-1 (PARP-1), which leads to necrotic cell death **(55, 56)**. Stimuli that directly or indirectly affect mitochondria, such as H_2O_2 and the DNA-alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), also induce PARP-1-mediated necrotic cell death **(57, 58)**. PARP-1 is a nuclear enzyme that transfers long-branched chains of poly(ADP-ribose) to a variety of nuclear proteins, including PARP-1 itself **(59)**. When DNA damage is moderate, PARP-1 participates in DNA repair processes **(60)**. However, excessive PARP-1 activation causes depletion of NAD^+ by catalyzing the hydrolysis of NAD^+ into nicotinamide and poly-ADP-ribose, leading to ATP depletion, irreversible cellular energy failure, and necrotic cell death **(55, 56, 61)**. MNNG-induced cell death requires activation of c-Jun N-terminal kinase (JNK) mediated by RIP1 and TRAF2, which function downstream of PARP-1 **(62)**. A similar signaling cascade is initiated after H_2O_2 exposure by the formation of a complex among RIP1, TRAF2, and JNK1 **(63)**. However, the mechanism by which intracellular molecules such as RIP1 and TRAF2 sense the activation of PARP-1 remains to be elucidated.

It has also been reported that hyperactivation of PARP-1 induces the release of apoptosis-inducing factor (AIF) from the mitochondrial intermembrane space by an unkown mechanism and is required for PARP-1-mediated cell death **(64)**. Moreover, mitochondrial depolarization and subsequent AIF translocation were not observed in $JNK1^{-/-}$ MEF cells after MNNG exposure **(62)**, indicating that JNK activation is required for the translocation of AIF to the nucleus, where it contributes to chromatin condensation and the generation of

large DNA fragments of about 50 kbp **(65)**. Furthermore, the release of AIF also contributes to a loss of function in the mitochondria by promoting complex I destabilization and concomitant ROS production, favoring necrotic cell death **(66)**. Consequently, necrosis induced by H_2O_2 and IR is reduced by the downregulation of AIF **(67, 68)** as well as by the inhibition and genetic knockout or knockdown of PARP-1 **(64, 69, 70)**. Of note, PARP-1 is inactivated by caspase-3-specific cleavage during apoptosis. This cleavage separates the DNA binding domain from the catalytic domain, resulting in its inactivation and thereby preventing DNA repair-induced cell survival **(54)**. As is also the case for caspase-8-mediated RIP1 cleavage, this is an example of an apoptotic signaling event that actively inhibits the initiation of necrotic cell death.

Mediators of Necrotic Cell Death

Calcium

Ca^{2+} transport through the plasma membrane and intracellular membranes is a tightly controlled process, and the regulated release of Ca^{2+} from the intracellular stores is an essential step in many cellular signaling events. Under pathological conditions, regulatory mechanisms may be overwhelmed and the intracellular Ca^{2+} concentration increases abnormally via two main routes: influx from extracellular pools through various channels and release from endoplasmic reticulum (ER) stores. The proper Ca^{2+} concentration positively regulates the global mitochondrial function, and thus any perturbation in mitochondrial or cytosolic Ca^{2+} homeostasis will have profound implications for cellular functioning (Fig. 27.2). When mitochondria are overloaded with Ca^{2+}, the mitochondrial permeability transition pores (MPTPs) open, causing mitochondrial permeability transition (MPT) **(71)**. MPTPs are multiprotein complexes that form large nonselective pores through both the inner and outer mitochondrial membranes, connecting the cytosol with the mitochondrial matrix **(72)**. Key structural components of this pore are the adenine nucleotide translocator (ANT) in the inner membrane, cyclophylin D (CypD) in the matrix, and the voltage-dependent anion channel (VDAC) in the outer membrane, but other molecules are also present **(73)**. MPT is accompanied by mitochondrial inner membrane depolarization, uncoupling of oxidative phosphorylation, matrix swelling, and outer mitochondrial membrane rupture [reviewed in **(74)**]. If the onset of MPT is widespread and involves most mitochondria in a cell, and glycolytic sources of ATP are inadequate, the cells become profoundly ATP-depleted. This will ultimately lead to organelle disruption and cell lysis. MPT can be inhibited by submicromolar concentrations of cyclosporin A (CsA), an immunosuppressive drug that inhibits CypD **(75, 76)**. In addition, CypD-deficient cells are less sensitive to MPT and to necrotic cell death induced by Ca^{2+} overload or oxidative stress, indicating that CypD is

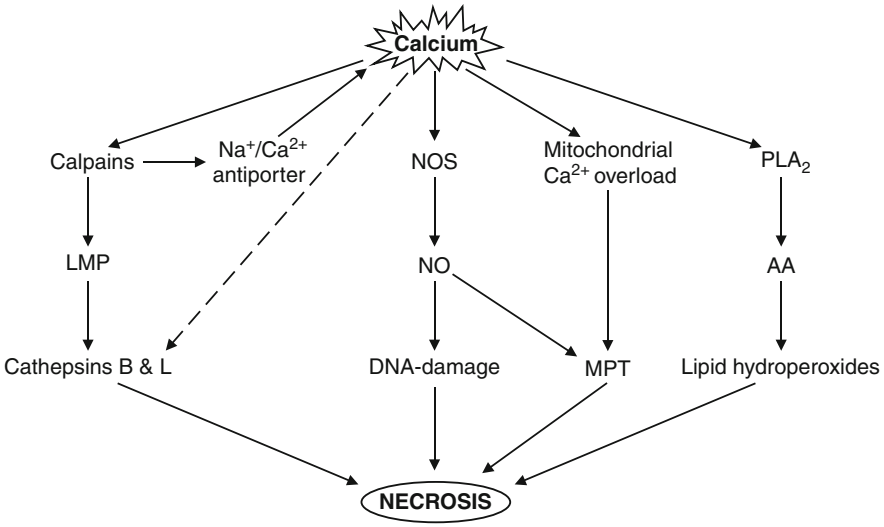

Fig. 27.2 Ca^{2+}-mediated signaling events leading to necrotic cell death. Elevated Ca^{2+} levels in the cytosol lead to the activation of calpains, which may contribute to the release of cathepsins B and L in the cytosol by causing LMP. Mitochondrial Ca^{2+} overload results in opening of the MPTPs and subsequent MPT. Ca^{2+} can induce NO-mediated DNA damage through the activation of NOS. Ca^{2+}-mediated PLA_2 activation leads to the formation of lipid hydroperoxides, which in turn disrupt organelle and cell integrity. All these Ca^{2+}-mediated signaling events contribute to the necrotic cell death process

a key regulator of MPT-related necrotic cell death **(77–81)**. However, these *in vivo* studies should be interpreted in terms of the role of CypD in cell death modulation, which could be independent of MPT regulation because MPTP can still open in CypD-deficient cells, providing that the Ca^{2+} load is permissive **(77–81)**. Even ANT-deficient hepatocytes still exhibit MPT and cell death in response to Ca^{2+} ionophores, demonstrating that ANT is not required for MPT **(82)**.

Ischemia results in a limited supply of oxygen and glucose to the affected tissue and the accumulation of metabolic waste products. If ischemia persists, ANT and several components of the mitochondrial electron transport chain (ETC) are damaged, causing ATP synthase to hydrolyze ATP instead of producing it **(83)**. To maintain ATP production during ischemic conditions, cells resort to anaerobic glycolysis using glycogen stores and glucose in the surrounding tissue **(84)**. This leads to acidosis due to the accumulation of lactic acid as a byproduct of anaerobic glycolysis, together with protons produced by ATP hydrolysis and the release of protons sequestered in acidic organelles, such as lysosomes and endosomes **(85, 86)**. The cell counteracts this pH drop by activating the Na^+/H^+ antiporter, leading to increased levels of intracellular Na^+. Because of the reduced ATP levels, the Na^+/K^+ ATPase cannot pump out this excess Na^+. Instead, the Na^+/Ca^{2+} antiporter pumps out Na^+,

leading to a progressive increase in intracellular Ca^{2+} **(87)**. Subsequently, Ca^{2+} enters the mitochondria through an electrogenic uniporter that acts as a channel and is pumped out again by a Na^{+}/Ca^{2+} antiporter **(88)**. As mitochondrial Ca^{2+} increases, the activity of the Na^{+}/Ca^{2+} antiporter becomes saturated and mitochondrial Ca^{2+} overload ensues **(89)**. Reexposure to oxygen following reperfusion results in a burst of ROS, which induces oxidative stress (see ahead). Moreover, at the onset of reperfusion, normal physiological pH is restored, which, together with oxidative stress, calcium overload, high phosphate concentrations, and low adenine nucleotide levels, makes the conditions ideal for opening of the MPTP **(89**, **90)**.

Besides affecting mitochondrial respiration, Ca^{2+} overload can activate lipases and proteases (see ahead) and neuronal nitric oxide synthase (nNOS), leading to excessive nitric oxide (NO) production. This pathway is involved in excitotoxicity, the pathological process by which cells die due to the release of excess neurotransmitters or in response to overexposure to excitatory amino acids, such as N-methyl-D-aspartate (NMDA) and glutamate **(91)**. Excitotoxins induce a massive cytosolic Ca^{2+} influx by releasing the ER Ca^{2+} pool and/or by increasing the influx of extracellular Ca^{2+} through plasma membrane transporters, resulting in stimulation of NO production. In the presence of superoxide ($O_2^{-\bullet}$), NO can generate peroxynitrite ($ONOO^{-\bullet}$), a potent inducer of MPT and DNA damage, resulting in the activation of PARP-1 and consequently caspase-independent cell death **(92)** (discussed earlier). NO also affects several components of the ETC. ROS production is enhanced by inhibition of complex I in the presence of a high concentration of Ca^{2+}, as well as by inhibition of complex IV; enhanced ROS production leads to impairment of ATP synthesis and cell function **(93)**. NO can also hamper caspase-3 activation by its nitrosylation, thereby inhibiting apoptosis and promoting necrosis **(94)**.

Reactive Oxygen Species (ROS)

ROS are generated by all aerobic cells as byproducts of a number of metabolite reactions. Mitochondria are the major intracellular source of ROS, which are generated predominantly at complexes I and III of the ETC **(95**, **96)**. However, membranes of the ER and nucleus also contain ETCs, from which electrons can escape and react with molecular oxygen to generate superoxide radicals. Physiological levels of ROS are involved in various biological processes, including cell proliferation **(97)**, activation of gene expression **(98)**, and defense against microorganisms **(99)**. To counteract the accumulation of harmful ROS, cells possess numerous ROS defense systems that include nonenzymatic antioxidants (e.g., glutathione and thioredoxin) and specific antioxidant enzymes [e.g., catalase, superoxide dismutase (SOD), and glutathione peroxidase] confined to specific subcellular locations. Oxidative stress is caused by a spatiotemporal imbalance of ROS production and detoxification. This imbalance

may be due to either the overproduction of ROS or a deficiency in the antioxidant systems. Oxidative stress leads to damage of cellular macromolecules, which is associated with a variety of pathophysiological conditions, including inflammation, apoptosis, necrosis, septic shock **(100)**, IR **(101)**, and neurodegeneration **(102)**.

ROS Generation and Necrosis

Mitochondria-derived ROS accumulates during TNF-induced necrotic cell death of L929 and MEF cells **(35, 103)**. This accumulation can be blocked by the lipophilic antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) **(35, 104)**. Both compounds decrease the levels of ROS and at the same time protect against TNF-induced necrosis of L929 and MEF cells **(35, 105)**, demonstrating the important role of mitochondria-derived ROS in this type of necrotic cell death. Consistently, the complex I inhibitor rotenone decreases TNF-induced ROS levels and cytotoxicity *in vitro* and *in vivo* **(104, 106)**.

Furthermore, the accumulation of mitochondrial ROS in MEF cells is dependent on RIP1, TRAF2, and FADD **(35)**. In agreement with this observation, RIP1 co-localizes with mitochondria upon TNF stimulation of THP-1 cells, as confirmed by enhanced RIP1 levels in the mitochondrial fraction of TNF-stimulated THP-1 cells **(107)**. Pretreatment of these cells with TNF or anti-Fas leads to translocation of zVAD-fmk to the mitochondria, where it covalently interacts with ANT. The results are decreased ADP transport, subsequent ATP depletion, and necrotic cell death. The interaction between ANT and zVAD-fmk seems to depend on RIP1 because the depletion of RIP1 by pretreatment with geldanamycin or RNA interference (RNAi) abolished this interaction and the suppression of ADP uptake. Although the induction of ROS was observed during this cell death process, it seems that it is not necessary for ATP depletion and cell death. On the other hand, the overexpression of CypD blocks TNF-induced necrotic cell death **(107)** but enhances necrosis triggered by Ca^{2+} overload or by ROS **(71)**. This indicates the involvement of different signaling mechanisms in necrosis, depending on the stimulus and cell type. Accordingly, CypD-deficient cells are largely protected against necrosis induced by Ca^{2+} overload or by ROS, but they are not less sensitive to TNF-induced necrotic cell death **(71, 77–79)**.

More recently, nonmitochondrial ROS production by the NADPH oxidase Nox1 was also found to contribute to TNF-induced necrotic cell death in L929 and $p65^{-/-}$ MEF cells **(108)**. Knockdown of Nox1 prevented both superoxide generation and cell death in response to TNF. In this pathway, Nox1 is activated by a RIP1-dependent signaling complex containing TRADD, Nox1, NOXO1, and the small GTPase, Rac1. As ROS are generated by Nox1 early after TNF treatment, this nonmitochondrial ROS might lead to the generation of mitochondria-derived ROS **(109)**. This connection between RIP1 and Nox1 has only been demonstrated in L929 cells, and neither oxidative

stress nor NADPH oxidase activity appeared to play a role in TNF-induced necrosis in FADD-deficient Jurkat cells **(110)**.

ROS generation was also shown to be involved in dsRNA-induced necrosis in human Jurkat cells and murine L929 cells **(45**, **104**, **108)**, but Nox1 did not seem to be involved **(108)**. Furthermore, treatment with BHA did not block this cell death pathway but shifted the response from necrosis to apoptosis **(45)**.

ROS-Mediated Damage of Biomolecules

ROS generated by mitochondria, or elsewhere in the cell, can cause damage to cellular macromolecules, including DNA, proteins, and lipids (Fig. 27.3). Oxidative damage to DNA leads to single and double DNA strand breaks, DNA-protein cross-linking, and oxidation of purines and pyrimidines **(111)**. This damage initiates the DNA damage response, during which p53 and PARP-1 are activated. The activation of p53 may cause apoptosis and cell cycle arrest, whereas the hyperactivation of PARP-1 leads to necrotic cell death (discussed earlier). Proteins too suffer from damage by oxidative stress. ROS can lead to the oxidation of sulfhydryl groups, reaction of proteins with aldehydes, protein-protein cross-linking, and protein fragmentation **(112)**. These modifications lead to loss of the normal functions of proteins and enhance their susceptibility

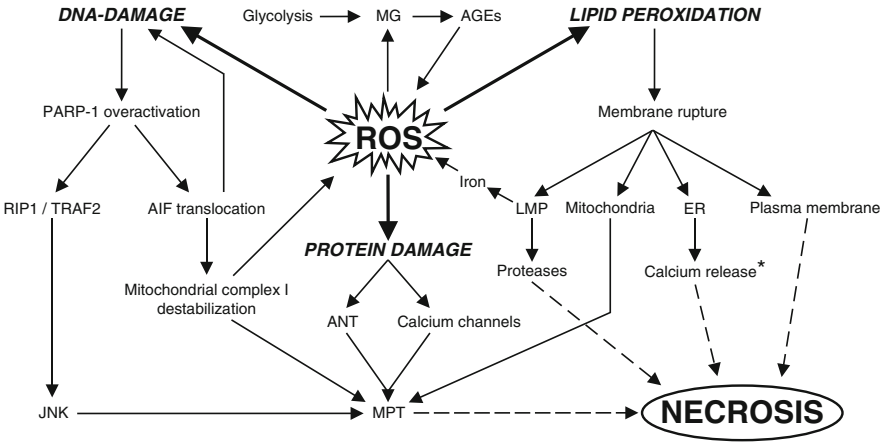

Fig. 27.3 ROS-mediated damage to proteins, lipids, and DNA. The ROS-mediated oxidation of ANT and Ca^{2+} channels contributes to MPT. DNA damage leads to the overactivation of PARP-1, which ultimately results in MPT through the upstream activation of JNK and the translocation of AIF to the nucleus. Lipid peroxidation results in loss of organelle and plasma membrane integrity. ROS contributes to enhanced MG levels and subsequent AGE formation. ROS formation is amplified through several positive-feedback loops. Mitochondrial dysfunction, AGE formation, and iron liberation upon LMP all enhance ROS production

* Ca^{2+} release upon ER rupture initiates a signaling cascade shown in Fig. 27.2.

to proteolytic degradation. Oxidation of the thiol group of ANT has been shown to promote MPT **(113)**. Ca^{2+} channels in ER and the plasma membrane are also affected by ROS, contributing to the necrotic cell death process **(114)**.

Other targets of ROS are the polyunsaturated fatty acid residues in the membrane phospholipids, which are extremely sensitive to oxidation **(115)**. Lipid peroxidation in mitochondria affects vital mitochondrial functions, such as oxidative phosphorylation, inner membrane barrier properties, maintenance of mitochondrial membrane potential, and the mitochondrial Ca^{2+} buffering capacity **(112)**. Lipid peroxidation also destabilizes the plasma membrane and intracellular membranes of lysosomes and ER, leading to intracellular leakage of lysosomal proteases and Ca^{2+}, respectively. Among the different ROS, H_2O_2 plays a particularly important role because it diffuses freely across cellular membranes and undergoes homolytic disintegration when interacting with iron in the Fenton reaction **(116)**. This reaction generates highly reactive hydroxyl radicals, which are among the most potent inducers of lipid peroxidation. Due to the degradation of iron-containing metalloproteins within the lysosomes, these organelles become rich in low-mass iron **(117)**. In addition, the redox-active form of lysosomal iron is promoted by the acidic pH (4.5–5.5) and by the presence of reducing agents such as cysteine **(118**, **119)**. Moreover, since lysosomes do not contain H_2O_2-degrading enzymes, such as catalase or glutathione peroxidase, every H_2O_2 molecule that diffuses into the lysosome will induce the formation of a hydroxyl radical **(120)**. Lysosomal rupture releases not only lytic enzymes in the cytosol, but also iron, which in turn causes site-specific induction of hydroxyl radicals. Therefore, redox-active iron needs to be transported safely and stored in a nonredox-active form, such as transferrin and ferritin. A recent study reported that ROS formation increased after TNF stimulation due to JNK1-dependent ferritin degradation, accompanied by an increase in the labile iron pool **(121)**. Accordingly, ferritin-deficient L929 cells, which store only small amounts of iron, show only a small increase in labile iron pool and concomitant ROS production when stimulated with TNF. As a result, these cells are less sensitive to TNF-induced necrotic cell death **(122)**. In addition, RIP1 was found to act upstream of the increase in the labile iron pool in this pathway **(122)**. As reported several times, treatment with iron chelators, such as desferrioxamine (DFO), almost fully prevents oxidative stress-induced lysosomal rupture, mitochondrial injury, and final cell death **(116**, **123**, **124)**. These findings reveal an important role for redox-active iron in ROS-mediated necrotic signaling.

Phospholipases and Lipoxygenases

Phospholipase A_2 (PLA_2) enzymes are important components in the regulation of various biological processes, including inflammation, cancer, and apoptosis **(125)**. PLA_2 comprises a family of esterases that hydrolyze the sn-2 ester bond in

phospholipids to release free fatty acids and lysophospholipids. Mammalian PLA_2 isotypes can be classified into three major subfamilies: secretory PLA_2 ($sPLA_2$), cytosolic Ca^{2+}-dependent PLA_2 ($cPLA_2$), and Ca^{2+}-independent PLA_2 ($iPLA_2$) **(126)**. $cPLA_2$ is an intracellular enzyme found in most cells and tissues. Upon Ca^{2+}-mediated translocation to cellular membranes, such as those of the nucleus, ER, and Golgi apparatus, it preferentially hydrolyzes arachidonate-containing phospholipids, resulting in the release of arachidonic acid (AA) **(125, 127)**. Another prerequisite for the translocation and activation of $cPLA_2$ is the phosphorylation of four serine residues by MAPKs and Ca^{2+}/calmodulin kinase-II **(125)**. ROS have also been reported to promote the activation of $cPLA_2$, with the subsequent release of AA **(128, 129)**. On the other hand, $cPLA_2$ activity has also been identified as another source of ROS during TNF-induced, caspase-independent cell death **(106)**. The released AA can be converted into lipid hydroperoxides by lipoxygenases (LOXs). LOXs are monomeric, nonheme, nonsulfur iron dioxygenases that catalyze the conversion of polyunsaturated fatty acids into conjugated hydroperoxides **(130)**. Lipid hydroperoxidation may lead to the disruption of organelles and the plasma membrane **(131, 132)** and thereby contributes to necrotic cell death. Several studies demonstrate a role for the PLA_2/LOX pathway in necrotic signaling processes. PLA_2 was activated after TNF treatment of L929 cells, and the overexpression of $cPLA_2$ sensitized TNF-resistant L929 variants to TNF-induced necrosis **(133, 134)**. We recently confirmed the involvement of the PLA_2/LOX pathway in TNF-induced necrosis by showing that several $cPLA_2$ and LOX inhibitors inhibit cytotoxicity **(104)**. In addition, during TNF-induced necrosis of L929 cells, $cPLA_2$ activity seemed necessary for the generation of ceramide, another mediator of necrotic cell death **(135)** (see ahead). $cPLA_2$ was also shown to play a major role in the oxalate- or oxidant-induced death of renal epithelial cells **(129, 136)**. Another study revealed an *in vivo* contribution of $cPLA_2$ during TNF/zVAD-fmk-induced lethal shock **(106)**. Additionally, some PLA_2 inhibitors can prevent ischemic cell death **(137, 138)**, and $cPLA_2$-deficient mice exhibit reduced injury after brain ischemia **(139)**.

Proteases

Several proteases have been implicated in necrotic cell death, including calpains and cathepsins. Calpains are intracellular cysteine proteases ubiquitously and constitutively expressed in mammalian cells. They can modulate a variety of physiological processes **(140)** but can also become important mediators of cell death **(141)**. Two subfamilies of calpains exist, μ- and m-calpains, requiring micromolar and millimolar concentrations of Ca^{2+} for their activation, respectively. Calpains are present in the cytosol as inactive precursors that are activated in response to increased levels of cytosolic Ca^{2+} **(142)**. Conditions of

cellular stress often result in elevation of cytosolic Ca^{2+} levels, such as during necrotic signaling, and this leads to calpain activation. Calpains then cleave the Na^+/Ca^{2+} antiporter in the plasma membrane, which normally pumps out excess Ca^{2+}, resulting in a sustained Ca^{2+} overload and irreversible Ca^{2+} deregulation via this positive-feedback loop **(143)**. Massive activation of calpains may also contribute to the release of cathepsins B and L in the cytosol by causing LMP, as proposed in the "calpain-cathepsin" hypothesis by Yamashima and colleagues **(144)**.

Cathepsins are proteases residing mainly in the lysosomes and synthesized as inactive precursors that undergo proteolytic activation at the optimal acidic pH **(145)**. At this pH, most cathepsins possess both carboxypeptidase and endopeptidase activities, but at neutral pH (e.g., in the cytosol), the endopeptidase activity predominates. Cathepsin B is the most abundant lysosomal protease and is required for the breakdown of phagocytosed molecules during cellular protein turnover **(145, 146)**. After ischemia in primate hippocampal neurons, μ-calpains are activated and localize at the lysosomal membrane, where they provoke its disruption, which leads to the release of cathepsins. In the cytosol, these cathepsins cleave molecules that are normally not exposed to these proteases, eventually leading to necrotic cell death. The cathepsin and calpain inhibitors CA-074 and E-64c can prevent this ischemia-induced necrotic cell death **(144, 147)**. The importance of calpains and cathepsins in necrosis is confirmed by a study in *C. elegans*, in which the RNAi-mediated knockdown of calpain-like CLP-1 and TRA-3 or cathepsin-like ASP-3 and ASP-4 resulted in reduced necrotic cell death of neurons **(148)**.

Lysosomal rupture is favored not only by Ca^{2+}-mediated activation of calpains, but also by ROS-induced lipid peroxidation, PLA_2 activation, and ceramide generation. The important role played by lysosomal membrane permeabilization (LMP) in necrosis is also suggested by the observation that the overexpression of Hsp70, a guardian of lysosomal integrity, protects against necrotic cell death induced by TNF, heat shock, or oxidative stress **(149–151)**. In addition, the mitochondria of cells overexpressing Hsp70 are also protected against IR injury, probably due to a decrease in ROS and lipid peroxidation, leading to preservation of mitochondrial function and ATP formation **(152)**. A study on oxidative stress-induced necrosis indicated that Hsp70 plays a protective role by preventing the release of iron from the lysosomes **(153)**. Pretreatment of Hsp70-depleted cells with desferrioxamine protected them against the oxidative stress. However, it remains unclear whether Hsp70 exerts this effect by influencing the generation of hydroxyl radicals in lysosomes or by stabilizing the lysosomal membranes or certain lysosomal membrane proteins **(153)**. In this respect, it has been shown that the C-terminal part of Hsp70 is sufficient for neuroprotection in response to IR, indicating that neither the ability to fold denatured proteins, nor interactions with co-chaperones, nor other proteins that bind the N-terminal half of Hsp70 are essential for the protective effect **(154)**.

Ceramide

The sphingomyelin (SM) pathway is a conserved signaling system in which the sphingolipid ceramide is a central molecule. Ceramide serves as an intracellular second messenger of stress signals initiated by certain cytokines, irradiation, or chemicals **(155)**. Ceramide generated by stress is mainly the product of neutral or acid sphingomyelinases (N- or A-SMase), although it can also be generated by *de novo* synthesis by ceramide synthase **(156)**. SMase hydrolyzes sphingomyelin, the major phospholipid in cellular membranes, in ceramide. However, ceramide also accumulates due to changes in its clearance by sphingomyelin synthase or ceramidases **(157)**. The accumulation of ceramide has been demonstrated in TNF-induced necrosis of L929 cells, which seemed to require PLA_2 activity **(135**, **158)**. Acid SMase-deficient cells displayed a substantially higher resistance to TNF/zVAD-fmk-induced necrosis than wild-type cells. Acid SMase inhibitors also partially protect or retard the necrotic response of L929, Jurkat, and NIH3T3 cells **(159)**. Furthermore, the addition of ceramide analogues was effective in mediating caspase-independent cell death in several other cell types **(160**, **161)**. In mitochondria, ceramide is able to block complex III activity, thereby increasing the production of ROS **(162)**. RIP1 seems to have an important role in the ceramide death pathway, because blockade of RIP1 function by genetic ablation, RNAi, or pharmacological intervention protected against TNF-induced generation of ceramide and caspase-independent cell death in all types of cells studied **(159)**. As RIP1 appears to be a central initiator of necrotic cell death, it is tempting to speculate that RIP1 phosphorylates and activates $cPLA_2$ **(125)**, which in turn mediates the generation of ceramide. However, elevated ceramide production is certainly not the only effect of RIP1 signaling in response to TNF, since the inhibition of ceramide accumulation was not as effective as RIP1 inhibition in protecting against cell death, indicating that other targets exist downstream from RIP1. Ceramide can be further converted to sphingosine by acid ceramidase. Due to its long hydrophobic tail and polar head containing a proton-trapping amino group **(163)**, sphingosine has detergent as well as lysosomotropic properties and causes lysosomal membrane rupture in a dose-dependent manner **(164)**.

Methylglyoxal

Glycolysis is the major biochemical pathway for the production of methylglyoxal (MG) in the cell. Two glycolytic metabolites, namely glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP), undergo spontaneous, nonenzymatic phosphate elimination to yield MG **(165)**. MG is nonenzymatically conjugated with glutathione, but it can also react with lysine, arginine, and cysteine residues of proteins **(166)**. The interaction of MG with proteins is rapid and initially reversible. However, this MG moiety can undergo further chemical

rearrangements, yielding complex adducts collectively termed advanced glycation end products (AGEs) **(167)**. This process is called nonenzymatic glycosylation or glycation. Importantly, the formation of AGEs is accelerated by oxidative stress **(168)**. On the other hand, MG, once bound to proteins, constitutes a very localized and continuous center of ROS production on the target protein **(169)**. For example, the MG-derived cation radical, its anion counterpart, and (in the presence of oxygen) also superoxide radicals may induce the cleavage of peptide bonds, the oxidation of bystander proteins, or the formation of intra- and intermolecular protein cross-links **(170)**.

These effects are counteracted by MG detoxification. The most important detoxification mechanism in mammalian cells is the glyoxalase pathway, in which glyoxalase 1 converts GSH-conjugated MG into S-D-lactoylglutathione. This product is then converted by glyoxalase 2 into the harmless and cell-permeable D-lactate, with the regeneration of glutathione **(171)**. Because MG and glyoxalase 1 activity are linked to the accumulation of ROS and to the level of glutathione, respectively, the glyoxalase pathway fulfills an important role in cellular systems where ROS production or the antioxidant defense is of importance.

In this respect, the conception of the role of MG as an inevitable byproduct of glycolysis has shifted recently; it is now considered an important second messenger in several cell biological processes **(172)**. Exogenously added MG causes ROS-dependent, JNK-mediated, and p38 MAPK-mediated apoptosis in different cell lines and increases cytosolic Ca^{2+} **(173–176)**. The mode of cell death triggered by MG can be linked to the antioxidant and detoxifying systems of the cell line: low glyoxalase 1 activity, together with lower amounts of GSH and decreased Cu/ZnSOD and catalase activity, favors apoptotic cell death, whereas higher activities of these enzymes together with higher amounts of GSH favor caspase-independent cell death **(177)**. A connection between MG and necrosis was recently uncovered. In L929 cells, TNF-induced necrotic cell death is characterized by an increase in the concentration of MG. This in turn induces the formation of an MG-derived AGE on a specific but unidentified target protein. In this model, TNF also stimulates PKA-dependent phosphorylation of glyoxalase 1. This phosphorylation does not affect the activity of glyoxalase 1. However, inhibition of the phosphorylation of glyoxalase 1 inhibits AGE formation, indicating that the phosphorylated form of glyoxalase 1 plays a role in the modification of proteins by MG. Furthermore, the formation of the MG adduct on the target protein is also inhibited when oxidative stress or glycolysis is inhibited. Inhibition of these two events, together with inhibition of phosphorylation of glyoxalase 1, retards cell death considerably, whereas exogenously added MG accelerates it **(178)**, suggesting that MG may function as a mediator in TNF-induced necrosis of L929 cells. These results further indicate that MG modification of proteins, oxidative stress, glycolysis, and phosphorylation of glyoxalase 1 are associated with necrotic cell death. MG can also induce necrosis in tubular renal cells *in vitro* **(176)**. When the glucose concentration is high, MG induces necrosis in HUVEC cells, but at lower concentrations of glucose, it induces apoptosis **(179)**.

Innate Immune Response Following Necrotic Cell Death

Because apoptotic cells display an "eat-me" label, such as the exposure of phosphatidylserine on the cell surface, they are rapidly recognized and taken up by professional and nonprofessional phagocytes by a zipper-like mechanism **(180)**. In contrast, the recognition and uptake of necrotic cells by macropinocytosis is slower and less efficient and occurs only after the loss of plasma membrane integrity **(180)**. Due to the containment of the apoptotic cells or particles, and the wave of caspase proteolytic activity, the rapid uptake of apoptotic cells is immunologically silent and does not provoke inflammation **(181)**. On the other hand, necrotic cells release their intracellular contents before phagocytes can clear them, resulting in the activation of proinflammatory and immunostimulatory responses **(182)**. High-mobility group box 1 (HMGB1) protein is one of the proinflammatory molecules passively released by necrotic cells **(183)**. It is a DNA-binding molecule involved in DNA bending, nucleosome stabilization, and transcriptional regulation **(184)**. However, HMGB1 remains bound to DNA in apoptotic cells, even during secondary necrosis **(183)**. Released extracellular HMGB1 binds with high affinity to receptors for advanced glycated end products **(185)**, TLR-2 and TLR-4, activates macrophages and dendritic cells, and promotes neutrophil recruitment **(186, 187)**. Necrotic cells also release heat-shock proteins (Hsp70, Hsp90, gp96) and calreticulin **(188)**, leading to the activation of antigen-presenting cells (APCs) **(189)**. Furthermore, large amounts of uric acid are released, resulting in activation of the inflammasome, which is involved in the maturation of the inflammatory cytokine IL-1β **(190, 191)**. Other molecules that become immunostimulatory when released from necrotic cells include mRNA **(192)**, genomic DNA **(193)**, nucleotides, and nucleosides **(194)**, all of which bind to TLRs and induce the maturation of APCs. In addition, necrotic cells can also induce an inflammatory response by actively secreting inflammatory cytokines due to the activation of NF-κB and MAPKs **(195)**. This observation is in agreement with the notion that necrotic cells retain the ability to synthesize proteins until the membrane completely loses its integrity, in contrast to apoptotic cells, in which translation is rapidly inhibited **(25, 196)**.

Molecular Interference with Pathology-Associated Necrotic Cell Death

As discussed in the previous sections, there is increasing evidence that regulated necrotic cell death occurs in several physiological and pathological conditions. Many studies have investigated ways to interfere with this necrotic cell death process at different molecular levels (MPT, LMP, ROS) in pathologies associated with necrotic cell death, such as IR injury of various tissues, neurodegenerative diseases, and TNF-induced shock (Table 27.1).

Table 27.1 Molecular interference with pathology-associated necrotic cell death

Necrotic event	Therapeutic	Molecular target	Pathology	Ref.
MPT	Propofol	Oxidative stress	IR injury rat heart	**(197)**
	Dantrolene	Blocker of Ca^{2+} release	IR injury heart and brain	**(198, 199)**
	Cyclosporin A	CypD	IR injury heart and brain	**(200)**
	Sanglifehrin-A	CypD	IR injury heart and brain	**(201)**
	3-aminobenzamide, BGP-15	PARP-1	IR injury heart and brain	**(203–205)**
	Necrostatin-1	RIP1	IR injury heart and brain	**(39, 110, 206)**
LMP	Hsp70 overexpression	Lysosomal guardian	IR injury heart and brain	**(208, 209)**
	Geldanamycin	Hsp70 upregulation	Cerebral stroke	**(210)**
			TNF-induced lethal shock	**(211)**
ROS	Clioquinol	Cu/Zn chelator	Neurodegenerative diseases	**(215)**
	Desferrioxamine	Iron chelator	Neurodegenerative diseases	**(216)**
	Curcumin, ginkgo extract	Antioxidants, anti-inflammatory	Neurodegenerative diseases	**(217, 218)**
	Coenzyme Q-10	Antioxidant	Neurodegenerative diseases	**(219)**
	BHA	Antioxidant	TNF/zVAD-induced lethal shock	**(106)**

As described earlier, Ca^{2+} overload and ROS production favor MPT during necrotic cell death. Thus, inhibiting the opening of MPTP by reducing oxidative stress or mitochondrial Ca^{2+} overload could protect from IR injury. Indeed, treatment with propofol, a free radical scavenger that lowers oxidative stress and/or inhibits Ca^{2+} channels, protects rat hearts from IR injury **(197)**. Mitochondria from the propofol-treated perfused hearts showed less damage to ETCs and a significant reduction in calcium-induced MPTP opening. Dantrolene selectively affects the ryanodine receptor on sarcoplasmic reticulum (SR) membranes, resulting in the blockade of Ca^{2+} release by the SR. Several studies illustrate reduced tissue injury of the ischemic heart and brain in the presence of dantrolene **(198, 199)**. Furthermore, and as mentioned earlier, treating mice with CsA, or with the more specific sanglifehrin-A, reduced IR injury of the heart and brain **(200, 201)**. As CypD is the target of these immunosuppressants, this observation was confirmed using CypD-deficient mice and CypD transgenic mice. Whereas the CypD-deficient mice were protected against

IR-induced heart and brain failure **(77–80, 202)**, the CypD-overexpressing mice showed mitochondrial swelling and spontaneous cell death **(78)**. PARP-1 inhibitors, such as 3-aminobenzamide or BGP-15, were also effective in preventing myocardial and cerebral damage following ischemia **(203–205)**. IR injury in the heart and brain can also be decreased by the administration of necrostatin-1 (Nec-1), which blocks RIP1-mediated necrotic cell death **(39, 206)**. However, this effect was not observed in CypD-deficient mice, indicating that the cardioprotective effect of Nec-1 depends on the presence of CypD, which delays the opening of MPTP in rat cardiomyocytes subjected to oxidative stress **(207)**.

Transgenic mice overexpressing Hsp70 are protected against myocardial and cerebral ischemia **(208, 209)**. Furthermore, Hsp70 upregulation by pretreatment with geldanamycin can protect the brain against stroke *in vivo* and astrocytes against ischemia *in vitro* **(210)**. In addition, a recent study reported that Hsp70 transgenic mice are protected against TNF-induced lethal shock **(211)**. Moreover, the induction of Hsp70 expression by the addition of zinc to the drinking water of wild-type mice conferred protection against TNF-induced death **(211)**. Similarly, BHA, a lipophilic food antioxidant, completely abrogated sensitization of TNF-induced shock in mice treated with zVAD-fmk **(106)**. Trolox, a water-soluble analogue of vitamin E, which can prevent lipid peroxidation, and dipyridyl, an iron chelator, both protected against H_2O_2-induced lipid peroxidation and the resultant necrotic death of cardiac myocytes, demonstrating the important role of ROS and redox-active iron in necrosis **(212)**.

In neurodegenerative diseases, such as AD, PD, and HD, oxidative stress is a major etiologic factor. During normal aging, the brain accumulates iron, copper, and zinc, which increase vulnerability to oxidative stress, for example, to ROS generated by the Fenton reaction **(213)**. These age-related neurodegenerative disorders are accompanied by the formation of abnormal protein-metal interactions that could contribute to neuronal damage. An example of such an aberrant protein-metal interaction in AD is the increased formation of β-amyloid peptide in the presence of iron, which leads to efficient generation of ROS [reviewed in **(214)**]. In agreement with the protective effect of iron chelation and ROS scavenging in necrosis, studies with metal-protein attenuating compounds (e.g. clioquinol) **(215)**, iron chelators (e.g. DFO) **(216)**, and natural antioxidants (e.g., curcumin, ginkgo extract) **(217, 218)** seem promising for the future treatment of patients with neurodegenerative diseases. In this respect, coenzyme Q-10 shows beneficial effects in several animal models of neurodegenerative diseases **(219)**. Coenzyme Q-10 acts both as an antioxidant and as an electron acceptor at the level of the mitochondria **(220)**.

On the other hand, the induction of necrotic cell death may be desirable, for example, during tumor therapy, because resistance to apoptosis is an important aspect of carcinogenesis. Some treatments used in cancer therapy can cause the necrotic death of tumor cells along with apoptosis [reviewed in **(221)**]. These include photodynamic therapy (PDT) and alkylating

DNA-damaging agents. PDT involves the administration of photosensitizing molecules to the tumor, such as porphyrins, chorines, or phthalocyanines **(222)**. Upon excitation, the photosensitizers generate ROS (primarily singlet oxygen), leading to death of the target tumor cells, even if they are resistant to apoptosis **(223)**. Depending on their localization, activated photosensitizers can cause lysosomal rupture with the concomitant release of lysosomal proteases **(224)** or the loss of the plasma membrane integrity **(225)**. However, the lethality of PDT appears to be largely due to the loss of mitochondrial membrane potential **(226)**. Accordingly, PDT-mediated cell death is suppressed by treatment with CsA **(227)**.

DNA-alkylating agents are the most widely used and effective chemotherapeutic drugs in cancer treatment **(228)**. As previously mentioned, hyperactivated PARP-1 is a crucial initiator of DNA damage-induced necrotic cell death because it depletes cytosolic NAD^+ and induces necrosis **(55**, **56**, **61)**. On the other hand, the inhibition of PARP-1 may contribute to tumorigenesis by inducing genomic instability **(229)**. However, PARP-1 inhibition can also contribute to cell death in tumor cells lacking other components of DNA repair **(230**, **231)**. Moreover, the hyperactivation of PARP-1 depletes cytosolic NAD^+ and induces necrosis **(61)**. Thus, depending on the context, either the hyperactivation or inhibition of PARP-1 can be therapeutically beneficial.

Concluding Remarks

Necrotic cell death has been extensively studied over the past decade. This has led to a considerable increase in our knowledge of stimuli, initiators, and mediators or executioners involved in necrotic cell death (Fig. 27.1). It is clear that necrosis is not the result of a single, well-defined signaling pathway, but rather the outcome of intricate interplay between several signaling events. Furthermore, depending on the stimulus and cell type, different signaling mechanisms are involved in necrosis. As RIP1 appears to be a central kinase in several of the described necrotic cell death pathways [reviewed in **(37)**], it can be considered a key molecule in the initiation of necrosis. The kinase activity of RIP1 is crucial for death receptor-mediated necrosis. During TNF-induced necrotic cell death, RIP1 appears to be responsible for the observed ROS accumulation, ceramide generation, and increase in labile iron pool, which together act to achieve the final necrotic outcome. Moreover, RIP1 is also involved in necrosis induced by DNA damage or by the triggering of TLR-3 and -4. Thus, the identification of RIP1 substrates would lead to a more mechanistic insight in the events downstream from RIP1 signaling events during necrotic cell death. That should bring to light other target proteins and could lead to development of new therapeutic strategies for interference with necrotic cell death.

This chapter makes it clear that calcium and ROS are the two main players in necrosis. Increased calcium enhances oxidative stress by increasing NO production or by affecting mitochondrial respiration, which leads to augmented ROS production. In addition, higher cytosolic calcium concentrations result in the activation of proteases (calpains and cathepsins), $cPLA_2$, and lipoxygenases, all of which are negative effectors of organelle and plasma membrane integrity. On the other hand, mitochondrial dysfunction and increased ROS production can modulate extramitochondrial calcium pools.

Depending on the cellular context and the intensity of the stimulus, either apoptosis or necrosis can often be initiated in response to the same stimulus. This redundancy helps to ensure that unwanted or damaged cells are eliminated when one of these two cell death pathways is hampered. This is important, for example, during infection, because several pathogens have evolved strategies to suppress apoptosis of the host cells in order to complete their replication cycle. In the presence of caspase inhibitors, cells often shift their response from apoptosis to necrosis when exposed to a certain death stimulus. Vice versa, the inhibition of ROS by the ROS scavenger BHA upon dsRNA stimulation has been shown to shift the response from necrosis to apoptosis, and a similar shift is observed after TNF stimulation of geldanamycin-pretreated L929 cells, in which RIP1 levels are strongly reduced. However, once a cell has selected the pathway to its death, the other pathway is often actively counteracted. During death receptor-induced apoptosis, RIP1 is cleaved by caspase-8, which suppresses its pronecrotic and prosurvival properties. In addition, PARP-1 is cleaved by caspase-3 during apoptosis in such a way that the resulting PARP-1 fragments can no longer be stimulated by DNA-strand breaks. During necrosis, apoptosis can be inhibited by PARP-1-mediated ATP depletion or NO-mediated nitrosylation of caspase-3, resulting in its inactivation.

The immunological consequences of necrotic and apoptotic cell death are quite different. Apoptotic cells are removed silently, whereas necrotic cells release their intracellular contents, resulting in the activation of proinflammatory and immunomodulatory responses that can function as a danger signal to alert the organism to the severity of the insult. However, the defective clearance of dying cells may contribute to the persistence of inflammation and to excessive tissue injury, which are associated with the development of several diseases, such as chronic obstructive disease, diabetes, atherosclerosis, and systemic lupus erythematosus.

This chapter illustrates the existence of a regulated and/or programmed form of necrosis, a pathway that is being unraveled by extensive research. Elucidating the necrotic cell death pathways in more detail is important because it could lead to the development of new therapeutic strategies for antitumor therapy, the treatment of neurodegenerative diseases, and the management of infections, and it may also be useful in other applications, such as organ transplantation.

Abbreviations

AA, arachidonic acid; AD, Alzheimer's disease; AGE, advanced glycation end product; AICD, activation-induced cell death; AIF, apoptosis-inducing factor; AMPA, 2-amino-3-propionate; ANT, adenine nucleotide translocator; APC, antigen-presenting cell; ASC, apoptotic speck protein containing a CARD; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; CsA, cyclosporin A; CypD, cyclophylin D; DED, death effector domain; DFO, desferrioxamine; DHAP, dihydroxyacetone phosphate; ER, endoplasmic reticulum; ETC, electron transport chain; FADD, Fas-associated death domain; FasL, Fas ligand; GAP, glyceraldehyde-3-phosphate; GSH, glutathione; HD, Huntington's disease; HMGB1, high-mobility group box 1; Hsp, heat-shock protein; IR, ischemia-reperfusion; JNK, c-Jun N-terminal kinase; LMP, lysosomal membrane permeabilization; LOX, lipoxygenase; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; MG, methylglyoxal; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; MOI, multiplicity of infection; MPT, mitochondrial permeability transition; MPTP, mitochondrial permeability transition pore; NALP3, NACHT/LRR/pyrin domain-containing protein 3; Nec-1, necrostatin-1; NF-κB, nuclear factor κB; NLR, NOD-like receptor; NMDA, N-methyl-D-aspartate; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; PARP-1, poly(ADP-ribose) polymerase-1; PD, Parkinson's disease; PDT, photodynamic therapy; PKA, protein kinase A; PLA, phospholipase A; PYCARD, PYD and CARD domain-containing protein; RIP1, receptor interacting protein 1; RLR, RIG-I-like receptor; ROS, reactive oxygen species; SM, sphingomyelin; SOD, superoxide dismutase; SR, sarcoplasmic reticulum; TLR, Toll-like receptor; TNF, tumor necrosis factor; TNFR1, tumor necrosis factor receptor 1; TRADD, TNF receptor-associated death domain; TRAF2, TNF receptor-associated factor 2; TRAIL, TNF-related apoptosis-inducing ligand; VDAC, voltage-dependent anion channel.

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Chapter 28
Caspase-Independent Mitotic Death

Katsumi Kitagawa

Abstract The spindle checkpoint ensures proper chromosomal segregation by monitoring kinetochore–microtubule attachment. A failure of this checkpoint causes aneuploidy, which leads to tumorigenesis. The cell death that prevents the aneuploidy caused by failure of the spindle checkpoint is yet unknown. We have identified a novel type of mitotic cell death, which we term caspase-independent mitotic death (CIMD). When BUB1 but not MAD2 is depleted, CIMD is induced by conditions that activate the spindle checkpoint [e.g., cold shock or treatment with microtubule inhibitors or 17-AAG (17-allylaminogeldanamycin)]. CIMD depends on the apoptosis-inducing factor (AIF) and endonuclease G (Endo G), which are effectors of caspase-independent cell death. CIMD also depends on p73, a homologue of p53, but not on p53. When BUB1 is completely depleted, aneuploidy occurs instead of CIMD. Therefore, CIMD may be the programmed cell death that protects cells from aneuploidy by inducing the death of cells prone to substantial chromosome missegregation.

Keywords Spindle checkpoint · Mitosis · Bub1 · Mad2 · Caspase-independent cell death (CIMD) · Chromosomal instability · p73 · Aneuploidy

Introduction

Errors in the molecular mechanisms that ensure accurate chromosome segregation during mitosis (e.g., chromosome nondisjunction and chromosome loss) result in imbalances in chromosome number, that is, aneuploidy. Aneuploidy is thought to contribute to the development of cancer **(1)** by causing the expression of recessive oncogenic phenotypes **(2)**.

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The spindle checkpoint (also called the mitotic spindle checkpoint, the mitotic checkpoint, or the spindle assembly checkpoint) ensures the fidelity of chromosome segregation by delaying mitotic progression via transient inhibition of the anaphase-promoting complex (APC), a ubiquitin-E3 ligase, in response to defects in kinetochore–microtubule attachment (Fig. 28.1). Defects in the spindle checkpoint result in aneuploidy **(3)**.

Genes required for the spindle checkpoint were first isolated from the budding yeast *Saccharomyces cerevisiae* and include *MAD1*, *MAD2*, and *MAD3* (mitotic arrest-deficient) **(4)**; *BUB1* and *BUB3* (budding uninhibited by benzimidazole, a microtubule-depolymerizing drug) **(5)**; and *MPS1* (monopolar spindle) **(6)**. Spindle checkpoint proteins and their functions are highly conserved between yeast and humans. [The human homologue of Mad3 is BUBR1 and the designations of other yeast and other human homologues are the same **(7)**.]

Several pieces of evidence suggest that the spindle checkpoint plays a role in tumorigenesis **(1)**. Mutations in human Bub1 homologues (BUB1 and BUBR1) were first found in subtypes of colorectal cancer cells that exhibited chromosome instability (CIN tumor cells) **(8)**. The CIN phenotype can cause mutations in spindle checkpoint genes **(9–11)**, decreased levels of spindle checkpoint proteins **(12, 13)**, and loss of spindle checkpoint activity **(14, 15)**. *Mad2*$^{+/-}$ mice have a high rate of lung tumor development after a long latency **(16)**. *Bubr1*$^{+/-}$ mice and *Bub3*/*Rae1* heterozygotes are more disposed to tumor development **(17, 18)**.

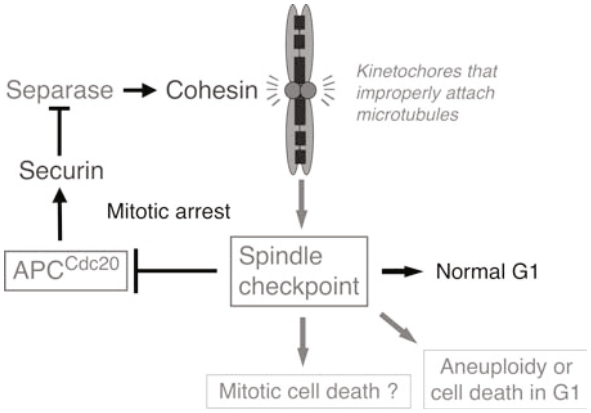

Fig. 28.1 The spindle checkpoint protects cells from chromosome missegregation caused by mitotic errors. When the checkpoint is activated by defects in kinetochore–microtubule attachment, it arrests the cell cycle by inhibiting the anaphase-promoting complex (APC). APC inhibition causes securin to accumulate, which in turn inhibits separase activity, which targets cohesin. As a result, cohesion is maintained and sister chromatids remain bound together. Therefore, cells are arrested in mitosis, thereby preventing aneuploidy. Defects in the mitotic spindle checkpoint result in cell death or aneuploidy, which may lead to tumorigenesis

The spindle checkpoint is the major target of mitotic alterations in cancer—38% of the mitotic regulators that are mutated in cancer are part of the spindle checkpoint, which suggests that this checkpoint plays a vital role in preventing malignant transformation **(19)**. Carter et al. found that 29 of the 70 genes included in a chromosome instability signature were mitotic regulatory genes **(20)**.

Apoptosis might play a role in preventing the aneuploidy caused by defects of the spindle checkpoint. Mutations in *bub1* cause chromosome missegregation and apoptosis in *Drosophila* **(21)**, and *Mad2*-null mouse embryos undergo apoptosis at E6.5-E7.5 **(22)**. In these cases, cell death seems to occur in the subsequent G1 phase, and p53 is likely to induce apoptosis when aneuploidy occurs. Mouse embryonic fibroblast lines carrying deletions in *Mad2* and p53 but not in *Mad2* can be established and have very high levels of chromosome instability **(23)**.

We have recently identified a novel type of cell death occurring during mitosis that is independent of caspase activation or p53. We therefore termed it *caspase-independent mitotic death* (CIMD). CIMD occurs in BUB1-deficient cells that have defects in kinetochore–microtubule attachment **(24)**. However, aneuploidy occurs instead of CIMD when BUB1 is completely depleted **(24)**. Thus, CIMD might be the cell death mechanism that protects cells from aneuploidy by inducing the death of cells prone to substantial chromosome missegregation.

Aneuploidy or Cell Death Occurs When the Spindle Checkpoint Fails

When cells cannot satisfy the spindle checkpoint after a long mitotic delay, they undergo one of the following cell fates: (1) some die in mitosis; (2) some exit mitosis but die by apoptosis in the subsequent G1 phase; or (3) some exit mitosis but are tetraploid and reproductively dead **(25)**. Microtubule inhibitors induce mitotic arrest by activating the spindle checkpoint and eventually causing cytotoxicity **(26)**. Several studies have described the cytotoxicity of microtubule inhibitors and resultant death as either apoptosis in G1 or reproductive death **(26)**. However, questions about cell death during mitosis have remained. Although some evidence suggests that apoptosis occurs during mitosis **(27–31)**, the relationship between apoptosis or programmed cell death during mitosis and the spindle checkpoint is unknown.

What happens when the spindle checkpoint fails? When cells have defects in kinetochore–microtubule attachment (e.g., when treated with microtubule inhibitors), the spindle checkpoint is activated and arrests the cell cycle in early mitosis (Fig. 28.1). If the spindle checkpoint is defective (Fig. 28.1), this arrest cannot occur. As a result, even though all chromosomes are not aligned properly, premature mitotic exit occurs, followed by substantial chromosome loss or gain [Fig. **28.2**(a) and Color Plate 14]. We have seen abnormal nuclei in *Mad2*-depleted HeLa cells treated with drugs that activate the spindle checkpoint:

nocodazole (microtubule-depolymerizing drug), paclitaxel (microtubule-stabilizing drug), and 17-allylaminogeldanamycin (17-AAG, an Hsp90 inhibitor that induces kinetochore defects) **(24, 32)**.

An interesting finding is that substantial Bub1 depletion does not reduce the drug-induced mitotic delay **(24)**, suggesting that the spindle checkpoint activity is not altered. However, in a colony growth assay, the treatments caused substantial lethality in Bub1-depelted cells **(24)**. Chromosomes in Bub1-depleted mitotic cells show terminal deoxynucleotide transferase dUTP nick end-labeling (TUNEL)-positive signals (Fig. 28.3 and Color Plate 15) **(24)**, suggesting that these cells are dead or dying in mitosis before chromosome loss or gain. Thus, mitotic delay is not affected, because cells are dead in mitosis, and not because the spindle checkpoint activity is not altered [Fig. **28.2**(b) and Color Plate 14]. TUNEL-positive chromosomes have been found in early mitosis

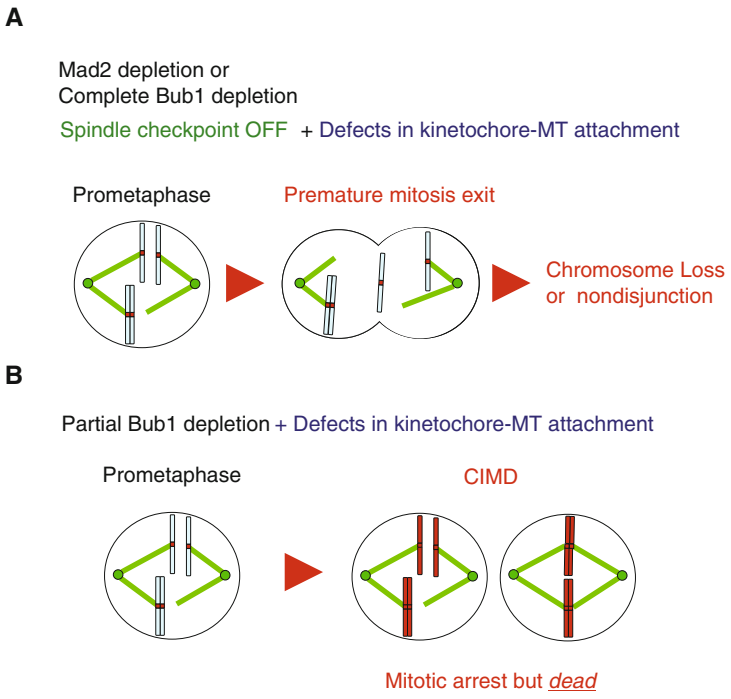

Fig. 28.2 (**a**) A model that describes how chromosome loss or nondisjunction occurs in spindle checkpoint-defective cells (MAD2-depleted cells or complete BUB1-depleted cells). In spindle checkpoint-mutant cells, the spindle checkpoint is not activated even if there are defects in kinetochore–microtubule attachment. No mitotic delay occurs, which results in the premature exit from mitosis. Thus, there is substantial chromosome loss or nondisjunction, and presumably cell death follows. (**b**) A model that describes the same scenario in partial BUB1-depleted cells. Here, defects in kinetochore–microtubule attachment induce lethal DNA fragmentation (CIMD). Because cells are still arrested in mitosis, the mitotic index remains unchanged. Therefore, the spindle checkpoint appears to be active.(*see* Color Plate 14)

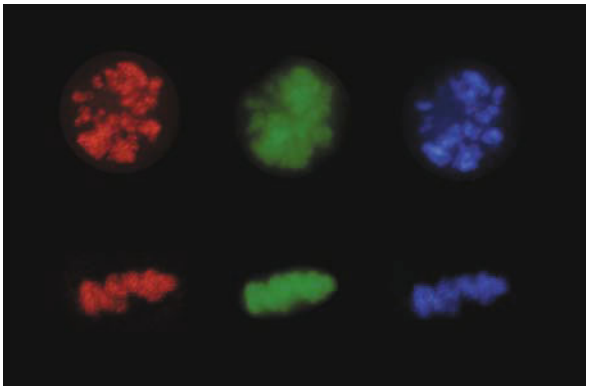

Fig. 28.3 CIMD occurs in BUB1-depleted cells in the presence of microtubule inhibitors or 17-AAG. HeLa cells that are BUB1-depleted and 17-AAG-treated exhibit DNA fragmentation (TUNEL-positive; *red*) during mitosis (*top row*, prometaphase; *bottom*, metaphase). Forty-eight hours after HeLa cells were transfected with BUB1 siRNA, they were incubated with 17-AAG (+17AAG, 500 nM) for 24 hours at 37 °C. Fixed samples were stained by using an *in situ* cell death detection system that contained TMR red (TUNEL-signal; *red*), an antiphosphorylated histone H3 mouse monoclonal antibody, and FITC-conjugated secondary antibodies (*green*). DNA was stained with DAPI (*blue*) to visualize (*see* Color Plate 15)

(prophase, prometaphase, or metaphase) only and not in late mitosis (anaphase or telophase) in these cells **(24)**. Because chromosomes are normally aligned (in metaphase) or not yet aligned (i.e., had not been lost or gained), there is no reason for cells to die at that stage. It is therefore possible that this mitotic cell death is programmed cell death and termed CIMD because DNA fragmentation is independent of caspase activity **(24)**.

Nocodazole or cold treatment, paclitaxel, and 17-AAG **(32)**, but not ICRF187 (a top II inhibitor), induce CIMD in BUB1-depleted cells **(24)**. Because all these treatments except ICRF187 induce defects in kinetochore –microtubule attachment, we conclude that defects in kinetochore–microtubule attachment activate the spindle checkpoint in normal cells but can induce CIMD in BUB1-depleted cells.

CIMD as Programmed Cell Death

There is additional proof that CIMD is programmed or active cell death. First, death occurs rapidly: DNA fragmentation starts 20 minutes after adding microtubule inhibitors or 17-AAG, and several hours later cells collapse **(24)**. Second, programmed cell death depends on factors that induce cell death actively, and the removal of these factors suppresses lethality. CIMD depends on the apoptosis-inducing factor (AIF) and endonuclease G (Endo G), both DNases,

and their depletion suppresses the lethality of CIMD **(24)**. Furthermore, CIMD depends not on p53, but on its homologue p73, a putative tumor suppressor and checkpoint protein **(24)**.

As transcriptional factors, p73 and p53 function in the same manner **(33)**. However, p73 has specific targets and specific roles **(33)**, and CIMD is probably one of them. At the G2/M transition, p73 is phosphorylated at Thr-86 by the p34cdc2/cyclin B complex **(34)**. This M phase-specific phosphorylation of p73 generally negatively regulates its transcriptional activity. However, there are p73-specific transcriptional targets during mitosis such as the cyclin-dependent kinase inhibitor Kip2/p57 **(35)**. Therefore, CIMD may be induced by p73-specific transcriptional target proteins during mitosis.

Low Levels of BUB1 Are Required for CIMD

Meraldi and Sorger reported that even very low levels of BUB1 remaining in the cell can induce mitotic delay, but when BUB1 is completely depleted, premature mitotic exit occurs **(36)**. Their finding is consistent with our observation that CIMD does not occur when BUB1 is almost completely depleted. Therefore, the remaining BUB1 appears to be required to induce CIMD **(24)**. Many cells having abnormal nuclei result when CIMD does not occur **(24)**; hence, CIMD probably protects cells from aneuploidy by killing those destined to have abnormal nuclei.

It is still not clear why the partial, but not the complete, depletion of BUB1 activates p73. A simple model could explains this: BUB1 kinase is activated because of defects in kinetochore–microtubule attachment, and BUB1 phosphorylates p73 to activate it, but binding BUB1 to p73 silences the transcriptional activity of p73. Therefore, only a certain level of BUB1 is needed to phosphorylate p73, one low enough to release phosphorylated p73 from BUB1 to bind DNA. However, unfortunately, immunoprecipitation or the two-hybrid system has been unable to detect the interaction between BUB1 and p73. Therefore, another approach is to assume that an unknown protein X exists, which is phosphorylated by BUB1 instead of p73. The phosphorylated protein X can bind to p73 to activate it. The overexpression of protein X should induce CIMD. To prove this model, unknown [such as BUBR1 **(37)**, BUB3 **(38**, **39)**, CENPE **(37)**, RAE1 **(40)**, and Blinkin **(41)**] and known proteins that physically interact with BUB1 should be tested as being the likely protein X.

Autophagy Occurs During CIMD

Our electron microscopy (EM) analyses have revealed that cells in which CIMD occurs have more autophagosomes, especially in those treated with 17-AAG and depleted of BUB1 **(24)**. Substantial numbers of holes or vesicles have been

observed in cells in which CIMD occurs **(24)**. Autophagy might generate these holes or vesicles via the autophagy/autolysosome pathway. Under certain conditions, autophagy can promote cell death, particularly if cells are triggered to die but apoptosis is not possible **(42–44)**. Importantly, these studies show that autophagy proteins are required to induce or execute cell death **(42–44)**. Therefore, future studies can determine whether autophagy is necessary to induce CIMD.

Other Types of Mitotic Cell Death

Apart from CIMD, there are two other types of mitotic cell death: mitotic catastrophe and mitotic apoptosis (Table 28.1). Mitotic catastrophe results from the combination of cell damage and deficient cell cycle checkpoints, in particular, DNA structure checkpoints and the spindle checkpoint **(45, 46)**. Mitotic catastrophe results in the formation of multinucleate and giant cells that contain uncondensed chromosomes **(46)**, which are not observed during CIMD but are similar to those observed in MAD2-depleted cells (Table 28.1). Castedo et al. defined the molecular events that characterize the two types of mitotic catastrophe **(45)**. The current opinion is that in the first type, the cell dies in a p53-dependent manner during or near metaphase, for example, as seen during the death of Chk2-inhibited or Plk2-depleted cells. In the second type, death occurs in a partially p53-dependent manner after failed mitosis and during the activation of the polyploidy checkpoint **(45)**. Mitotic catastrophe is also accompanied by chromatin condensation, mitochondrial release of proapoptotic proteins, caspase activation, and DNA degradation **(45)**. In contrast, CIMD occurs in a p53-independent manner. Nitta et al. reported that the spindle checkpoint is required for mitotic catastrophe induced by DNA-damaging agents **(47)**. Therefore, the features of CIMD differ from those of mitotic catastrophe (Table 28.1).

Table 28.1 Mitotic cell death

	CIMD	Mitotic catastrophe	Mitotic apoptosis
Caspases	No	Yes	Yes
p53	No	Yes	ND
p73	Yes	ND	ND
DNA fragmentation: TUNEL DNA ladder	Positive Yes—but mostly large molecules	Positive No	Positive Yes
DNA morphology	Normal condensed chromosomes	Multinucleate and uncondensed chromosomes	Bubble-shaped and fragmented nuclei
AIF and EndoG	Yes	ND	ND

Note: ND, not determined.

Several previous reports have described apoptosis during mitosis. Woods et al. reported that high concentrations of paclitaxel (>25 nM for HeLa cells) are very cytotoxic **(30)**. With 100–200 nM paclitaxel, p53-independent, TUNEL-positive cells appear to be in prophase and their morphology resembles that of cells undergoing typical apoptosis (i.e., nuclei bubble-shaped and fragmented). This appearance is different from that observed during CIMD. High concentrations of paclitaxel induce apoptosis by activating kinase pathways that include Akt and mTOR **(48)**. We have used paclitaxel at a low concentration (10 nM), sufficient to cause a substantial mitotic delay but not enough to induce apoptosis in most cells **(30)**. Burns et al. reported that several hours after treatment with paclitaxel or nocodazole, 80% of Snk/Plk2-depleted p53-positive cells contain active caspase-3 and that 4 N cells are positive for cyclin B **(28)**. Yang et al. reported that Mitosin/CENP-F depletion induces premature chromosome decondensation followed by cell death with caspase activation **(31)**. Deluca et al. reported that hNuf2-depleted cells exit directly from a delayed mitosis, exhibit apoptotic cell morphology, and contain DNA whose appearance resembled that seen in cells undergoing typical apoptosis **(29)**. Because hNuf2 depletion blocks stable kinetochore–microtubule attachment, one could argue that hNuf2 depletion-induced cell death occurs downstream of BUB1 depletion. However, if this were the case, BUB1 depletion itself should cause substantial CIMD, but this does not occur. In addition, Meraldi et al. showed that BUB1 is not required for the kinetochore localization of hNuf2 **(36)**. Therefore, hNuf2 depletion-induced cell death is unlikely to be CIMD, although further investigation is required to clarify this observation. None of the previously mentioned studies or other findings of mitotic cell death appear to exemplify the CIMD that is detected by the TUNEL assay during early mitosis and is independent of caspase and p53.

CIMD and Tumorigenesis

The physiological function of CIMD *in vivo* is not yet known. CIMD is induced in BUB-depleted cells by cold treatment (at 23 °C), which depolymerizes microtubules **(24)**. Cells in a human body can occasionally be exposed to this stress. We have not yet determined the pathway that leads to DNA fragmentation from Bub1 depletion, but defects or changes in other factors in this pathway could induce CIMD when cells are exposed to room temperature or lower. We hypothesize that CIMD protects cells from aneuploidy, which can lead to tumorigenesis; when CIMD fails, tumorigenesis increases in BUB1-deficient animals.

$Bub1^{+/-}$ mice do not exhibit spontaneous tumorigenesis **(49)**. However, Bub1 hypomorphic mice ($Bub1^{H/H}$ and $Bub1^{-/H}$) that express the Bub1 protein at 20–30% of the levels of $Bub1^{+/+}$ mice are more prone to spontaneous tumorigenesis than are $Bub1^{+/+}$ mice **(49)**. Therefore, CIMD may occur in

Bub1$^{+/-}$ but not in Bub1 hypomorphic mice. We are currently testing mouse embryonic fibroblast (MEF) cells derived from these Bub1 mutant mice **(40)** for CIMD. If CIMD suppresses spontaneous tumorigenesis in Bub1$^{+/-}$ mice, the reduction or removal of p73 in Bub1$^{+/-}$ mice should result in increased spontaneous tumorigenesis. These genetic analyses will help determine the *in vivo* function of CIMD.

Potential Targets of Cancer Therapy

The occurrence of CIMD in the presence of anticancer drugs such as microtubule inhibitors and 17-AAG **(26, 50, 51)** supports the hypothesis that it is a mechanism to kill cancer cells. We could use the CIMD assay (i.e., BUB1 depletion and TUNEL assay) to screen for drugs or inhibitors such as 17-AAG that induce defects in kinetochore–microtubule attachment (Fig. 28.4). Also, BUB1 kinase inhibitors should work synergistically with microtubule inhibitors and 17-AAG (Fig. 28.4). Thus, it would be useful to screen chemical compound or small molecule libraries for BUB1 kinase inhibitors. In addition, small interfering RNA (siRNA) screens to determine factors that affect CIMD will identify genes that function downstream of BUB1

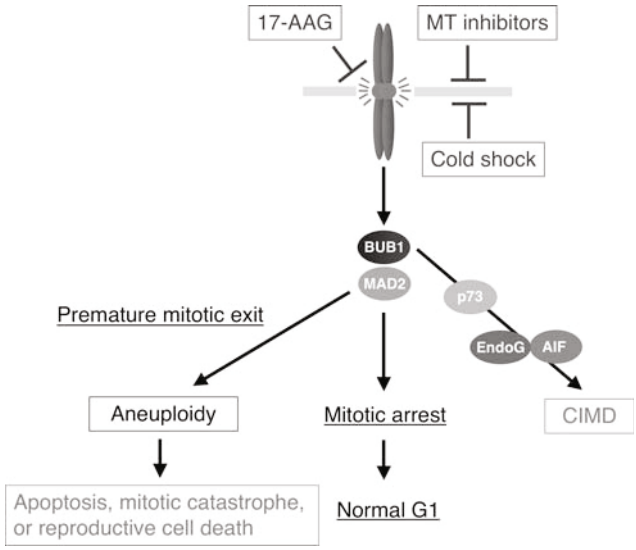

Fig. 28.4 Pathway showing how and where the treatments used in our studies act on our proposed model. When defects in the kinetochore-microtubule attachment occur in BUB1-depleted cells, the cells enter CIMD. If MAD2 is absent, chromosomes are lost or gained. The resulting aneuploid cells then enter the subsequent G1 phase, and apoptosis, mitotic catastrophe-like death, or reproductive death occurs. Thus, CIMD may be an alternative cell death system that prevents aneuploidy

(Fig. 28.4). Further investigation of the CIMD molecular pathway will contribute to identifying new candidates for anticancer drugs.

Determination of the Spindle Checkpoint Activity by Monitoring Mitotic Index Can be Ambiguous

The process of CIMD necessitates the reevaluation of the assessment of mitotic index when microtubule inhibitors or 17-AAG are used. When the spindle checkpoint activity of a mutant cell or a cancer cell line is tested, it may be wrong to conclude that the spindle checkpoint activity is intact even if the mitotic index remains unchanged. The TUNEL assay has to confirm that CIMD is not occurring. Bub1 levels are often reduced in several human cancers, including colorectal, esophageal, and gastric cancers **(12,52,53)**. Also, the alternation of other factors in this pathway could be the condition for triggering CIMD.

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Chapter 29
Lysosomal Proteases in Cell Death

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Abstract Apoptosis is a highly organized, energy-dependent program by which multicellular organisms eliminate damaged, superfluous, and potentially harmful cells. While caspases are the most prominent group of proteases involved in the apoptotic process, the role of lysosomes and, more particularly, of lysosomal cathepsins in cell death has only been recently unmasked. The release of cathepsins from the lysosomal lumen to the cytosol is a precondition for their participation in the regulation of apoptosis and has been described in response to a variety of death stimuli such as members of the cell-surface TNF receptor family, chemotherapeutic drugs, and nonreceptor-mediated apoptotic agents. This lysosomal membrane permeabilization often relies on the activation of the intrinsic apoptosis pathway, which involves mitochondrial membrane permeabilization and the consequent release of the proapoptotic mitochondrial proteins into the cytosol. These factors lead to caspase activation and, finally, cell death.

The aim of this chapter, emphasizing the role of lysosomal proteases in apoptosis, is to summarize past and recent findings that provide an insight into the mechanisms by which these hydrolases modulate apoptosis and also those that strongly argue for their role in the control of pathogenic cell dismantling.

Keywords Lysosome · Apoptosis · Cathepsins · Proteases · Lysosomal membrane permeabilization

Introduction

Most intracellular organelles have been reported to trigger cell death after stress stimulation and constitute potential therapeutic targets that could overcome the resistance of cancer cells to conventional DNA-damaging agents **(1**, **2)**.

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Among them, the lysosomes, which are membrane-bound cytoplasmic organelles containing over 50 soluble hydrolases (peptidases, proteases, lipases, glycosidases, nucleases, sulfatases, and phosphatases) that are capable of digesting macromolecules (i.e., nutrients, endocytosed, and phagocytized material and cellular components) and providing the cell with simple and reusable metabolites **(3)**. Most of these enzymes, optimally active at acidic pH, are located in the lysosome lumen, and the deficiency of some of them is directly linked to (neuro)degenerative human disorders named *lysosomal storage diseases* **(4)**. In addition, some heavily glycosylated proteins are also present in the lysosomal membrane. In mammalian cells, the vast majority of lysosomal hydrolases are tagged with mannose-6-phosphate. This terminal oligosaccharide chain allows the transport of glycoproteins from the Golgi apparatus into endosomes after ligation on mannose-6-phosphate receptors, whereas in endosomes, the acidic milieu causes the dissociation of this complex and the released receptor is recycled to the Golgi apparatus **(5)**. The proteolytic processing and maturation of the zymogens occur autocatalytically or through cleavage mediated by other lysosomal enzymes **(6)**.

The simplistic view of lysosomes as "nonspecific enzyme bags" is now reexamined due to the overwhelming number of observations that illustrate their implication in events of inflammatory and immune responses, in tumor angiogenesis, and also as cell death signal integrators in many controlled-death pathways **(7)**. Indeed, most apoptotic models rely on both caspase and cathepsin involvement, whereas others appear to be dependent exclusively on cathepsins, which can substitute for caspases, thereby eliciting caspase-independent apoptotic processes **(8)**. Another cell death process that appears to require lysosomal proteases is the alleged type II cell death, or autophagy, in which extensive degradation involving large portions of the cell is induced as part of programmed cell death [for more comprehensive reviews, see **(7**, **9)** and also Chapter **30**]. Lysosomal enzymes can also be involved in more defined necrotic cell death (see Chapter 27).

The aim of this chapter, emphasizing the role of lysosomal proteases in apoptosis, is to summarize past and recent findings that provide an insight into the mechanisms by which these hydrolases modulate apoptosis and also those that strongly argue for their role in the control of pathogenic cell dismantling.

Nature of the Signals Leading to Lysosome-Induced Cell Death

A number of *in vitro* and *in vivo* studies have indicated that lysosomes can mediate or modulate some cell death programs in response to a variety of death stimuli such as cell-surface death receptors of the TNF receptor family, lymphocyte B or T activators, chemotherapeutic drugs, nonreceptor-mediated apoptotic agents, p53 activation, oxidative stress, growth factor deprivation, or pathogens (see Table 29.1).

Table 29.1 Signals leading to lysosome-induced cell death

Stimulus	Cell type	Protease implicated	Reference
TNFα	Murine hepatocytes	Cathepsin B	**(28)**
	MCF-7 human breast cancer cells	Cathepsin B	**(70)**
	WEHI-s murine fibrosarcoma cells	Cathepsin B	**(25)**
	Ovarian cancer cells	Cathepsin B	**(111)**
	L929 murine fibrosarcoma cells	Cathepsin D	**(74)**
TRAIL	Hepatocellular carcinoma cells	Cathepsin B	**(164)**
	KMCH cholangiocarcinoma cells	Cathepsin B	**(165)**
	Oral squamous cell carcinoma	Cathepsin B and D	**(21)**
IFN-γ, anti-Fas, TNFα	HeLa human cervix carcinoma cells	Cathepsin B and L	**(23)**
Synthetic retinoid CD437	HL-60 human leukemic cells	Cathepsin D	**(119)**
Resveratrol	Colorectal cancer cells	Cathepsin D	**(166)**
Doxorubicin, etoposide	Murine fibroblasts	Cathepsin D	**(106)**
Etoposide	U 937 leukemia cells	Cathepsin D	**(167)**
Microtubule stabilizing agents	Human nonsmall cell lung cancer cells	Cathepsin B	**(26)**
Camptothecin	Namalwa B human lymphoma	Cathepsin B	**(168)**
Miltefosine	U 937 leukemia cells	Cathepsin B	**(166)**
α-tocopheryl-succinate	Murine fibroblasts	Cathepsin D	**(107)**
Ultraviolet radiation	Human keratinocytes	Cathepsin L	**(95)**
Ultraviolet B	Melanocytes	Cathepsin D	**(57)**
Hydrogen peroxide	Neuroblastoma cells	Cathepsin D	**(40)**
	Rat alveolar epithelial cells	Cathepsin D	**(116)**
	Rat astrocytes	Cathepsin D	**(115)**
	PC12 neuronal cells	Cathepsin D	**(117)**
Naphthazarin	Rat cardiac myocytes	Cathepsin D	**(113)**
Menadione	Pancreatic acinar cells	Cathepsin B	**(169)**
Staurosporine	Human fibroblasts	Cathepsin D	**(118)**
Sphingosine	Jurkat human lymphoma T cells	Cathepsin D	**(35)**
Type1-fimbriated *Escherichia coli* bacteria	Neutrophils	Cathepsin B and D	**(44)**
Biphosphinic palladacycle complex	K562 leukaemia cells	Cathepsin B	**(170)**
Silica	Murine alveolar macrophages	Cathepsin D	**(121)**
Low-frequency electromagnetic stimulation	Morris rat hepatoma cells	Cathepsin B	**(171)**
Permissive temperature (32 °C)	Murine lymphoma cell line	Cathepsin D	**(106)**
Bortezomib	Human pancreatic cancer cells	Cathepsin B	**(77)**

Table 29.1 (continued)

Stimulus	Cell type	Protease implicated	Reference
β-amyloid peptide	Rat cortical neurons	Cathepsin L	**(92)**
Bile salt	Rat hepatocytes	Cathepsin B	**(75)**
	Rat hepatoma cell line	Cathepsin D	**(83)**

Intracellular Mechanisms of Lysosomal Cell Death Induction

Receptor-Mediated Endocytosis

The plasma membrane withholds death receptors belonging to the TNF receptor superfamily such as TNFR1, Fas, and DR4/5. Considerable advances have been made in the characterization of intracellular signaling events emanating from these receptors **(10)**. They are expressed in almost all cell types and can signal both cell death and survival **(11)**. For TNFR1, upon binding of its ligand, its signaling appears to require the sequential formation of two distinct complexes **(12)**. The first complex, which comprises TNFR1, the adaptor TRADD, the kinase RIP1, and TRAF2, has been proposed to be rapidly formed at the plasma membrane and to promote survival via the activation of the transcription factor NF-κB. The second complex, which lacks TNFR1 but involves FADD and procaspases-8 and -10, has been reported to form in the cytoplasm and initiate apoptosis. However, some reports point out the importance of internalization of TNFR1 by endocytosis for further apoptotic signaling to occur. Indeed, experiments to suppress the internalization of TNFR1 either by pharmacological approaches or using mutants of TNFR1 carrying deletions of the internalization domain (TRID) have confirmed the need for ligand-induced internalization of TNFR1 for the initiation of the apoptotic program **(13**, **14)**. Subsequent to this, along the endocytic pathway, the endocytic vesicles containing activated TNFR1 complexes, named *TNF receptosomes*, fuse with trans-Golgi vesicles to form multivesicular endosomes. These internal vesicles and the fluid phase will ultimately be delivered to lysosomes.

Besides this endocytic model of apoptotic signaling by TNFR1, there is mounting evidence that following their cell-surface internalization, death receptors can trigger a lysosome-dependent death through the action of certain lysosomal proteases **(15–17)**. Indeed, endocytosis of ligand-activated TNFR1 results in the acidic sphingomyelinase-mediated generation of ceramide, which binds and activates cathepsin D by autocatalytic processing **(18)**. This leads to apoptosis via the cleavage of Bid in the acidic environment of the endo-lysosomal compartment **(19)**. The membrane-bound neutral sphingomyelinase was also involved in the connection between receptor and lysosomes, since this

phenomenom was abolished in murine FAN (factor associated with neutral sphingomyelinase activation)-deficient fibroblasts as well as in human cells overexpressing a dominant-negative form of this p55TNFR1-associated adapter protein **(20)**.

Similarly to TNFα, the death ligand TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), which binds to DR4 and DR5 receptors, was able to induce apoptosis in KMCH cholangiocarcinoma cells by activating JNK-dependent Bim, which in turn induces Bax-mediated permeabilization of lysosomes **(21)**. Finally, the involvement of lysosomal proteases upon ligation of anti-Fas antibodies to CD95/Fas death receptor has also been demonstrated in several carcinoma cells **(22, 23)**. However, these results have recently been challenged with the observation that anti-Fas-induced apoptosis in the presence of cycloheximide was not impaired in murine cathepsin B-deficient primary skin fibroblasts **(24)**.

Lysosomal Membrane Permeabilization

Perturbations of the lysosomal membrane function were observed in response to exogenous apoptotic stimuli, lysosomotropic toxins, and endogenous signal transducers. This so-called lysosomal membrane permeabilization (LMP) is an ill-defined process that leads to the release of lytic enzymes, from the lysosomal lumen to the cytosol, an event that is later followed by classical caspase-dependent apoptosis, caspase-independent apoptosis-like cell death (lysosomal proteases appear capable of executing cell death independently of the apoptotic machinery)] **(25, 26)**, or necrosis **(27)**. A quantitative relationship between the magnitude of lysosomal rupture (i.e., the amount of lysosomal hydrolases released into the cytosol) and the mode of cell death might explain the different outcomes following LMP. Indeed, a complete breakdown of the lysosomal membrane would result in unregulated necrosis **(27)**, whereas a limited release of lysosomal enzymes into the cytoplasm may trigger apoptosis **(28)**.

Whether lysosomal rupture is associated with cytosolic acidification has been poorly studied and needs to be clarified. However, several apoptosis-stimulating events such as the formation of the apoptosome, the activation of caspases **(29, 30)**, or the stimulation of leukocyte elastase inhibitor/leucocyte-derived DNase II (LEI/L-DNase II) **(31)** are enhanced by decreasing cytosolic pH. Recently, it has been shown that treatment of U937 cells with TNFα resulted in LMP, which was associated with an increased release of cathepsin D and cytosol acidification **(32)**. Moreover, treating cancer cells with inhibitors of vacuolar-type H^+ ATPase (V-ATPase) has been shown to induce the alkalinization of lysosomal pH, cytosol acidification, LMP, and caspase activation **(33)**.

How to Induce Lysosomal Membrane Permeabilization

Several candidates for mediating LMP have been proposed. Lipids are one, including sphingosine, which is generated from ceramide by the action of acidic ceramidase in TNFα-induced cell death signaling **(34)**. When generated in lysosomes, sphingosine could function as a detergent, permeabilize lysosomal membranes, and facilitate the relocation of some hydrolases to the cytoplasm. With low amounts of sphingosine, this phenomenon leads to mitochondrial perturbations and caspase activation, whereas higher concentrations are associated with a total rupture of lysosomes and necrosis **(35)**. This sphingolipid could also permeabilize *in vitro* the membrane of isolated lysosomes **(34)**. Moreover, in cells overexpressing a dominant-negative form of FAN, a decrease in sphingosine generation was associated to LMP suppression, supporting the possible implication of sphingolipids in lysosomal rupture **(20)**.

The destabilization of lysosomes by the oxidation of membrane lipids has been also reported when oxidative stress generates intralysosomal reactive oxygen species (ROS) through Fenton-type chemistry **(36, 37)**. Indeed, lysosomes are the organelles that exhibit the most important pool of redox-active iron in the cell and are particularly sensitive to oxidative stress **(38, 39)**. In hydrogen peroxide-treated human neuroblastoma cells, it has been shown that desferrioxamine, an iron chelator that abolishes the formation of ROS within lysosomes, prevented lysosome leakage and caspase activation **(40)**. Moreover, small interfering-RNA-induced inhibition of the lysosomal iron-binding protease lactoferrin affects carcinogen-triggered apoptosis **(41)**. Several oxidative stresses or stimuli that promote the production of intracellular ROS such as radiation **(42)** or hypothermia **(43)** induce time- and dose-dependent lysosomal rupture and the release of cathepsins in the cytosol, leading to apoptosis or necrosis depending on the magnitude of the insult **(44, 45)**. Finally, mitochondrial ROS can increase LMP and favor the release of lysosomal proteases such as cathepsin B, thereby creating a feedback loop between these two organelles **(46)**.

Another possible mechanism is the translocation of proapoptotic members of the Bcl-2 family to lysosomes, where similarly to mitochondria, these proteins could form pores and permeabilize the lysosomal membrane **(47, 48)**. Upon stimulation of fibroblasts with staurosporine or hepatocytes with TNFα, Bax or Bid has been proposed to translocate to the lysosomal membrane, leading to the release of cathepsin D **(49)** or B **(20, 50)**, respectively. Moreover, studies to suppress Bax activation either by pharmacological approaches or by small interfering-RNA-mediated silencing have confirmed that LMP is Bax-dependent **(21, 51)**. Conversely, Bcl-2, an antiapoptotic protein, has been reported to exert a protective effect by inhibiting oxidant-mediated lysosomal rupture **(47, 48)**.

Recently, it was found that a phosphorylated form of p53 could trigger permeabilization of the lysosomal membrane and apoptosis by forming

complexes with LAPF/Phafin-1, a lysosome-associated and apoptosis-inducing protein containing PH and FYVE domains **(52)**. The p53 inhibitor pifithrin-alpha and small interfering-RNA-mediated knockdown of p53 both prevented this permeabilization in stress-induced neuronal death **(53)**. However, further studies are needed to determine whether p53 could form complexes with Bcl-2 members to act on the lysosomal membrane, as it was demonstrated for the control of mitochondrial membrane destabilization **(54)**.

Finally, the possibility that lysosomal proteases themselves may permeabilize the lysosomal membrane cannot be ruled out. Indeed, cathepsin B-defective cells show a decreased sensitivity to TNFα-induced LMP, suggesting an active role of this enzyme in the induction of lysosomal rupture **(34)**. Moreover, the serine protease inhibitor Spi2A, which also inhibits cathepsin B, partially blocks lysosomal membrane leakage. NF-κB may inhibit the lysosomal pathway of apoptosis by increasing Spi2A transcription **(55)**.

Conversely, the negative control of the death-associated LMP has been linked to the 70-kDa heat-shock proteins (Hsp70). In many tumor cells, Hsp70-1, one of the eight highly homologous members, has been localized to membranes of the endosomal/lysosomal compartment and reported to inhibit LMP after treatment with cytotoxic agents **(56, 57)**. Recently, depletion of another member, Hsp70-2, has been shown to result in lysosomal destabilization, leading to cathepsin-dependent cell death **(58)**. These effects could be prevented by Hsp70-binding protein 1 (HspBP1), a protein that interacts with Hsp70 and antagonizes the prosurvival activity of Hsp70 **(59)**.

The grasp of the above-described mechanisms will open new and exciting possibilities, including control of lysosome stability and new therapeutic strategies based on the lethal effect of lysosomal proteases.

Lysosomal Proteases

Nature of Proapoptotic Lysosomal Proteases

Lysosomal proteases have long been regarded as housekeeper enzymes engaged in organelle and macromolecule digestion inside the lysosomal/autolysosomal compartment, but recently a body of evidence suggested that some lysosomal proteases participate in apoptotic pathways triggered by different stimuli. Among them, cathepsins are the best-characterized lysosomal proteases. They are divided into three subgroups according to their active-site amino acid that confers catalytic activity. Most of them are cysteine-proteases (cathepsins B, C, F, H, K, L, O, S, V, W, and X/Z), two are aspartyl proteases (cathepsins D and E), and one is a serine protease (cathepsin G) **(3)**. The nature of cathepsins involved in the execution of cell death is cell-type- and stimulus-dependent and probably depends on the availability of specific substrates. Whereas lysosomal enzymes generally exhibit an acidic optimum pH, their ability to cleave

cytosolic targets at a neutral pH can be questioned. Nonetheless, different studies have demonstrated that cysteine cathepsins are still active at neutral pH, making them potentially harmful if present outside their acidic environment **(60)**, although their lifetime is likely quite limited under these conditions **(61)**.

Cysteine Cathepsins

Cysteine cathepsins belong to the papain subfamily of cysteine peptidases and are ubiquitously expressed. Most of them are monomeric proteins consisting of two domains, referred to as the R (right) and L (left) domains **(62)**. They share a conserved active site that is formed by cysteine, histidine, and asparagine residues **(63)**.

They exhibit an endopeptidase activity, but some exceptionally also demonstrate exopeptidase activity. For instance, cathepsin B cleaves within polypeptide chains but also possesses a dipeptidyl-carboxypeptidase activity **(64)**. In addition, cathepsin C, which exhibits a tetrameric structure, is not an endopeptidase, but only a dipeptidyl-aminopeptidase **(62, 65)**].

In the cytosol, cysteine cathepsins are inhibited by cystatins, which represent a protective barrier against the spontaneous destabilization of lysosomal membranes **(66)**. Cysteine cathepsins are essential in the maintenance of homeostasis, but a direct or indirect role of these lysosomal proteases and, more particularly, cathepsins B, C, and L in cell death has been demonstrated **(60)**.

Cathepsin B

Cathepsin B is the most abundant lysosomal protease. It is synthesized as a 48-kDa precursor that undergoes proteolytic processing to active single-chain (31-kDa) and double-chain (25- and 5-kDa subunits) glycosylated forms **(67)**. The involvement of cathepsin B in apoptotic signaling has been shown in cathepsin B knockout hepatocytes that display resistance to TNFα-induced mitochondrial release of cytochrome c, caspase activation, and apoptosis **(68)**. Moreover, immortalized murine embryonic fibroblasts from cathepsin B- and L-deficient mice are highly resistant to TNFα plus cycloheximide **(69)**.

The implication of cathepsin B in DNA damage or TNFα-induced apoptosis has also been reported in tumor cells **(25, 70–72)**. This has been correlated with the production of sphingosine, which may be responsible for the release of the lysosomal protease in the cytosol **(34)** and a decrease in the activity of sphingosine kinase-1, a key enzyme that regulates levels of sphingosine and its phosphorylated form sphingosine 1-phosphate **(71, 73)**. In addition, the human homologues of SETA binding protein 1 **(74)**, bikunin and TSRC1 **(72)**, were also reported to interact with cathepsin B and to influence TNFα-induced apoptosis.

Cathepsin B is also involved in apoptotic cell death triggered by TRAIL **(21)**, bile acid **(75, 76)**, proteasome inhibitor **(77)**, microtubule stabilizing agent **(26)**, bacillus Calmette-Guérin **(78)**, interleukin-24 **(79)**, and cold ischemia-warm

reperfusion **(80)**. Moreover, this protease is also involved in neuronal cell death **(81**, **82)**. Finally, pharmacological and endogenous inhibitors as well as antisense nucleotides of cathepsin B protected cells from apoptosis **(25**, **81**, **83)**.

However, in some cells, this protease is not positively correlated to cell death but might be pivotal for survival, as shown with pharmacological inhibitors such as CA-074 and the broad-spectrum cysteine cathepsin inhibitor E-64, which lead to human neuroblastoma cell death **(84)**. Moreover, the synthetic cysteine cathepsin inhibitor CATI-1 kills human leukemia and lymphoma cells independently of caspases, p53, Bax, and MAPK **(85**, **86)**. Finally, the deficient activity of cathepsins B found in *I-cell disease* fibroblasts **(87)** or in mammary carcinoma cells isolated from cathepsins B knockout mice **(88)** does not account for their resistance to TNFα-induced apoptosis.

Cathepsin C

Studies using cathepsin C knockout mice have demonstrated that this aminodipeptidase was the principal hydrolase that converts the neutral serine proenzyme granzyme B into its enzymatically active 32-kDa form in the granules of cytotoxic T and natural killer lymphocytes **(89)**. Cathepsin C is believed to indirectly activate caspases and lead to target cell apoptosis through granzymes. Indeed, it was reported that *in vitro* granzyme B cleaves numerous substrates after aspartate residues, such as procaspases-3, -7, -8, and -10, and that in cells external granzyme B induces cell death predominantly through caspase-3 activation **(90)**. In addition, cytotoxic lymphocytes of mice lacking cathepsin C exhibit severe defects in the induction of target cell apoptosis, and this defect is comparable to that observed in perforin- or granzyme A- or B-deficient effector cells **(89)**.

However, these results have been challenged with the recent observations that not all of the granzyme B activity is eliminated in cathepsin C-deficient mice and that the effector lymphocytes from this knockout mice could induce apoptosis of target cells by a mechanism that was indistinguishable from classic apoptosis **(91)**.

Cathepsin L

Different studies have indicated that cathepsin L can modulate the apoptotic process. Indeed, similarly to cathepsin B, murine cathepsin L-deficient immortalized embryonic fibroblasts were found to be resistant to TNFα-induced cell death **(69)**. Moreover, based on the inhibition of cell death by pharmacological inhibitors of cathepsin L, the involvement of this protease in β-amyloid-induced apoptosis in rat cortical neurons **(92)**, supraoptimal activation-promoted apoptosis in T lymphocytes **(93)**, or prolactin-induced spermatogonial apoptosis in newt testis **(94)** has been hypothesized. It was also shown that UV irradiation-induced apoptosis was reduced in transgenic mice overexpressing hurpin, a selective inhibitor of cathepsin L **(95)**.

However, in glioma cell lines expressing a cathepsin L antisense, the apoptotic rate by either intrinsic or extrinsic stimuli was increased, whereas the overexpression of this enzyme seemed to protect the cells from apoptosis **(96, 97)**. Increased apoptosis in the absence of cathepsin L could be rescued by inhibition of the aspartic protease cathepsin D. Indeed, it was proposed that cathepsin L contributes to control death receptor-induced apoptosis via proteolysis of cathepsin D **(98)**.

Finally, it has been suggested that purified cathepsin L from rat liver could activate cytosolic caspase-3 **(99)**; however, other studies have demonstrated that main effector caspases are poor substrates for cysteine cathepsins **(100, 101)**. The proapoptotic role of cathepsin L thus remains to be clarified using additional approaches.

Aspartyl Cathepsins

Cathepsin D is the predominant lysosomal aspartic acid protease **(102)** and is expressed in all human tissues. However, its expression can considerably fluctuate depending on the cell type **(103)**. The structure of this endopeptidase is very similar to that of other aspartyl proteases of the pepsin family **(104)**, but unlike them, this protease is generally formed by two subunits of 48 and 32 kDa **(105)**. Its processing could be regulated through the products of two lipid hydrolases, namely lysosomal sphingomyelinase and ceramidase **(18, 19)**. In contrast to cysteine cathepsins, mammalian cells do not contain known inhibitors of cathepsin D.

Several studies have demonstrated that cathepsin D plays a pivotal role in cell death signaling by inducing or inhibiting this process. First, cells isolated from cathepsin D-deficient mice were found to be more resistant to stress-induced cell death signaling **(106–108)** than cells from their wild-type littermates. Second, the genetic knockdown of cathepsin D in endothelial cells was shown to inhibit H_2O_2-induced cell death **(109)**, whereas the ectopic expression of this protease in HeLa cells could trigger cell death in the absence of any external stimulus **(23)**. In accordance with this observation, microinjection of this enzyme was able to induce per se caspase-dependent apoptosis in fibroblasts **(110)**. Finally, numerous studies have shown that pepstatin A, an aspartic protease inhibitor, could partially block apoptosis induced by several stresses, including Fas **(23)**, IFN-γ **(23)**, TNFα **(19, 111)**, camptothecin **(112)**, naphthazarin **(113, 114)**, hydrogen peroxide **(109, 115–117)**, staurosporine **(118)**, the synthetic retinoid CD437 **(119)**, sphingosine **(35)**, bleomycin **(120)**, silica **(121)**, or the permissive temperature in a murine lymphoma cell line **(106)**. Unfortunately, pepstatin is not entirely specific and is also known to inhibit cathepsin E, pepsin, and renin.

Nevertheless, some reports have challenged this concept and have shown that cathepsin D is not directly implicated in cell death signaling. Indeed, murine cathepsin D-deficient and wild-type immortalized embryonic fibroblasts were equally sensitive to TNFα-induced cell death **(69)**. Furthermore,

pepstatin A did not protect the cells from etoposide-, anti-CD95-, serum deprivation-, or TNFα-induced cell death **(111**, **122**, **123)**. It did not suppress Bid cleavage or caspase activation induced by photodynamic therapy in murine hepatoma cells **(124)** and has been described to trigger apoptosis in human neuroblastoma cell lines **(84)**.

Finally, the possibility that the apoptotic effect of cathepsin D could be independent of its catalytic activity has emerged **(123**, **125)**. Indeed, wild-type and mutated catalytically inactive cathepsin D overexpressing cancer cells were equally sensitive to the lethal effects of etoposide, suggesting that during the apoptotic process, this lysosomal protease interacts with a member of the apoptotic machinery but does not cleave specific substrates **(125)**. This concept is in agreement with the fact that cathepsin D exhibits very little or no activity on protein substrates at neutral pH, probably because of deprotonation of both the active site Asp residues **(126)**.

Other Proteases

A potential role in the regulation of apoptosis for other lysosomal proteases or lysosomal proteins that modify other proteins has also been reported. For instance, the well-known serine endopeptidase chymotrypsin B has been found to be released from the lysosomes in the cytosol of TNFα-treated rat hepatoma cells, an event that preceded mitochondrial alterations and apoptosis **(127)**. Two other lysosomal hydrolases, CLN1 and CLN2, which encode Palmitoyl Protein Thioesterase1 (PPT1) and Tripeptidyl Peptidase-I (TPP1), respectively, are also known to modulate some cell death programs. Indeed, the deficiency of these lysosomal enzymes is responsible for devastating human lysosomal disorders called neuronal ceroid lipofuscinoses that are characterized clinically by early visual loss and massive apoptotic neuronal death **(128)**.

PPT1 removes the palmitic acid linked to the cysteine residue of S-palmitoylated proteins such as H-Ras/p21 and subunits of small G proteins **(129)**. It is a housekeeping enzyme present in many tissues but is especially abundant in the spleen, brain, and testis **(129)**. Its deficiency **(130)**, or its inhibition following either antisense construct expression or administration of pharmacological inhibitors **(131**, **132)**, was associated with increased apoptosis. Moreover, PPT1 deficiency has been linked to ER stress as evidenced by the activation of the unfolded protein response, caspase-12 (or caspase-4 in humans) and caspase-3 **(133**, **134)**. The same group reported that caspase-9 is activated following the increased production of reactive oxygen species and the disruption of calcium homeostasis **(135)**. However, studies on cultured mammalian cell models or in *Drosophila* have yielded contradictory results. Indeed, the overexpression of PPT1 in *Drosophila* during visual system development led to apoptosis of photoreceptors, suggesting that a tight regulation of this enzyme activity is necessary for neuronal survival **(136)**. We recently observed that PPT1 could be an important regulator of TNFα-induced

apoptosis in nonneuronal cells (C. Tardy, T. Levade, and N. Andrieu-Abadie, unpublished observations).

How the deficiency of TPP1, a ubiquitously distributed pepstatin-insensitive exotripeptidase, with little endoproteolytic activity, results in neurodegeneration is totally unknown. It has been proposed that TPP1 overexpression protects neural cell lines against apoptosis **(137)**. However, we recently demonstrated that the apoptosis defect, reported in fibroblasts derived from patients affected with I-cell disease, having a deficient activity of almost all lysosomal hydrolases, was partially corrected when the activity of TPP1 was restored in these mutant cells **(87)**. These data suggest that TPP1 may indeed regulate apoptosis. However, how this lysosomal protease is connected with apoptosis has never been studied.

How Are Lysosomal Proteases Connected to Classical Apoptotic Pathways?

LMP often relies on the activation of the intrinsic apoptosis pathway, which involves mitochondrial membrane permeabilization with the consequent release of the proapoptotic mitochondrial proteins into the cytosol **(28**, **110**, **118**, **138–142)**. One of the links that connect lysosome leakage to mitochondria might be the proapoptotic Bcl-2 homologue Bid **(19**, **20**, **44**, **101**, **142)**. Bid plays an important role as the substrate/target of lysosomal enzymes in cytochrome c release-mediating cell death **(101)**. The proteolytic activation of Bid can be catalyzed by different proteases: caspase-8, many isoforms of cathepsins, granzyme B, and calpains, which cleave Bid at Asp **(59)**, Arg **(65)**, Asp **(75)**, and Gly **(70)**, respectively. Additionally, it was recently reported that chymotrypsin B, purified from rat liver lysosomes, might be involved in apoptotic regulation through cleavage of Bid mapped at Phe **(67**, **127)**. However, Bid is not the only cellular target of lysosomal enzymes, as ablating it in cystatin B/Bid-double-deficient mice did not rescue neuronal apoptosis **(82)**.

In T lymphocytes, cathepsin D can activate Bax, another Bcl2 proapoptotic family member. This activation leads to the permeabilization of the outer mitochondrial membrane, the release of AIF from the mitochondria, and apoptosis **(139)**. The same observation has been made in H_2O_2-treated neuroblastoma cells **(40)**. Consistent with these studies, selected compounds were demonstrated to induce LMP and the release of cathepsin D into the cytosol but were unable to lead to apoptosis in Bax-deficient colon carcinoma cell lines **(143)**, confirming that Bax is an essential component of the death pathway triggered by cathepsin D.

Moreover, arachidonic acid **(144)**, caspase-2 **(50**, **77)**, and cathepsins themselves have also been directly or indirectly implicated **(28)** to trigger mitochondrial alterations downstream of LMP.

Finally, whereas evidence supports participation of the mitochondria in transmitting lysosomal death signal, the mechanisms by which LMP and lysosomal proteases lead to mitochondrial disruption need to be clarified.

Therapeutic Implications

Lysosomal proteases are multitasking enzymes. They present opposing roles in apoptosis depending on their localization (extra- or intracellular) and their implication in pathological conditions such as cancer or neurodegenerative disorders.

Can Tumor Progression Be Modulated via Lysosomal Proteases?

Drug development studies for cancer treatment point to a new tumor-suppressive role for lysosomal proteases. Indeed, therapeutic strategies using lysosomotropic toxins, inhibitors of the vacuolar H-ATPase, or HSP-70 might be used to exploit LMP and the role played by the lysosomal enzymes in apoptosis **(7)**. Cancer cells frequently contain high levels of lysosomal proteases, and the LMP pathway could be a therapeutic opportunity to target tumor tissue more specifically. However, it was reported that in invasive cancer cells, the localization of lysosomes often shifts from a perinuclear to a peripheral pattern, with the subsequent translocation of lysosomal proteases to the cell surface and their secretion into the extracellular space **(7**, **145)**. By degradating some components of the extracellular matrix such as type IV collagen, laminin, and fibronectin, secreting enzymes may participate in tissue invasion, motility, and angiogenesis **(63**, **145**, **146)**. Thus, combined pharmaceutical approaches using noncell-permeable inhibitors of lysosomal proteases and lysosomotropic agents will suppress extracellular lysosomal hydrolase activity. This would block their tumor-promoting actions without affecting LMP, thereby allowing tumor cell death.

Two approaches are available with the therapeutic potential to induce LMP: The first is the formation of reactive oxygen species (ROS) by irradiation. Upon illumination with specific wavelengths, photosensitizing molecules can generate ROS, notably singlet oxygen, leading to lysosomal membrane damage. Second, lysosomal destabilization can be induced by lysosomotropic compounds such as chloroquine **(147)** and L-leucyl-L-leucine ester **(148)**.

However, cancer cells have developed strategies to counteract lysosomal destabilization and its consequences. For instance, the translocation of HSP70 to the inner leaflet of the lysosomal membranes where it might block LMP was induced by several stress agents **(56)**. Conversely, the depletion of HSP70 in human tumor cells specifically triggers LMP and cathepsin-dependent cell death *in vitro* and in tumor xenografts **(149**, **150)**.

Role of Lysosomal Proteases in Neurodegenerative Diseases

The duality in the role of lysosomal proteases in apoptosis is also highlighted in neurodegenerative diseases. Studies on animals harboring a defect in one of the lysosomal proteases have provided important insights into the possible role of these enzymes in apoptosis. In many cases, dysregulated or deficient lysosomal protease activity results in severe pathological conditions associated with an exacerbated cell death in different tissues, notably the brain.

The double knockout of cathepsins L and B results in massive apoptosis of selected neurons in the cerebral cortex, the cerebellar Purkinje, and granule cell layers, leading to lethality **(151)**. Likewise, the targeted deletion of cathepsin D in mice is associated with an extensive neuropathology similar to that observed in brains of patients with neuronal ceroid lipofuscinoses **(102**, **152**, **153)**, with morphological and biochemical features of apoptosis and autophagic stress **(154)**. At day 26 of life, newborn mice died because of atrophy of the intestinal mucosa and consequent anorexia. An increased photoreceptor cell death was also shown in a transgenic mouse line expressing an enzymatically inactive form of cathepsin D **(155)**. Moreover, selective neuronal apoptosis was also observed in cathepsin D-deficient sheep, which are affected with a congenital form of neuronal ceroid lipofuscinosis **(156)**. However, it was recently shown that neuron death and neurodegeneration were not impaired in the double knockout of Bax and cathepsin D, suggesting that alterations in the macroautophagy-lysosomal degradation pathway could mediate neuron death in the absence of Bax-dependent apoptosis **(154)**.

In addition, the targeted disruption of PPT1 or TPP1 in mice causes a neurological disorder closely resembling human ceroid lipofuscinoses. Neuronal loss and apoptosis were prominent in the brain of PPT1- or TPP1-deficient mice, which likely led to the premature death of these animals **(157**, **158)**.

Finally, the fact that a defect in a lysosomal proteolytic enzyme often results in neuronal cell death is further corroborated by the observation that genetic defects of cystatin B, a cytosolic inhibitor of lysosomal cysteine proteases, and of cathepsin A, another lysosomal cathepsin, also lead to neurodegenerative diseases **(159**, **160)**.

Conclusion

Studies on the role of lysosomal proteases in cell death may appear paradoxical when comparing the results obtained *in vitro*, on cultured cells, with the symptoms observed in the diseases that are dependent on the function of these enzymes. Indeed, lysosomal proteases can either prevent apoptosis, as described under physiological conditions with knockout mice experiments **(102**, **152**, **153**, **161)**, or promote apoptosis in cells treated with cytotoxic agents. However, it is conceivable that the role played by these enzymes differs according to the tissue or cell type. For instance, differences in processing and trafficking have been reported for

PPT1 in neuronal versus nonneuronal cells **(162)**. This phenomenon was also observed for nonlysosomal apoptotic proteins, such as the serine protease HtrA2/Omi, which functions as an antiapoptotic protein in stress-induced neuronal cell death in contrast to its role in other somatic cells **(163)**.

In addition, lysosomal proteases can either induce death in cancer cells when they are translocated in the cytoplasm of these cells or participate in tumor invasion when they are released in the extracellular space.

Although the function of lysosomal proteases in apoptosis is not yet fully understood and obviously needs further investigation, one can reasonably admit that these proteases modulate cell death. Therefore, understanding how the distribution of lysosomal proteases influences apoptosis appears to be an important challenge to developing novel treatments.

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Chapter 30
Autophagy and Cell Death

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Abstract Autophagy is an evolutionarily conserved biological phenomenon related to protein degradation and organelle turnover. Three types of autophagy have been defined: macroautophagy, microautophagy, and chaperone-mediated autophagy, which differ the way of in the delivery of substrates to the lysosome. In macroautophagy, substrates are wrapped in a double membrane structure, called the autophagosome. The formation of the autophagosome and its fusion with the lysosome are genetically controlled by a series of autophagy molecules and are activated in response to a number of environmental cues. Much has yet to be learned about the signaling pathway and the molecular mechanisms about this process. Autophagy is important to multiple cellular functions, particularly for nutrient and energy balance, and the turnover of cellular substances. The relationship of autophagy with cell death is complicated and may be context-dependent. Whereas the nature of autophagic death has yet to be carefully defined, it seems that autophagy may, in fact, be a key regulator of both apoptosis and necrosis. In this context, the roles of macroautophagy in both prosurvival and prodeath have been identified. Understanding the circumstance in which autophagy affects cell functions and therefore cell viability is critical for the future intervention of this process to control cancer, tissue injury, and other disease processes.

Keywords Autophagy · Macroautophagy · Cell death · Apoptosis · Necrosis · Atg molecules · Bcl-2 family proteins · Starvation · Metabolic stress · Mitophagy · ER stress · Hypoxia

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Introduction

Two major protein degradation systems are present in eukaryotic cells: the proteasome and the lysosome. They differ in their functional significance and the type of substrates they take in for degradation. In the lysosome system, the degradation of extracellular materials is mediated by endocytosis (heterophagy), whereas the degradation of intracellular components is mediated by three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) **(1–7)**, which differ in how the cytoplasmic materials are delivered to the lysosome. In macroautophagy, the content is sequestered in a double-membrane structure called the *autophagosome*, which subsequently fuses with the lysosome. In microautophagy, the content is directly taken up by the lysosomes through membrane invagination, whereas in CMA, the content binds to Hsc70 and its co-chaperones. The complex then binds to LAMP2a on the surface of the lysosome. The substrate protein is then transported into the lysosome **(6)**. This chapter discusses macroautophagy (hereafter referred to as autophagy) and its relationship with cell death in mammalian cells.

The term *autophagy* comes from Greek, meaning "self-eating." Autophagy as a biological phenomenon was first systemically described by de Duve and Wattiaux 40 years ago **(1)**, although it seems that the phenomenon, as a process of bulk segregation of cellular constituents, was reported as early as in 1957 in mammalian cells **(8)**. Unlike the ubiquitin proteasome system, autophagy is responsible for the degradation of long-lived proteins and is the only system that can degrade organelles, such as mitochondria **(4**, **9)**. Although autophagy has long been recognized, progression of the study was slow due to a lack of understanding of its molecular mechanisms. The breakthrough came in the 1990 s when the phenomenon was studied in yeast. The powerful yeast genetics allows the identification of multiple genes required for autophagy **(10**, **11)**. These works have since revolutionized the field and brought the research of autophagy into the molecular era.

Basic Autophagy Machinery

Autophagy is evolutionarily conserved and operates in plants, yeast, *C. elegans*, *Drosophila*, and mammals. A large portion of the molecular machinery of autophagy is conserved in these organisms **(11)**. Currently, 31 autophagy-related genes (ATG) have been identified since the first gene, Atg1, was discovered from a genetic screening in yeast **(12**, **13)**. Detailed discussions of these genes, particularly those of yeast, can be found in several recent reviews **(14**, **15)**. A brief summary of the mammalian system is given below.

The core autophagy machinery seems to be built around two ubiquitin-like conjugation systems **(3)** (Fig. 30.1). In one system, the ubiquitin-like protein

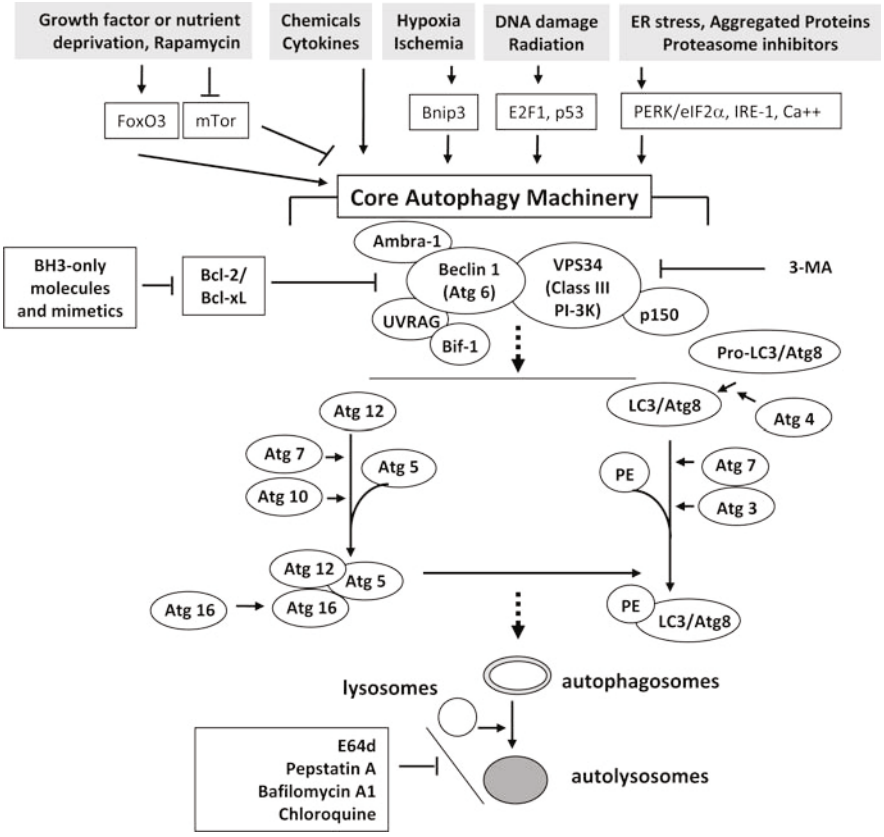

Fig. 30.1 Activation of macroautophagy in mammalian cells. Macroautophagy in mammalian cells could be activated by multiple signals. Some of them are listed at the top of the diagram. The signaling to the core autophagy machinery is only partially revealed, and remains largely unknown to many of the stimuli. The effects of mTOR, Bnip3, calcium, and the unfolded protein response components are among the better-understood mechanisms. The Beclin-1/Atg6 complex, composed of Beclin-1, VPS34 (the class III PI-3 kinase), and its regulatory partner, p150/VPS15, Ambra-1, UVRAG, and its binding partner, Bif-1, seems to act upstream by responding to the stimulation. The activity of this complex can be regulated by a number of other factors, including the Bcl-2 family proteins, and some pharmacological agents that suppress VPS34 enzymatic activity. It is not entirely clear how the Beclin-1 complex positively regulates the two conjugation systems: the Atg12-Atg5-Atg16 and the PE-LC3/Atg8 complexes, which form in the presence of Atg7, an E1-like enzyme, and Atg10 or Atg3, E2-like enzymes. These two complexes are required for the formation of an autophagosome (AV), which is a double-membrane structure. During the formation of AV, the cytosolic materials to be degraded are enclosed within the structure. Autophagosomes are then fused with the lysosomes to form autolysosomes, which degrade the enclosed materials. Both the fusion process and the lysosome activity can be blocked by multiple chemicals as indicated, which can thus block autophagy at a later stage

Atg12 was first activated by Atg7, a ubiquitin-activating enzyme (E1)-like protein, and then transferred by Atg10, a ubiquitin carrier protein (E2)-like protein, to Atg5 through a covalent bond. The Atg5-Atg12 complex interacts with Atg16 to form a multimer complex, which is localized to membranes of early autophagosomes. It seems that the assembly of this system is independent of autophagy activation. Thus, the complex appears to provide the necessary platform for autophagy activation.

In another system, the microtubule-associated protein 1 light chain 3 (LC3), or GATE-16 or GABARAP, all mammalian homologues of the yeast Atg8, is first cleaved by Atg4 to expose the conserved Gly^{120} at its C-terminus. LC3 is then conjugated to phosphatidylethanolamine (PE), also via Atg7, and Atg3, another ubiquitin carrier protein (E2)-like protein **(3**, **16)**. The unconjugated form of LC3 (called LC3-I) is in the cytosol, while the conjugated form (called LC3-II) targets to the autophagosomal membrane **(16)** with the assist of the Atg5-Atg12-Atg16 complex **(17)**. This association of LC3-PE to the autophagosomes is considered important for the membrane extension of the autophagosome and the eventual enclosure of the membrane to form the vacuoles. The Atg5-Atg12-Atg16 complex is recycled, while the LC3 complex stays on the membrane until it is degraded by the lysosome. LC3 is thus widely used as a marker for monitoring the autophagy process.

Several other key autophagy genes are important to the initiation and regulation of autophagy. Beclin-1, a mammalian homologue of Atg6, is particularly important. Beclin-1 forms a complex with VPS34, p150/VPS15. VPS34 is a class III PI-3-kinase that is required for autophagy and can be suppressed by 3-methyladenine (3-MA) and also Wortmannin. In yeast, Atg6 also binds to Atg14, which has not been identified in mammalian cells. However, several prosurvival Bcl-2 family proteins **(18)** and Ambra-1**(19)** are found in mammalian cells that can also interact with Beclin-1. However, Bcl-2 suppresses, but Ambra-1 promotes, Beclin-1/VPS34 activity, and therefore autophagy. While the exact mechanism is not clear, the recent finding that Beclin-1 contains a BH3-only domain **(18**, **20**, **21)** suggests that Bcl-2 may sequester Beclin-1 from its interaction with VPS34. Finally, while UVRAG has not been found to be instrumental in yeast autophagy, it seems important for mammalian autophagy due to its interaction with Beclin-1 **(22)**. Another recently discovered molecule, Bif-1, promotes autophagy by interacting with UVRAG **(23)**. Thus, there are extensive molecular interactions at the early stage of the formation of autophagosomes (Fig. 30.1).

Activation of Autophagy

A key regulatory of autophagy in yeast and mammalian cells is the TOR complex **(5**, **24)**. A functional TOR activity would suppress autophagy. Because the TOR pathway is central to the signaling of growth and energy metabolism,

autophagy is intimately coupled with growth and energy control (Fig. 30.1). It can be affected by the upstream signaling of the TOR pathway. For example, growth factors, insulin, and the class I PI3-kinase and Akt will all suppress autophagy by activating Tor **(5)**. On the other hand, suppressing TOR function with rapamycin could induce autophagy.

At the moment, it is not clear how TOR suppression could cause autophagy. In yeast, this seems to be coupled with the activity of the Atg1-Atg13 complex. Although the mammalian cells express two Atg1 homologues, ULK1 and ULK2, they do not seem to possess the Atg13 homologue. Furthermore, genetic deletion of ULK1, unlike that of Atg5 or Atg7, does not result in global defects in autophagy, but specifically affects mitochondria autophagy during erythrocyte maturation **(25)**. It is possible, however, that the Beclin-1 complex would be among the first to be activated by the signals from the TOR suppression.

Several other signaling pathways have been defined in mammalian cells, some of which are likely mTOR-independent (Fig. 30.1). One such pathway can be characterized as calcium-calpain-Gsα–mediated **(26)**. In a number of cases, the induction of autophagy in mammalian cells seems to be related to the transcriptional activation of a number of molecules that are either involved in the core process of autophagy or involved in yet-to-be defined manners. Several transcription factors can be involved. In muscle cells, activation of the FoxO3 transcription by the suppression of the IGF-1-PI-3 K-Akt pathway could in turn enhance the transcription of several autophagy genes, including LC3 and Bnip3 **(27, 28)**. Bnip3 could be also transcriptionally activated by HIF-1, which is induced during hypoxia **(29, 30)**. Bnip3 is responsible for the autophagy induced by hypoxia and ischemia. Furthermore, E2F1 can be responsible for the upregulation of Atg1, Atg5, LC3, and DRAM, which can be important for DNA damage-induced autophagy **(31)**. DRAM is a lysosomal membrane protein participating in autophagy that had previously been identified as a p53 transcriptional target **(32)**. Whereas nucleic p53 could promote autophagy via upregulating DRAM, a recent study has also indicated that cytoplasmic p53 can suppress autophagy by unknown mechanisms **(33)**.

Finally, ER stress caused by chemicals and misfolded protein aggregates can induce autophagy, which helps to relieve ER stress **(34–39)**. In this case, it seems that the unfolded protein response (UPR) pathways, PERK/eIF2alpha and IRE-1/JNK, may be responsible for the autophagy induction. The PERK/eIF2alpha pathway could promote Atg12 expression, which may provide a mechanism of autophagy induction in this case **(35)**. ER stress could elevate the intracellular calcium level, which in turn can activate calmodulin-dependent kinase kinase beta to promote the activity of AMPK, leading to mTOR suppression and autophagy **(40)**. Alternatively, calcium can induce the phosphorylation of PCKθ, which seems to specifically participate in ER-stress-induced, but not in amino acid deprivation-induced, autophagy **(41)**. In this case, the activation of PKCθ is independent of either mTOR or the UPR.

Functional Roles of Autophagy in Mammalian Cells

It is now clear that autophagy is activated under and regulated by many physiological and pathological conditions and, in turn, affects these processes **(4**, **6**, **7**, **42)**. Thus, autophagy is inevitably associated with the pathogenesis of many human diseases **(6**, **43)**. It is required for normal development and participates in the clearance of apoptotic cells during embryogenesis **(42)**. In adults, autophagy seems to be involved in the extension of life span and in protecting cells from stress response, such as starvation. The autophagic degradation of cellular constituents can efficiently recycle essential nutrients to sustain basic biological processes. Thus, autophagy is important for the regulation of energy and nutrient metabolism **(5**, **24)**. Autophagy is commonly known to be activated by amino acid starvation (in mammalian cells) or nitrogen deprivation (in yeast cells). Under these conditions, autophagy is activated to degrade proteins and recycle amino acids to meet the cell's energy requirement **(5**, **10**, **24**, **44)**. This function of autophagy in meeting the energy and nutritional needs of cells was evolutionarily conserved. Genetic deletion of Atg5 in mice led to perinatal death due to the lack of sufficient nutrients in the cardiac and diaphragm muscles for their vital functions **(45)**.

In addition, the degradation of mitochondria, peroxisomes, endoplasmic reticulum (ER), or ribosomes by autophagy is most likely associated with cellular homeostasis as well as changing metabolic needs **(7**, **13**, **46)**. The ability of autophagy to degrade misfolded proteins is an important beneficial function in the pathogenesis of conformational diseases **(6**, **37)**. Autophagy is also employed as a defense mechanism to clear up intracellular microbes, misfolded proteins, and damaged organelles **(7)**.

The role of autophagy in cancer development and cancer therapy has been an area of intense study in recent years **(47**, **48)**. In the first study aiming to understand the function of Beclin-1/Atg6, a mouse stain deficient in this gene was constructed. While Beclin-1-null mice were embryonic lethal, Beclin-1 heterozygous mice were normal at the beginning, but developed multiple tumors later on, suggesting that Beclin-1 is a haplo-insufficiency tumor suppressor **(49)**. The loss of heterozygosity of Beclin-1 is frequently seen in breast and ovarian cancers **(50)**. While the mechanism of how Beclin-1 serves as a tumor suppressor is not known, it is possible that its proautophagy function is important in maintaining cellular homeostasis by removing damaged organelles, such as mitochondria **(51)**. Damaged mitochondria may become a major source of intracellular free radicals that could cause genomic instability and tumors. As a consequence, autophagy may suppression tumorigenesis by preventing genomic instability **(48**, **52)**.

Autophagy in the Regulation of Cell Death

The relationship between autophagy and cell death has been hotly debated in recent years. There are ample observations indicating that the two processes are intimately connected. Evidence for the role of autophagy in promoting cell

survival or in promoting cell death is compelling in both cases **(53, 54)**. In addition, it seems that the regulation of autophagy, apoptosis, and necrosis can be coupled so that one type of mechanism may activate or inactivate the other.

Programmed cell death was initially classified into several categories, primarily based on the ultrastructural morphology of the dying cells **(55)**. While one category of PCD demonstrates features of apoptosis, another category shows the accumulation of autophagosomes **(56)**. The latter was classified as autophagic death. However, considerable controversy exists regarding whether the autophagy process actually promotes cell death or instead is a reactive process that may actually provide protection **(53–56)**. These concerns were not just for the developmental biology process, but also for many pathological processes where autophagy is induced significantly in response to cytotoxic or metabolic stress.

With the understanding of the molecular machinery of autophagy, it is now easier to address these issues. Thus, by inhibiting the key autophagy genes through genetic deletion, RNAi-mediated knockdown, or pharmacological interventions, one may determine whether cell death is suppressed, enhanced, or not changed at all. In this way, the influence of autophagy on cell death could be determined. Indeed, depending on the circumstances, both pro- and anti-death functions of autophagy could thus be identified.

Autophagy Promotes Cell Survival

There are numerous conditions in which autophagy clearly plays a prosurvival role. In neonatal mice, autophagy is required for the endogenous generation of nutrients in such energy-dependent organs as the heart and diaphragm as the newborn adapts to taking in nutrients from an exogenous source, i.e., milk **(45, 57)**. Autophagy deficiency due to the deletion of key autophagy genes, such as Atg5, can thus lead to the premature death of newborn mice. A similar dependency of survival on autophagy during starvation has been demonstrated in *Drosophila*, which occurs primarily in the nutrient-sensing organ, the fat body **(58, 59)**. At the cellular level, the importance of autophagy in survival during nutrient or growth factor deprivation can also be shown in mammalian cells **(44, 60)** and yeast cells **(61)**.

Autophagy is also important for cellular survival under other stressful conditions. In mammalian cells, autophagy could be activated in response to metabolic stress, ischemia, or hypoxia **(48, 62)**. The suppression of autophagy can result in increased cell death. Cytotoxic agents, including many chemotherapeutic agents, such as proteasome inhibitors **(39)**, ER stressors **(34–36, 39)**, DNA-damaging agents **(31, 32, 63)**, and histone deacetylase inhibitor **(64)**, can all activate autophagy, likely in response to the damage caused by these agents. Under pathological conditions, such as the accumulation of misfolded proteins,

autophagy is required for the cellular clearance of these proteins and survival **(37)**. In *C. elegans*, limited food, high temperature, a highly dense population, and mutation in the insulin-like growth receptor (*daf-2*) could all cause development arrest in the form of dauer diapause, which is specialized for survival under these adverse conditions. Autophagy is required for dauer entry and therefore for the survival of the worm **(65)**.

Autophagy promotes cellular survival through its basic function of degrading intracellular components. In the nutrient/growth factor depletion/deficiency condition, autophagic degradation recycles the cellular proteins and glycogen to provide amino acids and glucose for ATP generation **(44)**. In cells under DNA damage or metabolic stress, autophagy may play an important role in removing damaged organelles, such as mitochondria, to reduce the cellular ROS level and maintain genomic stability **(48, 62)**. The clearance of misfolded proteins resulting from ER stress, proteasome inhibition, or genetic mutation is another important function of autophagy in maintaining cellular viability **(37)**.

The mechanisms of autophagy induction under these different conditions are not all well defined. Growth factor deprivation seems to be linked to the downregulation of the Akt signaling, which leads, on one hand, to the suppression of the Tor signaling and, on the other hand, to the activation of FoxO3, a transcription factor that can cause the upregulation of several autophagy genes (discussed earlier). Both events can lead to autophagy activation.

Autophagy induced by the deprivation of nitrogen in yeast is also critically related to the suppression of Tor signaling **(61)**. However, amino acid deprivation in the mammalian cells does not seem to be completely dependent on the mTOR pathway **(5)**. The PERK/eIF-2α signaling, part of the UPR initiated at the ER, can also contribute **(66)**. This pathway, together with another UPR pathway orchestrated by IRE-1, is also involved in autophagy initiated by the misfolded proteins and ER stress **(35, 36, 39)**. However, in response to proteasome inhibitors, which also cause ER stress and the accumulation of misfolded proteins, only the IRE-1 pathway is required **(39)**. ER stress can lead to calcium release, which in turn can activate calmodulin kinase kinase beta and AMPK to suppress mTOR **(40)**. Interestingly, in these cases, cytoplasmic p53 can suppress the induction of autophagy, thereby ensuring its rapid degradation following the induction **(67)**.

Autophagy-mediated prosurvival function could suppress either apoptosis or necrosis. In many apoptosis-competent cells, autophagy could co-exist with apoptosis, and the suppression of autophagy increases apoptosis **(35, 36, 39, 44, 60, 63, 64)**. However, in apoptosis-incompetent cells, caused by the deletion of key proapoptosis genes, such as Bax and Bak, or the overexpression of anti-apoptosis genes, such as Bcl-2 or Bcl-xL, or the use of caspase inhibitors, the inhibition of autophagy often leads to necrosis **(48, 68, 69)**. Many agents could induce both apoptosis and necrosis, although apoptosis can be a dominant type of death, and autophagy can suppress both types. The latter suggests that autophagy acts at the upstream level, where the death stimulation is derived,

so it can mitigate the cause for both apoptosis and necrosis. This thinking is consistent with the idea that the clearance function of autophagy is responsible for its prosurvival function by removing the "damaged" cellular content.

Autophagy Can Participate in Cell Death

Although early studies largely employed morphological criteria to define autophagic death, which is subject to verification, more recent works are based on molecular evidence to substantiate the role of autophagy in promoting cell death.

A typical example for autophagy to participate in developmentally regulated programmed cell death is the degradation of the salivary glands during *Drosophila*'s pupal stage, which is triggered by the steroid hormone ecdysone. Cell death is accompanied with both autophagy and apoptosis features, and determining whether or not autophagy promotes cell death had previously been confusing. A recent study using autophagy gene mutants now indicates that cell death can be significantly inhibited, suggesting that autophagy can promote cell death **(70)**. Furthermore, autophagy is activated by growth arrest and can induce cell death in the absence of caspase activation. In this case, it seems that autophagy and apoptosis independently contribute to the death. Indeed, a combined inhibition of both pathways increased the suppression of the gland degradation **(70)**.

In mammalian cells, autophagy can contribute to cell death under several stressful conditions. Thus, autophagy can be induced in response to certain chemotherapeutic drugs **(71**, **72)**, radiation **(73)**, hypoxia **(74)**, ischemia in the brain **(75)**, cytokines such as INF-γ **(76)**, and ligands such as HIV-1-encoded envelope glycoproteins **(77)**. In these cases, the deletion or RNAi-mediated knockdown of key autophagy genes can significantly reduce cell death, while overexpressing these genes can promote it.

How autophagy promotes cell death is not entirely clear. Although it may be tempting to assume that excessive self-digestion could lead to the depletion of key molecules or organelles essential to the process, the mechanism of killing may be as diverse as the stress signals that induce autophagy in the first place. The autophagic machinery may be interfaced with the apoptotic machinery or the necrotic mechanism to promote apoptosis or necrosis. For example, it was found that in INF-γ-induced autophagic death in HeLa cells, Atg5 can bind to FADD and activate caspase-8 and downstream caspases as if there were a death receptor engagement **(76)**. In another case, when Atg5 was overexpressed, it could be cut by calpains. The 24-Kda cleaved Atg5 N-terminal fragment (aa 1-193) is then translocated to the mitochondria, where it binds to Bcl-xL and inactivates it, resulting in cytochrome c release and cell death **(71)**. In these cases, autophagy seems to link to the classical apoptosis pathway; therefore, the death is actually mediated by the apoptosis machinery.

However, in other cases, autophagy-induced cell death is activated by the loss of the ability to mount an effective apoptosis. Thus, in apoptosis-deficient fibroblasts lacking both Bax and Bak, treatment with etopside **(72)** or prolonged

ER stress **(78)** caused autophagy. Similarly, radiation-induced cell death would switch from apoptosis to autophagic death if caspase activity could be blocked **(73)**. The molecular mechanism is not clear in this type of autophagy-mediated nonapoptotic death, although the role of JNK has been implicated **(72)**. It is likely that cell death in these circumstances is necrotic **(78)**. Similarly, ROS-induced autophagic death can also be necrotic **(79)**. Autophagy-promoted necrosis has also been reported in *C. elegans* **(80)**.

Interestingly, a number of BH3-only Bcl-2 family proteins or their binding partners, when overexpressed, can directly induce cell death with contributions from the autophagic process. Bnip3 is a BH3-only Bcl-2 family protein that has been shown to induce autophagic death in a number of cases. A more detailed discussion of this molecule is given in the following section, since autophagy induced by this molecule can also be prosurvival in other cases **(81)**. Other BH3-only molecules that can induce autophagic death include Apolipoprotein L1 (ApoL1) **(82)** and Bik **(83)**. Finally, hSpin1, a human homologue of the *Drosophila* spinster (spin) gene, can induce nonapoptotic death, which can be inhibited by Bcl-xL **(84)**. The latter effect may not be related to apoptosis regulation but to a direct physical interaction. This type of nonapoptotic autophagy-mediated death is necrotic **(84)**.

One potential mechanism by which BH3-only molecules can induce autophagy is through competitive binding to Bcl-2 or Bcl-xL to disrupt the interaction of the latter with Beclin-1 (Fig. 30.1). Beclin-1 is an important autophagy molecule and interacts with multiple other molecules, such as the class III PI-3 kinase VPS34, UVRAG, and Ambra-1, to promote autophagy **(85)**. However, Beclin-1 also possesses a conserved BH3 domain of the Bcl-2 family proteins and, in fact, can interact with multiple antideath Bcl-2 family members, such as Bcl-2 and Bcl-xL **(85)**, which suppress the function of Beclin-1 as a proautophagy molecule. However, this interaction can be disrupted by the phosphorylation of Bcl-2 or by other BH3-only molecules, such as Bad, or BH3 mimetics, such as ABT737 (see Chapter 2) **(85)**. As a result, Beclin-1 can be desuppressed and can in turn promote autophagy. Indeed, in a human leukemia cell line, HL60, simply knocking down Bcl-2 can cause autophagy, which seems to contribute to the accompanying cell death **(86)**. In the case of Bik-induced autophagic death, the loss of Bcl-2 is a prerequisite **(83)**. Likewise, ApoL1-induced autophagy depends on its BH3 domain, which may thus be involved in the competitive binding with Bcl-2 to release Beclin-1.

Factors That May Affect Whether Autophagy Presents a Prosurvival or Prodeath Effect

The relationship between autophagy and cell death can be quite complicated and may be affected by many factors. Autophagy likely evolves as a physiological process but can be diverted to a prodeath pathway under pathological

conditions. A very unique example of autophagy in promoting cellular injury has recently been reported in Atg5-deficient mice **(87)**. Autophagy has been found to be required for the activation of trypsinogen to trypsin under normal conditions. The enzyme is harmful to tissue if its activation and release are not properly controlled, which can be a cause of pancreatitis. Notably, because of autophagy's role in promoting enzyme maturation, it participates both in normal pancreatic functions, essentially of a "prosurvival" nature, and in pancreatitis in pathological conditions, essentially of a "prodeath" nature.

The level of autophagy could determine the outcome. In *C. elegans*, it has been suggested that only the physiological level of autophagy during starvation is prosurvival and that excessive autophagy could be prodeath **(88)**. Excessive autophagy can cause cellular atrophy and the deficiency of vital cellular components.

The presence of compensatory mechanisms, such as chaperone-mediated autophagy (CMA), may also determine whether the inhibition of macroautophagy renders cells to be more sensitive or more resistant to certain stressful signals. Thus, murine fibroblasts prepared from Atg5-deficient embryos were more sensitive to death receptor-initiated death, but were more resistant to menadione- and UV radiation-induced death due to increased CMA **(89)**.

In a more general way, whether autophagy can be prosurvival or prodeath can also be dependent on both the agents used for stimulation and the status of the cells. Thus, autophagy induced by ER stress **(38)** or proteasome inhibitors (Ding and Yin, unpublished observation) is protective in tumor cells but indifferent or detrimental in nontransformed cells. In contrast, ROS-induced autophagy is prodeath in cancer cells but may not be so in nontransformed cells **(79)**.

Cell death and autophagy can be mediated by the same molecule, but through separate mechanisms. Thus, while autophagy and cell death may seem to be coupled by the same molecule, their relationship can be more complicated than a simple interpretation. This point may be best illustrated in the case of Bnip3, a BH3-only Bcl-2 family protein originally identified as an E1B19K and Bcl-2 interacting molecule **(90)** (see Chapter 2). Bnip3 is usually localized at the mitochondrial outer membrane. Unlike other BH3-only molecules, its transmembrane (TM) domain, but not its BH3-domain, is required for its activity and membrane targeting **(91, 92)**. The expression of Bnip3 is often low in normal conditions, but it can be rapidly induced in adverse conditions, in particular, in hypoxia or ischemia **(29, 30, 93, 94)**. Bnip3 can be transcriptionally activated by HIF-1 **(29, 30)** or FoxO3 **(27)** but repressed by NF-κB **(95)** or Rb/E2F **(96)**.

The increased expression of Bnip3 is often accompanied by apoptotic or necrotic cell death, and autophagy, in cell lines subjected to hypoxia **(29, 74, 96)**, or treated with ceramide or arsenic trioxide **(97, 98)**, or in cardiac myocytes subjected to ischemia-reperfusion injury **(99, 100)**. While both cell death and autophagy can be attributed to Bnip3, in most studies, direct evidence that autophagy contributes to cell death is limited, e.g., with tumor cells under hypoxia **(74)**. In contrast, in a cardiac myocyte line under simulated ischemia-reperfusion

conditions **(99)**, or in murine embryonic fibroblasts subjected to hypoxia **(101)**, autophagy was found to be important for cell survival. In both cases, it was found that this protective effect is due to the Bnip3-promoted autophagic removal of damaged mitochondria (mitophagy) **(99**, **101)**, which is necessary to prevent increased levels of reactive oxygen species **(101)**. Interestingly, a close homologue of Bnip3, Bnip3L/Nix, can also be induced by hypoxia and has been shown to be required for mitophagy during erythrocyte maturation **(102**, **103)**.

Could the different roles of Bnip3-mediated autophagy be related to different cell types under different treatments or be related to other factors? The mechanism by which Bnip3 induces autophagy has not been completely elucidated, although one study indicated that Bnip3 could bind to and inactivate Rheb, therefore inactivating mTOR during hypoxia **(104)**. As mTOR is a major factor of autophagy suppression, the negative effect of Bnip 3 on mTOR can be expected to induce autophagy. Bnip3 is thus considered to be required for both hypoxia-induced autophagy and hypoxia-induced mTOR repression. Alternatively, Bnip3 may compete with Bcl-2 for binding to Beclin-1 and thus can cause the derepression of Beclin-1, which in turn promotes autophagy **(101)** (Fig. 30.1).

It is not completely clear whether cell death and autophagy are mechanistically coupled in the case of Bnip3. It is possible that Bnip3-induced cell death may not be related to autophagy but instead goes through a separate mechanism. Some studies suggested that cell death is related to mitochondria permeabilization by its dimerized TM domain with or without the participation of Bax and Bak **(105**, **106)**. It has been postulated that Bnip3's killing activity may be secondary to prolonged hypoxia, ischemia-reperfusion injury, and anaerobic glycolysis, which results in acidosis **(81)**. Notably, acidosis has been found to stabilize Bnip3 and to increase its association with the mitochondria and its killing ability **(107)**. Furthermore, hypoxia or ischemia does not induce cell death in cardiac myocytes in the absence of acidosis **(108)**. Thus, it is possible that Bnip3 may primarily induce autophagy, as a protective mechanism at the early stage of hypoxia/ischemia, but induce cell death at the later stage when acidosis occurs, which promotes Bnip3 interaction with the mitochondria and membrane permeabilization.

Finally, when considering the cell death's relationship with autophagy, one may need to be aware that sometimes the same phenomenon may be subjected to different interpretations, as the mechanisms of the action are not always clear, particularly at the beginning. A pan-caspase inhibitor, z-VAD, is often used to suppress caspases and, therefore, caspase-mediated apoptosis. However, this chemical could inhibit other types of proteases, notably, the lysosomal cathepsins **(69)**. The application of z-VAD to several types of cells resulted in the increased accumulation of autophagic markers and necrotic cell death that seemed to be attributable to autophagy **(109)**. However, further investigation indicated that the increased autophagosomes are not due to an increase in the induction, but to a reduction in the degradation of the autophagosome because

of the lysosomal inhibition by z-VAD **(69)**. Thus, the increased cell death is related to reduced, rather than increased, autophagy, which was confirmed by a subsequent study indicating that autophagy was prosurvival rather than prodeath following z-VAD treatment.

The Potential Clinical Significance of Modulating Autophagy to Control Cell Death

Understanding the various conditions under which autophagy may be prosurvival or prodeath can have practical benefits in controlling the disease process. This topic is particularly attractive for cancer therapy, where the goal is to eliminate tumor cells by promoting cell death. Both properties of autophagy in regulating cell death have been explored for this purpose. Thus, numerous reports have indicated that suppressing autophagy during treatment with chemotherapeutic agents such as alkylating drugs, proteasome inhibitors, and histone deactylase inhibitors **(39**, **63**, **64)** could enhance apoptosis in various types of tumor lines. Conversely, promoting autophagy by the combined use of rapamycin has been found to enhance radiation therapy **(73)**. The future challenge is to define the conditions under which autophagy plays a specific role in cell death, promotion, or inhibition. It is possible to take advantage of this dichotomic characteristic of autophagy for maximal benefits. For example, ER-stress inducers **(38)** and proteasome inhibitors (Ding and Yin, Molecular Cancer Therapeutics, inpress, 2009) can induce the prosurvival function of autophagy in the tumor cells, but they induce the prodeath function in normal cells. Thus, the suppression of autophagy *in vivo* may specifically enhance death in the tumor cells, but reduce death in the normal cells under these circumstances.

The prodeath activity of autophagy during ischemic injury in the brain **(75)** may be suppressed to reduce organ damage. Other than directly targeting the autophagy genes, it may be possible to act on specific targets if the mechanism of autophagic death is known, such as the inactivation of Bcl-xL by the cleaved Atg5 **(71)**. On the other hand, autophagic activity can be enhanced for the removal of misfolded proteins, such as the mutant huntingtin, as seen in Huntington's disease, and the alpha-1 antitrypsin Z mutant **(26**, **37**, **110)**. The prosurvival function of autophagy could thus be explored in these conformational diseases.

Conclusion

Autophagy is an evolutionarily conserved physiological process that degrades various cellular contents. Its functions are thus interfaced with cellular survival and cell death. Depending on the context, autophagy activity could contribute to either cell survival or cell death. Only in very limited cases are the mechanisms by

which autophagy modulates cell death understood. But both apoptosis and necrosis can be modulated by autophagy, and excessive digestion by autophagy can also lead to cellular atrophy and direct death. Future work should focus on understanding individual cases so that this character of autophagy can be explored for cancer therapy, control of tissue injury, and treatment of conformational diseases.

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Part V
Approaches to the Study of Apoptosis

Chapter 31
Analysis of Apoptosis: Basic Principles and Protocols

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Abstract Apoptosis is a distinct form of cell demise. Originally defined by cellular morphology, apoptosis can now be characterized at the molecular, biochemical, and cellular levels. The detection of apoptosis has become more important, not only in the interests of scientific investigation, but also because of its significance in clinical practice. For example, since apoptosis has been implicated in a variety of devastating diseases such as cancer, therapies targeting apoptosis are being developed. To evaluate the effectiveness of the treatment, one would have to assess the apoptotic response before, during, and after the therapy. For the typical apoptosis, a set of characteristics in cell structure and biochemistry has been well defined. In combination, these provide the basis for apoptosis detection in a given setting. The methodology for analyzing these characteristics is as diverse as the research subjects. Several books devoted to the methodology of apoptosis analysis have recently been published **(1–3)**. Readers may find detailed experimental protocols in these books. This chapter provides an overview of the basic approaches used in analyzing apoptosis, the principle and the basic methodology, in order to provide a quick guide for readers to use when deciding which methods are available and appropriate for their own study. We start with the determination of cell viability and the morphology of dying cells. We then discuss approaches for examining apoptotic changes on the cell membrane, in the cytosol, and in the nucleus. We also summarize some of the common sources of reagents for apoptosis research.

Keywords Cell viability · Microscopy · TUNEL assay · Caspase · Mitochondria

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Determination of Cell Viability

Introduction

Regardless of how and when it takes place, apoptosis indicates the death of a cell. Cells undergoing apoptosis end up with a compromised metabolism, function, and capacity of proliferation: the loss of viability. Therefore, the assessment of cell viability should be considered one of the primary criteria for apoptosis. Commonly used approaches to measure cell viability can be classified into three categories. The first group of methods relies on the fact that living cells maintain the integrity of their plasma membranes. The second group of methods is based on the ability of a cell to sustain specific metabolic functions, while the third group of methods measures the cellular capacity to divide and proliferate.

General Procedures

Measurement of Plasma Membrane Integrity by Vital Dye Exclusion Assay

Vital dyes can be roughly classified into two types. By their physical properties, the first group is not permeable to plasma membranes and therefore does not enter intact cells. As a result, these dyes do not stain viable cells, but can enter cells with compromised plasma membranes and bind to internal structures or molecules, labeling these cells with color or fluorescence. Good examples in this class include trypan blue, propidium iodide, and ethidium homodimer. The second class of vital dyes is permeable to plasma membranes; usually, after entering the cell, the dyes are modified and trapped inside in the presence of intact plasma membranes. Therefore, unlike dyes in the first class, these dyes stain viable cells but not those with broken membranes. Good examples for these dyes include fluorescein diacetate and Calcein-AM **(4)**.

To do the assay, for adherent cells, cells are rinsed twice with phosphate buffer solution (PBS) and incubated with PBS containing a vital dye for a couple of minutes. The cells are subsequently rinsed with PBS to remove extracellular dye prior to examination by microscopy. For cell suspension, cells are collected by centrifugation and rinsed with PBS. The cells are subsequently resuspended into PBS containing vital dye. After minutes of staining, extracellular dye is removed by centrifugation. Cells are resuspended in PBS for microscopic examination.

Measurement of Cytosolic Leakage

This method is based on the consideration that viable cells with intact plasma membranes are able to preserve cellular contents, particularly macromolecules such as proteins. Once the plasma membrane is broken, cytosol is leaked into the extracellular space. A classic and still commonly used indicator for cytosolic

leakage is the release of intracellular enzymes such as lactate dehydrogenase (LDH). LDH is a ubiquitous 136-kDa cytosolic protein with enzymatic activities to catalyze the following reaction: pyruvate + NADH = lactate + NAD^+. NADH exhibits fluorescence at an excitation wavelength of 360 nm, with emission at 450 nm. The velocity of decreases in Ex360-nm/Em450-nm fluorescence in the reaction indicates the conversion of NADH to the nonfluorescent NAD and therefore LDH activity. Breakdown of the plasma membranes leads to the release of LDH into the incubation medium. The amount of LDH present in the extracellular space can therefore be used as an index of cell leakage or death **(5)**.

The basic procedure is as follows. The incubation medium is subjected to brief centrifugation to remove cellular debris. The resultant supernatant is added to enzymatic reaction buffer containing pyruvate and NADH. NADH fluorescence at Ex360 nm/Em450 nm is monitored during the reaction. The velocity of decreases in NADH fluorescence indicates the conversion of NADH to nonfluorescent NAD+ and therefore LDH activity. Parallel dishes of cells are lysed with 0.l% Triton X-100 to determine total LDH activity. The LDH activity obtained from the cell incubation medium is divided by the total LDH activity to calculate the percentage of LDH release.

Clonogenic Activity

The ability of cells to divide and form colonies is called *clonogenic activity*. For proliferative cells, the clonogenic activity is a functional measurement of cell viability. Cellular clonogenic activity depends on not only the integrity of plasma membranes but also a complex set of cellular functions involving energy production, macromolecule synthesis, and mitotic division.

The basic procedure is as follows. For adherent cells, cells are collected and separated into individual cells by trypsinization and vigorous pipetting. The cells are then plated at a very low density (200–1,000 cells/100-mm tissue culture dish). The cultures are maintained for a period of about five cell divisions. Colonies can be counted directly or after MTT (0.5 mg/ml in PBS) staining, under a microscope. For cell suspensions, cells are collected by centrifugation and cell clumps are broken up by pipetting. Individual cells are then plated in 0.1–0.2% agarose at low densities. The cultures are maintained and colonies are counted.

Comments

(a) The methods just described measure the integrity of plasma membranes. While an intact plasma membrane is a prerequisite for a cell to live, it does not necessarily indicate that the cell is viable. In the case of apoptosis, usually the integrity of plasma membranes is not compromised until the late stage of the so-called secondary necrosis. Thus, these measurements are not very useful in early-stage apoptosis detection; rather, they are utilized when examining cell injury accompanied by membrane damage.

(b) The dose and exposure time of vital dyes should be titrated for specific types of cells. Too much exposure may lead to cytotoxicity and nonspecific permeabilization.
(c) Vital dye staining can be used in conjunction with flow cytometry. This provides a quantitative assessment of cell viability.
(d) For an enzymatic measurement of cytosolic leakage, one has to be sure that the treatment does not interfere with the enzyme directly. Proper controls should be considered.
(e) Obviously, a clonogenic activity assay cannot be utilized to assess the viability of nondividing or postmitotic cells. For example, primary cultures of neurons usually do not proliferate when grown *in vitro*.

Morphological Examination

Introduction

Apoptosis was originally defined by a sequence of morphological features **(6)**. Despite recent progress in apoptosis research at the biochemical and molecular levels, morphological changes are still considered the gold standard for apoptosis.

By light microscopy, apoptosis is characterized by the condensation of nuclear chromatin and shrinkage of the cell. After condensation, chromatin becomes segregated against the nuclear membrane. At this stage, the nucleus appears to be shrunken and condensed as well. After the initial phase, the cell detaches from the neighboring tissues and enters the fragmentation stage. Cellular fragmentation is characterized by the formation of apoptotic bodies, or blebbing. In the apoptotic bodies, fragments of nucleus and cellular organelles including mitochondria are usually found. *In vivo*, the apoptotic bodies are rapidly absorbed through phagocytosis by macrophages or neighboring cells. In *in vitro* experimental models where phagocytosis is not available, the apoptotic cells will eventually undergo degradation by a process similar to necrosis, releasing the cellular contents into the incubation medium. This degradation is also called *secondary necrosis* **(6)**.

Electron microscopy is used to examine ultrastructural features of apoptosis. Typical ultrastructural alterations include the condensation of cytoplasm, fragmentation of the nucleus, aggregation of dense masses of chromatin beneath the nuclear membrane, protrusion of the cell surface, or formation of apoptotic bodies.

General Procedures

Standard procedures for light microscopy and transmission electron microscopy are recommended. A light microscope with phase contrast is helpful in documenting the stereotypical morphological changes during apoptosis. Nuclear changes are also frequently shown following fluorescent dye staining.

Comments

(a) Morphological features are considered the most reliable criteria in defining apoptosis.
(b) Light microscopy provides a quick, convenient, and on-site method for monitoring the progression of apoptosis. It can be semiquantitative if the images of representative fields of cells are captured. Electron microscopy provides the definitive morphological evidence of apoptosis; however, it does not provide quantitative data and thus cannot be used to compare or quantify the number of apoptotic cells between experimental conditions.
(c) Microscopic quantitation is subject to individual bias. The development of apoptotic morphology is highly heterogeneous among the cells. A consensus must be reached between investigators as to what is considered apoptotic.
(d) Flow cytometry may also be used for cell morphological examination based on the analysis of light-scattering properties of the cells. The forward scatter is commonly used to approximate cell size, while the side scatter generally correlates with cell granularity. In combination with the membrane integrity measurement described earlier, the different morphological features of apoptotic and necrotic cells can be detected **(7)**.

Alterations in the Plasma Membrane

Introduction

A noticeable change in the plasma membrane of apoptotic cells is the redistribution of phospholipids. In normal cells, phospholipids are present asymmetrically across the plasma membrane. While phosphatidylcholine and sphingomyelin are located mainly in the external leaflet, most of the phosphatidylethanolamine and the phosphatidylserine (PS) are restricted to the inner leaflet of the plasma membrane. Early during apoptosis, the lipid asymmetry is lost, resulting in the appearance of PS on the cell surface **(8)**. The measurement of the membrane lipid redistribution therefore provides another indication of apoptosis. The commonly used approach to detect membrane lipid redistribution monitors the appearance of PS on the cell surface. When PS is exposed on the cell surface, it can bind to Annexin V in a Ca^{2+}-dependent manner. Annexin V conjugated with fluorophores or biotin is available from commercial sources. One of the popular conjugates is FITC-Annexin V, which binds PS on the apoptotic cell surface and labels the cells with green fluorescence.

General Procedures

A basic method has been described **(9)** and can be modified in various experimental models. Briefly, for adherent cells, rinse the cells with PBS, add FITC-Annexin V prepared in the binding buffer (2.5 mM $CaCl_2$, 150 mM

NaCl, 10 mM HEPES, pH 7.4), and incubate for 2–5 minutes at room temperature. Rinse cells thoroughly with binding buffer to remove free FITC-Annexin V for microscopic examination. For cell suspension, collect cells by centrifugation and resuspend the cells in FITC-Annexin V in binding buffer. After 2–5 minutes of staining, wash off the unbound FITC-Annexin V with binding buffer. The cells are then examined under a fluorescence microscope.

The detection of PS exposure by Annexin V binding is also frequently used to quantify apoptosis via flow cytometry. Assay kits are commercially available. For example, the Annexin V-FITC apoptosis detection kit from BD PharMingen has been used in our studies **(10, 11)**. Briefly, cells are harvested by centrifugation at 1,000 × *g* for 5 minutes. After being washed in PBS containing 2% BSA, the cells are resuspended in binding buffer (10 mM HEPES-NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$) at a final density of 1–2 × 10^6 cells/ml. The single-cell suspension of 100 µl (1–2 × 10^5 cells) is incubated with 5 µl of Annexin V-FITC and 5 µl of propidium iodide for 15 minutes at room temperature in the dark. Finally, the mixture is diluted with 400 µl of binding buffer and analyzed with flow cytometry to determine the percentage of cells that have Annexin V-FITC staining or have staining of both Annexin V and propidium iodide.

Comments

(a) Binding of Annexin V can be used as a marker only in cells with intact plasma membranes. If the integrity of plasma membranes is compromised, Annexin V enters cells and may become associated with PS from inside, labeling the cells regardless of the mode of cell death. Therefore, to identify apoptosis, another staining such as PI that probes plasma membrane integrity should be included.
(b) Binding of Annexin to PS requires Ca^{2+}. Thus, care has to be taken not to significantly reduce Ca^{2+} concentration in the binding buffer.

Changes in the Cytosol

Introduction

A number of apoptotic changes occur in the cytosol; the most important events perhaps are the activation of caspases and the cleavage of multiple cellular proteins by the activated caspases. Other cellular changes involve the regulatory proteins such as the Bcl-2 family proteins. The background information on caspases and the Bcl-2 family proteins is given in Chapters 1 and 2, respectively.

A general discussion of the approaches that can determine these changes follows here.

Caspases

The activation of caspases is arguably a biochemical hallmark of classic apoptosis. There are several ways to detect caspase activation. Caspases are a family of cysteine proteases that cleave their substrates after aspartate residues. Existing as zymogens (also called procaspases) within unstimulated cells, caspase activation depends on the cleavage of the zymogen to form the large and small subunits, which in turn form an active heterotetramer complex. Thus, caspase activation can be determined by Western blots using anticaspase antibodies to examine whether the zymogen is converted into cleaved active forms. Alternatively, the enzymatic activities of caspases can be measured using synthetic fluorogenic or chromatogenic substrates. Finally, the caspase's activation status can also be determined by examining the cleavage of endogenous substrates, such as PARP (see Chapter 1).

General Procedures

Western Blot for Detecting Caspase Cleavage and Caspase Substrate Cleavage

Cells are harvested and washed in cold PBS. The cytosol can be prepared by a number of different methods. However, for detecting caspase substrate cleavage, the most effective way is to lyse the cell in a buffer containing 10% glycerol, 2% SDS, 0.003% bromophenol, and 5% 2-ME (added immediately before analysis) **(12)**. Proteins are separated by a standard SDS-PAGE followed by Western blot with specific antibodies against the caspase of interest. Commonly examined caspases are caspases-3, -7, -8, and -9. The antibodies against several pro- or cleaved caspases are also available from commercial sources. Alternatively, antibodies against caspase substrates, such as PARP, which is cleaved from 116 Kda to 85 Kda during apoptosis, can be used for the Western blot. There is a selectivity of certain substrates for some caspases, based on the recognition site (Chapter 1).

Measurement of Caspase Activities Using Synthetic Substrates

The caspase recognition site is composed of a tetrapeptide sequence that differs from one type of caspase to another. For example, caspases-3, -6, and -7 mainly recognize the motif of DEVD, whereas caspase-8 prefers the sequence of IETD. Other commonly used caspase substrates include YVAD for caspase-1, VDVAD for caspase-2, WEHD for caspase-5, and LEHD for caspase-9. These tetrapeptides can be synthesized and conjugated to a report group, which can be cleaved by the corresponding caspases for release. The

activity of the measured caspases is proportional to the amount of the released report group. The most commonly used report groups include p-nitroanilide (pNA; colorimetric detection by absorbance at 405–410 nm), 7-amino-4-methylcoumarin (AMC; fluorometric detection at an excitation/emission wavelength of 380/460 nm), and 7-amino-4-trifluoromethylcoumarin (AFC; fluorometric detection at Ex360 nm/Em530 nm).

To measure caspase activity, cells are first lysed in a caspase assay buffer (20 mM PIPES, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, pH 7.2) **(13)**. Alternatively, cells can be extracted in a lysis buffer containing 1% Triton X-100, 1 mM DTT, 25 mM HEPES, 115 mM NaCl, 1 mM KH_2PO_4, 4 mM KCl, pH 7.4, with a protease inhibitor cocktail **(14, 15)**. About 20 to 50 μg of proteins are used in each assay, mixed with 20 to 50 μM of the substrates in a volume of about 200 μl. The reaction is then monitored by the fluorometric or colorimetric detection at the specific wavelengths. The values reflect the relative caspase activity, which can be converted to specific activity with proper standards. Commercial kits for caspase activity measurement are now widely available.

Measurement of Caspase Activation by Immunostaining or with Fluorescent Substrates in Tissues and Cells

To detect the activation of caspase in a specific anatomic location, one can perform immunohistochemical or immunofluorescence staining on formalin-fixed paraffin sections with antibodies specifically against the activated caspases, which usually target to the subunits, but not the zymogen. Antibodies against activated caspases-3, -7, and -9 are now commercially available (e.g., from Cell Signaling Technology and BD PharMingen). A standard protocol is used for immunostaining **(16, 17)**. Briefly, after being fixed in 4% paraformaldehyde, the tissue sections or cells are incubated in a blocking buffer containing 2% normal nonimmune serum. The primary antibody is the antiactivated caspase, and the secondary antibody can be conjugated to either HRP or a fluorophore for either histochemical detection or fluorescent detection. It is possible to doubly stain the section with a cell-specific antibody to determine the type of cells that are undergoing apoptosis and therefore contain the activated caspases. A good example of such an application can be found in recent studies **(18)**.

To detect caspase activation in cultured cells, other than Western blot, one can also take advantage of several recently developed cell-permeable fluorescent substrates. One type of such substrates with the tradename of PhiPhiLux (Oncogene Research Products) is a peptide of 18 amino acids, with caspase recognition motifs in the center, and two fluorophores covalently attached near the termini. The two fluorophores quench each other in the native molecule because of intramolecular interactions until caspase hydrolysis breaks the peptide linkage. Thus, the substrates fluoresce in response to caspase activation **(19)**. Since these products are cell-permeable, they can label the cells undergoing

apoptosis, which can be quickly identified and quantified by fluorescence microscopy or flow cytometry.

Comments

Caspase activation is determined mainly based on the cleavage of the zymogen and by measuring the proteolytic activities. By combining proper identification methods, one can not only detect caspase activation qualitatively and quantitatively, but also localize the cells in a tissue. One needs to be aware, however, that the synthetic substrates used for measuring various caspase activities are not absolutely specific for a particular type of caspases. Thus, DEVD-AFC may detect caspase activities better to be described as DEVDase activities, which could include those of caspase-3, -7, or -6.

Bcl-2 Family Proteins

The status of the Bcl-2 family proteins can be examined from two aspects: the expression level and the activation status. For the former, a Western blot or Northern blot could be performed. For the latter, the approach will depend on the individual members. Commonly used assays can determine Bad phosphorylation, Bid cleavage, translocation of Bax, Bid, Bad, or Bim from the cytosol to the mitochondria, and the formation of Bax or Bak oligomers.

General Procedures

All the assays can be based on immunoblotting using antibodies specific to individual members. The preparation of cell fractions will vary depending on the nature of the experiments. If subcellular localization is not relevant, whole-cell lysate can be collected by lysis with 1–2% SDS. Cells need to be thoroughly solubilized, as many Bcl-2 family proteins are membrane-bound. If subcellular localization is part of the examination, then cellular components are subfractionated to obtain the mitochondria and the cytosol fractions. One fractionation method is by homogenization and differential centrifugation, which is discussed in a later section. Another commonly used fractionation method is to use low concentrations of digitonin to specifically permeabilize the plasma membrane (to release cytosol) but not the mitochondrial membrane. This method is convenient. Briefly, cells are incubated for a few minutes in an isotonic buffer (250 mM sucrose, 10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, and 1 mM EGTA, pH 7.1) containing 0.05% digitonin. The digitonin-soluble fraction is collected as cytosol and the insoluble part as the membrane-bound organellar fraction **(20**, **21)**.

These fractions are then resolved on SDS-PAGE followed by Western blot with specific antibodies to individual members to determine whether there is a change in the expression level, a cleavage product (such as p15 of Bid), or a phosphorylation

event. Antibodies specific for phosphorylated Bad are commercially available (e.g., Upstate Biotechnology and Cell Signaling Technology). Translocation from the cytosol to the mitochondria is indicated by the appearance of the molecule, such as Bid or Bax, in the mitochondrial fraction following apoptosis stimulation, which would be present mainly in the cytosol in healthy cells.

To determine whether Bax or Bak forms oligomers that are important to their functions, cross-linking agents are used **(22–24)**. For Bax oligomerization, both a membrane-permeable agent, Disuccinimidyl suberate (DSS), and a membrane-impermeable agent, Bis (sulfosuccinimidyl) suberate (BS^3) (Pierce Chemicals), have been used successfully **(22, 23)**. For Bak oligomerization, Bismaleimidohexane (BMH, Pierce Chemicals) has been used successfully **(24)**. For the detection of Bax oligomerization **(22)**, the mitochondrial fraction (0.5 mg of protein) is suspended in an isotonic buffer (200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM HEPES, pH 7.5), and BS^3 (in 5 mM sodium citrate buffer, pH 5.0) or DSS (in DMSO) is added to a final concentration of 10 mM. After 30 minutes of incubation at room temperature, the cross-linker is quenched by 1 M Tris-HCl, pH 7.5, to a final concentration of 20 mM. The membranes are then lysed in RIPA buffer and cleared by centrifugation at $12{,}000 \times g$ before analyzed by SDS-PAGE and Western blot with an anti-Bax antibody. For Bak oligomerization, 10 mM BMH in DMSO is added to the mitochondria pellet for 30 minutes at room temperature. The mitochondria are pelleted again and resuspended in protein sample buffer for SDS-PAGE and Western blot with an anti-Bak antibody **(24)**. In both cases, oligomerization can be initiated by an activating molecule, such as Bid, and can be visualized as a high-molecular-weight species **(25)**.

Comments

The subcellular localization of Bax can be confusing, as in some cultured cell types, a portion of Bax is already present in mitochondria, although in many other cell types, including primary cells, it is generally present only in the cytosol **(26, 27)**. It is possible that the not-so-optimal culture condition may cause Bax accumulation in mitochondria even in the absence of an apparent apoptotic signal. Thus, Bax activation should be indicated by a combination of criteria, including molecular oligomerization.

Mitochondrial Changes

Introduction

Mitochondria are recognized as the most important organelle involved in apoptosis initiation and regulation. Significant changes can be observed in mitochondrial physiology and morphology during apoptosis. In general, the apoptotic changes can be divided into two categories: release of apoptotic

proteins and alteration of mitochondrial functions. The released apoptotic proteins include cytochrome c, Smac/DIABLO, HtrA2/Omi, Endonuclease G, and AIF, in addition to some nonapoptotic proteins (see Chapter 6). The redistribution of these proteins can be determined by Western blot, ELISA, or immunofluorescence staining. A major test for mitochondrial functions related to apoptosis is the determination of the transmembrane potentials. Other assays have been developed to determine the mitochondrial permeability transition, mitochondrial generation of free radicals, mitochondrial redox status (e.g., detection of reduced glutathione or manganese-superoxide dismutase), mitochondrial calcium content, and mitochondrial pH changes [for reviews, see **(28–30)**].

Mitochondrial Release of Cytochrome c

Cytochrome c release is perhaps the most common parameter to be examined to determine whether or not the mitochondrial pathway is activated. It is usually determined by Western blot or ELISA using the cytosol, or by immunofluorescence staining of cells. In addition, to test whether an agent is capable of directly inducing cytochrome c release from mitochondria, the agent can be incubated with isolated mitochondria, and the release of cytochrome c into the incubation buffer can then be determined by Western blot or ELISA **(11)**.

General Procedures

Subcellular Fractionation

Cells are harvested, washed with cold PBS, and then resuspended in a hypotonic buffer (10 mM HEPES, pH 7.4, 38 mM NaCl) containing a cocktail of protease inhibitors **(31)**. Cells can be disrupted with a Dounce homogenizer, with a polytron homogenizer, or by repeat passing through a fine needle. Alternatively, isotonic buffers can be used to avoid breakage of mitochondria during homogenization. A commonly used buffer contains 200 mM mannitol, 70 mM sucrose, 1 mM EGTA, and 10 mM HEPES, pH 7.5 **(22)**. A simple KCl-based buffer (150 mM KCl plus 5 mM Tris-HCl, pH 7.4) has also been used successfully **(32)**. It requires pilot tests to determine the best way to disrupt a maximal number of cells without damaging the mitochondrial membranes, which could be cell-type-dependent and empirically determined. The lysates are then centrifuged at 600–1,000 $\times$ *g* to remove the undisrupted intact cells and nuclei. The supernatant is further centrifuged at 10,000–20,000 $\times$ *g* to pellet the heavy membranes, which are enriched for mitochondria. The method has also been used to subfractionate the mitochondria and cytosol in fresh or frozen tissues **(16, 33)**. This preparation of mitochondria should be sufficiently good for the general use described in this chapter. The mitochondria can then be resuspended in an isotonic buffer with energizing agents (250 mM sucrose, 10 mM

HEPES, 1 mM ATP, 5 mM sodium succinate, 0.08 mM ADP, 2 mM K_2HPO_4, pH 7.5) **(34)**. As discussed earlier, another commonly used fractionation method is to incubate cells with low concentrations of digitonin to collect the soluble part as a cytosolic fraction and the insoluble part as a membrane-bound organellar fraction **(20**, **21)**.

Western Blot Analysis

Western blot is conducted with both the supernatant and the pellet fractions with a proper anticytochrome c antibody. A control of the cytosol proteins can be β-actin, and that for the mitochondrial fraction can be cytochrome c oxidase subunit IV. The appearance of cytochrome c ($\sim$12 Kda) in the supernatant fraction and/or a reduction of cytochrome c content in the pellet fraction indicate cytochrome c release from mitochondria.

ELISA

The supernatant is prepared as described above and subjected to ELISA analysis for the detection of cytochrome c using one of the commercial kits (e.g., Oncogene Research Products or R&D Systems). The advantage of this assay is that the released proteins can be easily quantified for comparisons among samples.

Immunostaining

The subcellular (re)distribution of cytochrome c can also be visualized following immunofluorescence staining. Cells are washed and fixed with 4% paraformaldehyde. The primary anticytochrome c antibody applied should be able to recognize the native conformation of the molecule, such as the clone 6H2.B4 from BD PharMingen. After the application of the secondary antibody conjugated with a fluorophore, cells can be observed under a fluorescence microscope. Normal cells would have a perinuclear punctuated staining pattern, consistent with the mitochondrial localization of cytochrome c. In apoptotic cells, the staining may either assume a diffuse cytoplamic distribution or become very faint or invisible. To further differentiate the mitochondria and cytosol locations, cells can be co-stained with anti-Hsp60 antibodies or with a mitochondria-specific dye, such as MitoTracker, before fixation. Labeling of mitochondria can also be achieved by expressing MitoRed, a red fluorescent protein in mitochondria **(35)**.

Comments

These methods can be applied based on different situations. If there are plenty of cells, and it is easy to subfractionate the cells, then Western blot or ELISA can be a first choice for qualitative or quantitative analysis. Otherwise,

immunostaining can be selected, which can be applied where fewer numbers of cells are available for the study. It is, however, important to determine the percentage of cells with a particular cytochrome c staining pattern for a direct comparison among different samples **(35)**.

Mitochondrial Transmembrane Potentials

The transmembrane potential ($\Delta\Psi_m$) has been analyzed to indicate mitochondrial function during apoptosis. The potential is about 180 mV (negative inside). The potentials can be determined with lipophilic cations, which accumulate inside the mitochondria in a potential-dependent way. Commonly used fluorescent lipophilic cations include Rhodamine-123 (Rh123), 3,3′-dihexiloxocarbocyanine iodide [$DiOC_6(3)$], tetramethyrhodamine methyl ester (TMRM), and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1), which can be analyzed by flow cytometry or fluorescent microscopy. Nonfluorescent probes are also used, such as tetraphenyl phosphonium ion (TPP^+), which can be analyzed with a specific electrode **(36)**.

General Procedures

The procedures are fairly straightforward and can be applied to both cultured cells and isolated mitochondria, although only the former is discussed here **(37, 38)**. The probes can be directly added into the cell culture at their optimal concentration [**10** μg/ml JC-**1**, 0.**5** μM TMRM, or **20** nM $DiOC_6(3)$] and incubated for 15 minutes at 37 °C in the dark. Cells are then harvested and subjected to flow cytometry analysis. Alternatively, cells can be observed directly under a fluorescent microscope. For probes such as TMRM, the fluorescence intensity is directly coupled with the potentials: The higher the potentials, the greater the fluorescence intensity. On the other hand, JC-1 shifts reversibly from a monomeric form to an aggregated form upon membrane polarization. The emission wavelength also changes from 530 nm to 590 nm, accordingly, when excited at 488 nm. Thus, cells with a lower $\Delta\Psi_m$ would be detected in the green channel, and cells with a higher $\Delta\Psi_m$ will be detected in the orange/red channel. The quotient between green and red fluorescence provides an estimate of the mitochondrial potentials of the population, which are independent of the mitochondrial mass. It is still possible to measure the percentage of positive cells in each channel and the mean fluorescence intensity **(39)**.

It would be ideal to set up control cells that are perfectly healthy, with polarized mitochondria and control cells with depolarized mitochondria. The latter could be obtained by adding into the culture either the K^+ ionophore, valinomycin (100 nM or more), or a mitochondrial uncoupler, carbonyl cyanide

p-(trifluoromethoxy) phenylhydrazone (FCCP, 250 nM) or carnonyl cyanide m-chlorophenyl hydrazone (CCCP, 10 μM). The chemicals can be added 15 minutes before the addition of the potential probes and will completely dissipate the potentials, thus providing a good negative control for the assay.

Comments

There are many discussions about the use of different probes for the measurement of $\Delta\Psi_m$, concerning the specificity, the sensitivity, and the potential interference with mitochondrial function **(37**, **38**, **40)**. Rho123 is a classical probe. However, for cells containing mitochondria with different maturation states, as in a continuously growing cell line, Rh123 binding to the mitochondria may not be consistent with the potentials, but may be affected by the available binding sites **(38)**. It may also not be sensitive enough to a smaller change in potentials **(41)**. $DiOC_6(3)$ is another commonly used probe. However, its incorporation can be affected by the plasma membrane potentials. It may also be redistributed into the endoplasmic reticulum in cells with depolarized mitochondria. Thus, cells may maintain the fluorescence intensity even when mitochondria are depolarized **(41)**. Adjusting the concentration of $DiOC_6$ (3) to a low concentration (20 nM) may lessen some of the concerns **(37)**. Chloromethyltetramethyl-rosamine (CMTMRos) or MitoTracker, which had also been used for detecting membrane potentials, is now found to bind to mitochondria irrespective of the magnitude of the potentials **(42)**. Furthermore, it can induce permeability transition once entering into the mitochondria **(42)**. Thus, it is no longer recommended for measurement of $\Delta\Psi_m$. Instead, both TMRM and JC-1 are considered to be much more suitable for this purpose, for they are specific to the mitochondria, are sensitive to the potential changes, and do not interfere with the mitochondrial functions.

Changes in the Nucleus

Introduction

During apoptosis, the cell nucleus undergoes dramatic morphological changes. These include chromatin condensation, peripheral margination, nuclear shrinkage, and subsequent fragmentation **(6)**. The changes are apparently driven by biochemical processes that become active during apoptosis. Key proteins responsible for maintaining nuclear structure and chromatin integrity are proteolysed during apoptosis, releasing specific fragments. In addition, DNA breaks, including internucleosomal cleavage, are generated and have been recognized as a biochemical hallmark for apoptosis **(43)**.

Nuclear Condensation and Fragmentation

Nuclear condensation and fragmentation in apoptotic cells can be visualized by light- and electron microscopy (as discussed earlier). An alternative approach is to stain the nucleus and its fragments with fluorescent dyes that bind DNA. Two commonly used dyes are Hoechst (bisbenzimide) and DAPI (4′, 6′-diamidino-2-phenyindole, dilactate). Upon staining, the apoptotic nucleus exhibits much stronger fluorescence than the nucleus of normal control cells. Intense staining of the apoptotic nucleus may result from the increased permeability of the dyes and higher binding to DNA due to an altered chromatin configuration.

General Procedures

For adherent cells, cells are rinsed with PBS and incubated with PBS containing ~1 μg/ml of Hoechst or DAPI for a few minutes. The cells are subsequently rinsed with PBS to remove extracellular dye prior to examination by fluorescence microscopy. For cell suspension, cells are collected by centrifugation and rinsed with PBS. The cells are subsequently resuspended into PBS containing Hoechst or DAPI. After two minutes of incubation, extracellular dye is removed by centrifugation and resuspension of the cells for fluorescence microscopy.

Comments

The nuclear staining methods are easy to perform and do not take much time. They can confirm apoptotic nuclear morphology and can also be used for semiquantitative purposes. Under certain conditions, the nuclei of necrotic cells also display intense Hoechst or DAPI staining. This is due to increased permeability of the dyes to the necrotic cells with broken plasma membranes. Therefore, the integrity of plasma membranes should be examined simultaneously, for example, by PI staining.

DNA Content Staining by Propidium Iodide

An impressive degenerative process during apoptosis is the degradation of nuclear DNA, resulting in decreases in DNA content in the cell or hypoploid. This provides the basis for sorting and quantification of apoptosis by measuring cellular DNA content with flow cytometry.

General Procedures

A good protocol could be found in **(44)**. Briefly, cells are collected and resuspended in ice-cold PBS. Cells are then fixed by the dropwise addition of two volumes of cold methanol during gentle vortexing. Stain the cells with PBS

containing 10–20 μg/ml of propidium iodide (PI), 0.1% Triton X-100, 2 mM EDTA, and 2 U/ml of DNase-free RNase for 30 minutes. Cells are then analyzed by flow cytometry.

Comments

PI staining in this method is conducted in the presence of Triton X-100, which permeabilizes the plasma membrane. Triton X-100 incubation ensures the same PI exposure to DNA in different cells, regardless of the original plasma membrane integrity. This is different from PI staining detection of the plasma membrane integrity, where no detergent is included (discussed earlier in this chapter).

Not all hypoploid populations represent apoptotic cells. Cellular fragments and debris can be counted in the apoptotic population, resulting in overestimation. To avoid this problem, it is necessary to utilize rigid gating and threshold setting in FACS to exclude cellular fragments or debris. In addition, necrosis could also result in nonspecific DNA degradation, which can also be recognized by PI staining as hypoploid cells.

DNA Fragmentation

Internucleosomal DNA Cleavage

One of the first biochemical hallmarks identified for apoptosis was the cleavage of DNA at internucleosomal sites. This specific cleavage leads to the formation of nucleosomal fragments of 180–200 bp in length, which, upon electrophoresis, resolve into a "DNA ladder" **(43)**. Specific endonucleases, including DNA fragmentation factor (DFF) or caspase-activated DNase (CAD), are activated during apoptosis and are mainly responsible for this type of DNA breakdown **(45)**. Although a late event in apoptosis, internucleusomal DNA cleavage has frequently been used as an indicator for apoptosis.

General Procedures

Cells are lysed with a hypotonic buffer containing 0.5% Triton X-100, 20 mM EDTA, and 10 mM Tris-HCl (pH 7.4). The lysates are centrifuged at 14,000 × *g* for 20 minutes. The supernatant is collected and subjected to proteinase K and RNase digestion, followed by phenol-chloroform extraction. DNA fragments in the aqueous phase are precipitated with ethanol and subjected to 1.5% agarose gel electrophoresis. After electrophoresis, DNA in the gel is stained with ethidium bromide and visualized under UV transmission **(46)**.

Comments

Internucleosomal DNA cleavage is not always associated with apoptosis. A similar pattern of DNA breakdown has been shown in cells with necrotic morphology, although necrosis usually leads to nonspecific DNA degradation, which more often exhibits as a smear in gel **(46, 47)**.

DNA degradation appears to be a late event in apoptosis. In certain apoptotic models, typical apoptosis develops in the absence of internucleosomal DNA cleavage **(48)**.

DNA fragmentation is a rather qualitative than quantitative measurement of apoptosis. The amount of fragmented DNA is not proportional to the frequency of apoptosis. For example, the same apoptotic cells release more DNA fragments as apoptosis develops into later stages **(49)**.

Detection of DNA Breaks by Histochemical Methods (TUNEL)

Based on DNA breakdown in apoptotic cells, two histochemical techniques for apoptosis detection have been developed. The methods are commonly used to examine apoptosis *in vivo* or in tissue sections. The first method, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling, or TUNEL, utilizes the enzyme TdT to incorporate biotinylated dUTP onto the 3′ ends of fragmented DNA. The second method, *in situ* end labeling, or ISEL, utilizes the enzyme DNA polymerase I or its Klenow fragment to fill in recessed 3′ ends of DNA fragments with biotinylated dUTP. For both methods, incorporated biotinylated dUTP can be revealed by fluorescence microscopy after reaction with fluorescein isothiocyanate-conjugated avidin. Alternatively, it can be detected with avidin-conjugated horseradish peroxidase and the HRP substrate 3,3′-diaminobenzidine tetrahydrochloride (DAB).

General Procedures

Tissue sections or cells are first fixed with 10% formalin or 4% paraformaldehyde in PBS, followed by a partial digestion with proteinase K. This step is to reduce fixation-induced protein-DNA cross-linkage and increase the accessibility of DNA to TdT and dUTP-biotin. The endogenous peroxidase is inactivated by incubation of the tissues in 3% hydrogen peroxide. Tissue sections are then incubated with a mixture of the TdT enzyme and dUTP-biotin for 1 hour at 37 °C to label the 3′ ends of the broken DNA. After the wash, fluorescein isothiocyanate-conjugated avidin can be directly applied for the diction. Alternatively, avidin-HRP conjugate can be added, followed by the HRP substrate, DAB.

Comments

The general procedure for ISEL is similar to that of TUNEL assay; instead of TdT, DNA polymerase I or Klenow fragment is used to incorporate biotinylated dUTP. The methods detect DNA breakdown but may not always distinguish the mode of

cell death. Positive staining has been shown for cells with either apoptotic or necrotic DNA damage. In some cases, it may be able to discriminate the necrosis pattern from the apoptosis pattern. In the former, the staining is much more diffusive than in the latter, which often exhibits a nuclear pattern of staining **(50)**.

For a technical control, parallel tissue sections should be processed without enzyme exposure. For example, in TUNEL assay, a control staining of tissue omitting TdT incubation should be included. As a note, tissue sections can be co-stained with a cell-specific marker or antibody to determine the type of cells undergoing apoptosis **(33)**.

Resources for Apoptosis Research

Some of the commonly used reagents for apoptosis detection, including antibodies, assay kits, and fluorescent dyes, are summarized in Table 31.1. The available mouse genetic models have been summarized in Table 15.1. A good

Table 31.1 Commonly used reagents for apoptosis detection

Viability assay	Vital dyes: trypan blue, propidium iodide, ethidium homodimer, Calcein-AM, 7-AAD (7-amino-actinomycin D)
	Cytosolic leakage: LDH kit
	ADP/ATP ratio assay kit
Changes on the plasma membrane	Phospholipid redistribution: Annexin V apoptosis detection kit
	Death receptors and adaptor proteins: Fas ligand ELISA kit, antibodies against death receptors and adaptors such as TNF-α, TNFR, Fas, FasL, TRAIL, FADD, TRADD, RIP, TRAF1-6
Mitochondrial changes	Intermembrane apoptotic protein release: cytochrome c translocation kit, antibodies for cytochrome c, Smac, AIF, endonuclease G
	Mitochondrial transmembrane potentials: Rhodamin-123 (Rh123), 3,3′-dihexiloxocarbocyanine iodide (DiOC6), tetramethyrhodmine methyl ester (TMEM), 5,5′,6,6′-tetraethylbenzimidazo-carbocyanine iodide (JC-1)
	Mitochondria redox change: Glutathione apoptosis detection kit, MgSOD ELISA kit
Changes in the nucleus	Nuclear condensation and fragmentation: Hoechst dyes, DAPI
	DNA fragmentation: DNA ladder detection kit, TUNEL kit
	DNA content staining kit
Changes in the cytosol	Caspase activation: antibodies for pro-/cleaved caspase (from caspase-1 to caspase-14)
	Endogenous caspase substrate cleavage: PARP assay kit, etc.
	Synthetic caspase substrate: Ac-DEVD-AFC, Ac-IETD-AFC, Ac-LEDH-AFC, Ac-WEHD-AFC, Ac-YVAD-AFC, Ac-VDVAD-AFC, etc.
	Caspase activity assay kits
	Bcl-2 family proteins: antibodies to detect the expression and activation of Bcl-2 family proteins such as Bid, Bax, Bak, Bad, Bim, Bik, Noxa, PUMA, Bmf, Bnip3L, phospho-Bad, phosphor-Bik, phosphor-Bim, Bcl-2, Bcl-xL, Mcl-1, etc.

database for apoptosis-related molecules can be found at http://www.apoptosis-db.org, and for caspase substrates, http://www.casbah.ie.

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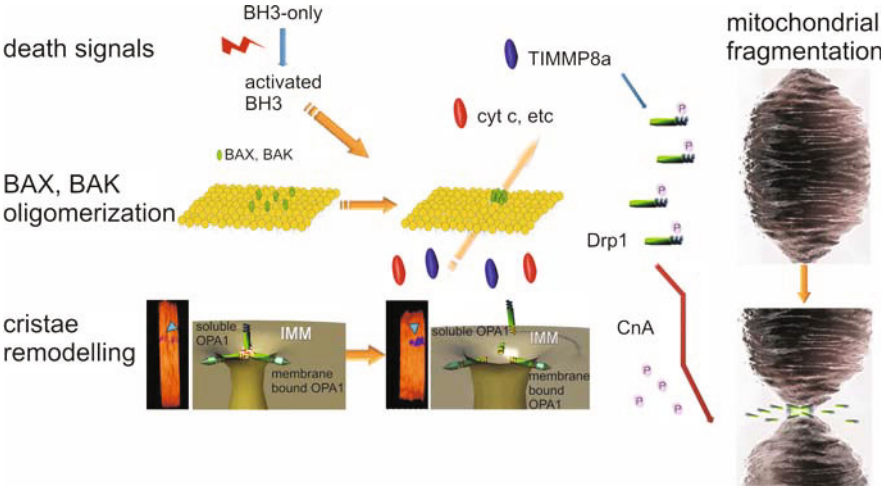
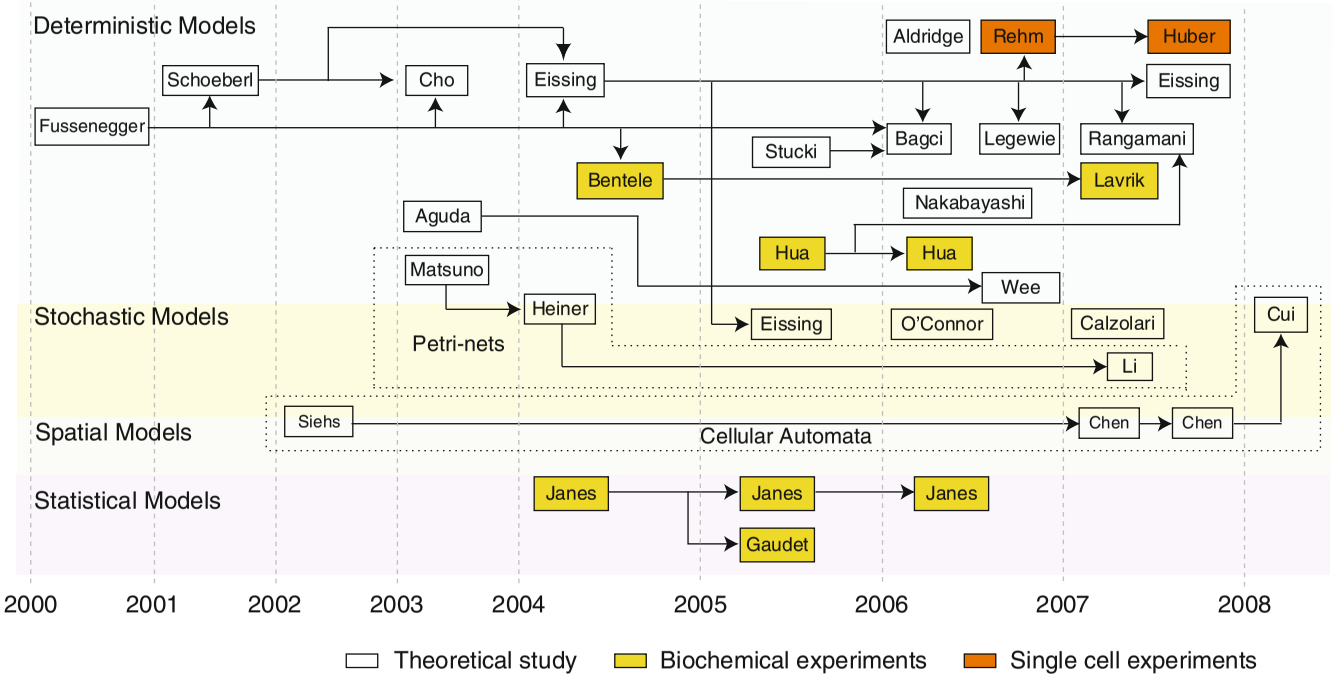
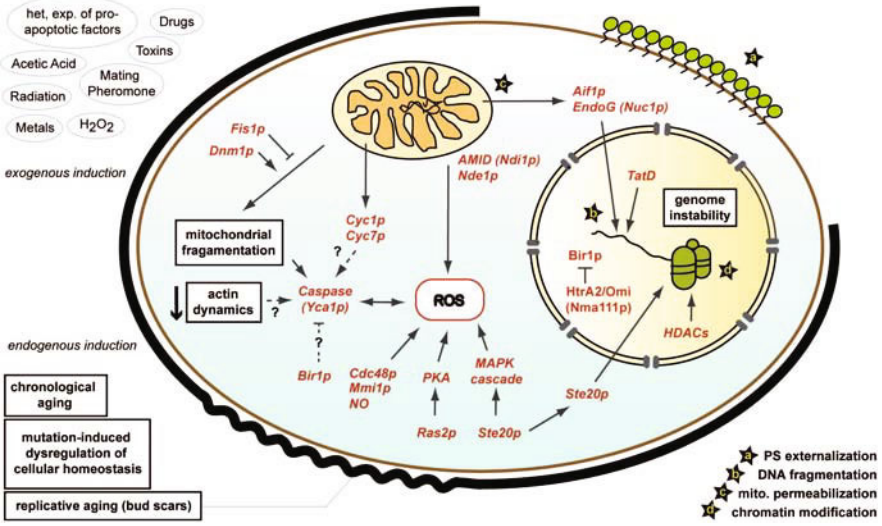
Color Plates



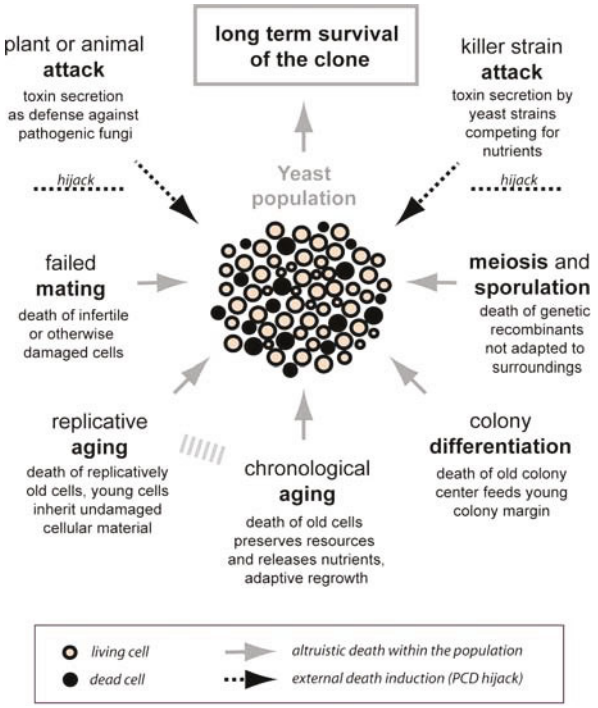
Color Plate 1. Schematic representation of the amplificatory loop at the mitochondrial level in response to an apoptotic stimulus. Three main interconnected mitochondrial steps are represented: (1) oligomerization of Bax and Bak, which generates a physical pathway for the efflux of proteins across the outer mitochondrial membrane; (2) Opa1-controlled remodeling of the cristae, leading to the redistribution of cytochrome c in the intermembrane space; (3) activation of mechanisms that cause mitochondrial fragmentation, following calcineurin (CnA)-dependent dephosphorylation of Drp1, or interaction of the latter with TIMMP8a, a component of the import machinery of mitochondria that is released together with cytochrome c. (Chapter 6, Fig. 1; *see* discussion on p. 167)



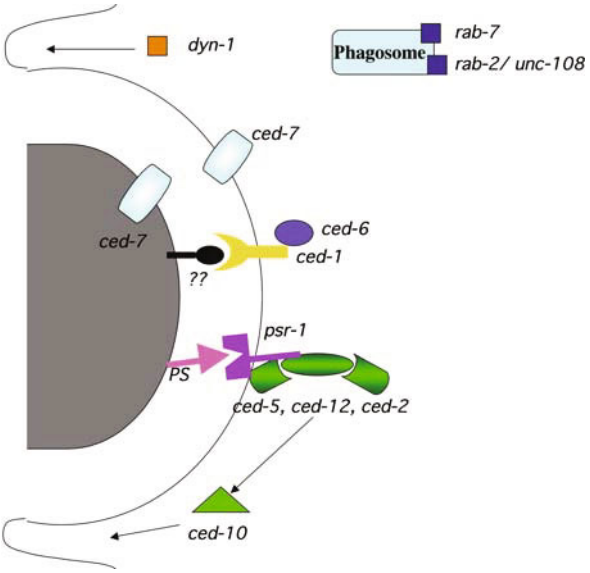
Color Plate 2. History of apoptotic systems modeling. Published systems biology studies were classified into four methodological categories and ordered chronologically. *Arrows* indicate an influence or logical connection between different studies with respect to the adoption of biological or methodological information (Chapter 12, Fig. 2; *see* discussion on p. 287)



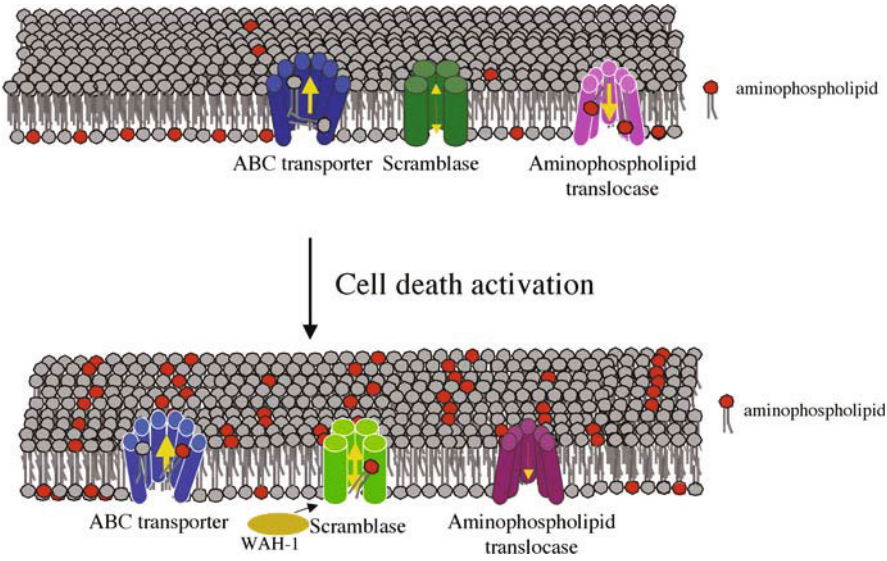
Color Plate 3. The molecular machinery of yeast apoptosis. Exogenous and endogenous induction of yeast apoptosis leads to the activation of the basic molecular machinery of cell death, which is configured by conserved apoptotic key players such as the yeast caspase Yca1p, the yeast homologue of mammalian HtrA2/OMI (Nma111p), or the apoptosis-inducing factor Aif1p. Furthermore, it involves complex processes like histone modification, mitochondrial fragmentation, cytochrome c release, and cytoskeletal perturbations (Chapter 14, Fig. 1; *see* discussion on p. 334)



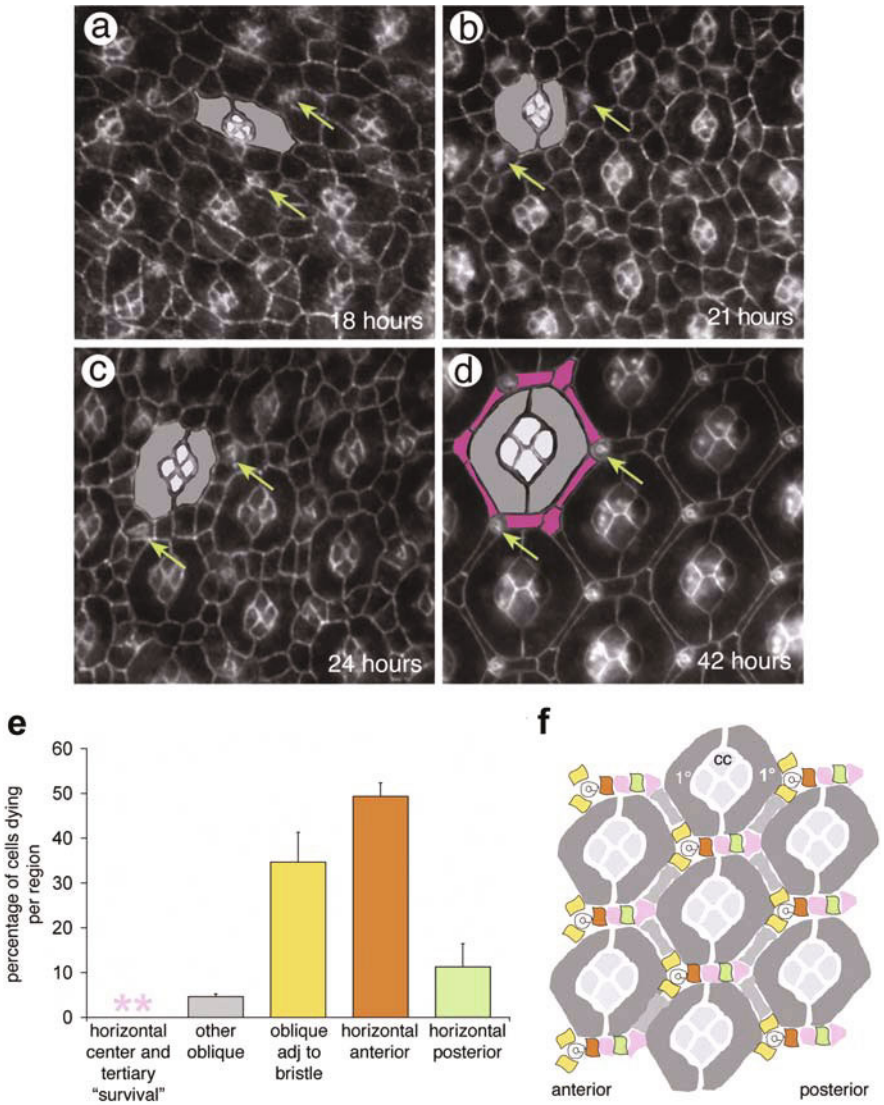
Color Plate 4. Physiological scenarios and yeast apoptosis. A wild-type yeast population promotes its own long-term survival and spreading of the clone by eliminating unfertile, damaged, or genetically unadapted individuals. Death in the population may also be triggered by toxins from nonclonal enemy strains or higher eukaryotes that hijack the PCD machinery of yeast (Chapter 14, Fig. 2; *see* discussion on p. 339)



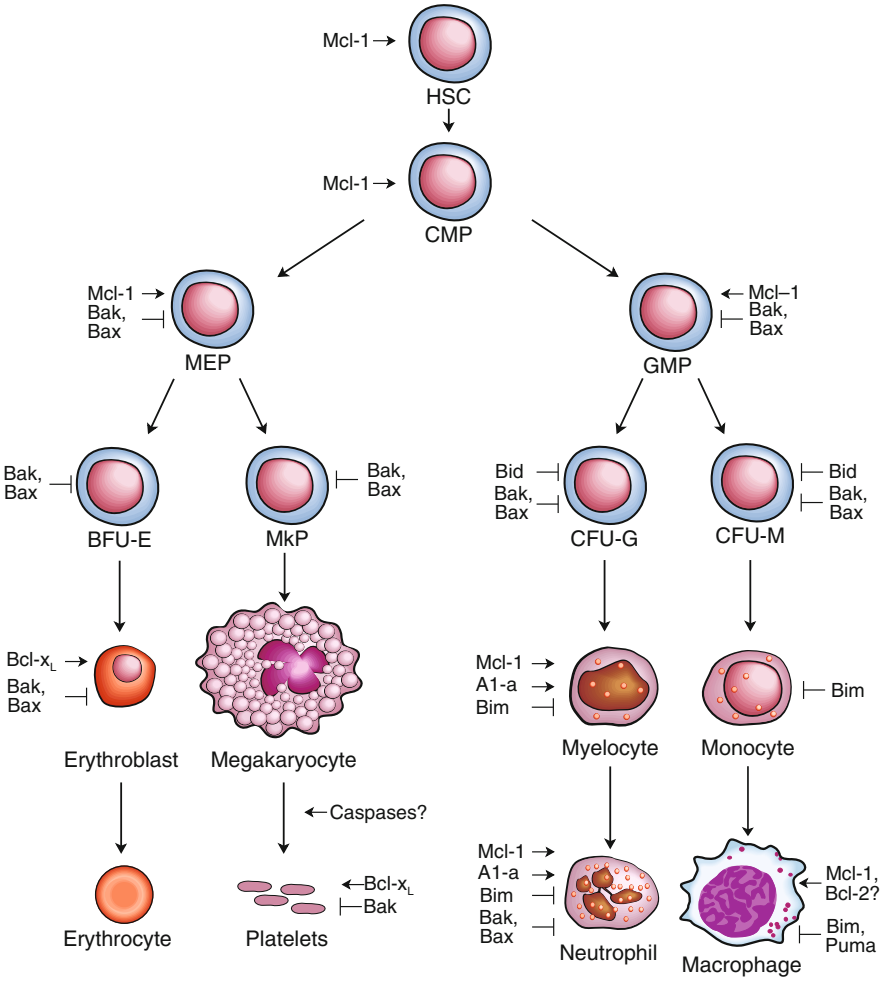
Color Plate 5. The molecular model for the cell corpse engulfment process. Two partially redundant pathways mediate the engulfment process. CED-1 and CED-7 act on the surface of engulfing cells to mediate cell corpse recognition and to transduce the engulfing signal through CED-6 to the cellular machinery of the engulfing cells for engulfment. CED-7 also acts in dying cells. DYN-1 acts in the CED-1 pathway to promote the delivery of intracellular vesicles to the phagocytic cups and the maturation of phagosomes. RAB-2 and RAB-7 mediate lysosome fusion with phagosomes and are important for the degradation of internalized apoptotic cells. The CED-2/CED-5/CED-12 ternary complex mediates the signaling events from externalized phosphatidylserine (PS)/PSR-1 and other unidentified engulfing signal(s) and receptor(s) to activate CED-10 during phagocytosis (Chapter 15, Fig. 5; *see* discussion on p. 364)



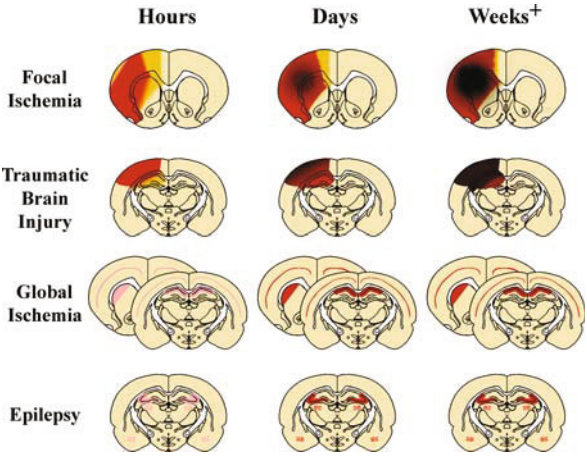
Color Plate 6. Molecular model of PS externalization during apoptosis. Phospholipid asymmetry is maintained through the action of three classes of proteins: scramblases, ABC transporters, and aminophospholipid translocases. In a living cell, scramblases are not activated and show little to no activity, ABC transporters are only used to maintain lipid balance between the two bilayers, and the aminophospholipid translocases transport any externalized PS and PE to the inner leaflet. During apoptosis, scramblases are activated, for example, by WAH-1 released from mitochondria, randomly scrambling phospholipids on the membrane, ABC transporters may be activated to transport specific lipids to the outer leaflet, and the aminophospholipid translocase is inactivated, leading to PS externalization on the outer leaflet (Chapter 15, Fig. 6; *see* discussion on p. 366)



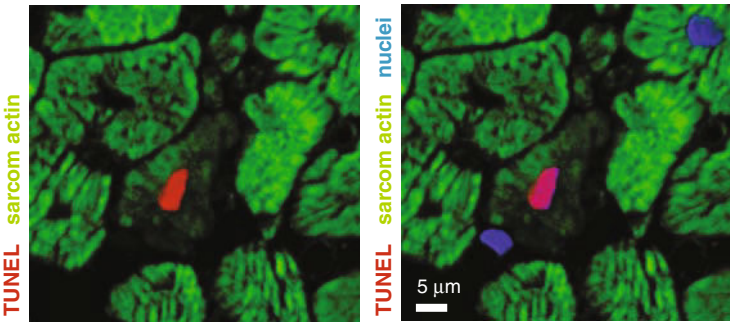
Color Plate 7. Live imaging demonstrates that cell death in the pupal eye is temporally and spatially regulated. (**A**–**D**) Pupal retinae of developmental ages as shown with cell boundaries outlined in *white*. For clarity, an ommatidium is shaded (primary pigment cells are *dark gray* and cone cells are *light gray*) in each panel and bristle groups are indicated by *green arrows*. IOCs account for the remainder of cells. In (D), the remaining IOCs after death are *colored pink*. (**E**) The percentage of cells observed dying graphed relative to specific regions. The shading correlates to the regions in (**F**). Two pink asterisks indicate that no cells were observed to die in these positions. (F) Schematic of the pupal retina, with each *shaded region* corresponding to a position in which cells will either be more likely to live (*pink and green*) or die (*orange and yellow*). Figure adapted from **(**139**)**. (Chapter 16, Fig. 4; *see* discussion on p. 389)



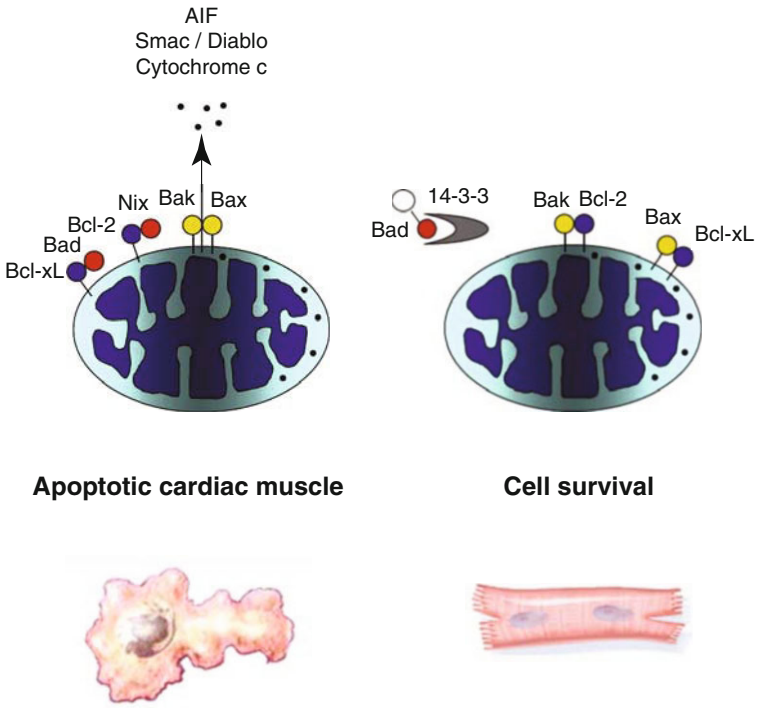
Color Plate 8. Bcl-2 family proteins regulate multiple aspects of hematopoiesis. Schematic representation of the non-lymphoid hematopoietic hierarchy. Proposed pro-apoptotic (⊥) and antiapoptotic (↑) functions for Bcl-2 family members are indicated. Hematopoietic stem cell (HSC), common myeloid progenitor (CMP), megakaryocyte/erythroid progenitor (MEP), granulocyte/macrophage progenitor (GMP), burst-forming unit-erythroid (BFU-E), megakaryocyte progenitor (MkP), colony-forming unit granulocyte (CFU-G), colony-forming unit macrophage (CFU-M) (Chapter 19, Fig. 1; *see* discussion on p. 443)



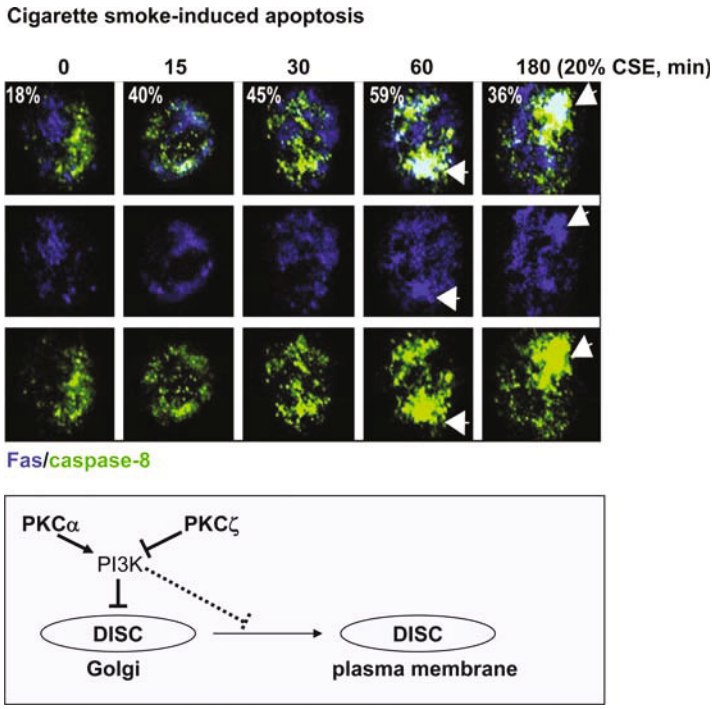
Color Plate 9. Progression of cell death in multiple models of acute neuronal injury. The progression of cell death varies across cell death models. Focal ischemia and traumatic brain injury display acute injury within hours of the insult, marked by the presence of necrotic morphology within the core of the injury (*black regions*), and progressing rapidly to inflammation (*dark red regions*). Delayed cell death, with morphological features of apoptosis or mixed morphologies, is found in regions proximal to the core of the injury and occurs over days to weeks (*red regions*). Global ischemia and kainic acid-induced epilepsy affect similar overall regions in both hemispheres of the brain, but in differing subregions. Cell death is more delayed compared to focal ischemia or traumatic brain injury and presents hallmarks of programmed cell death, with limited necrotic phenotypes. The figure represents moderate injury models; the range and severity of cell death are highly dependent on the degree of toxicity and the species or method used to induce injury. (Chapter 20, Fig. 1; *see* discussion on p. 462)



Color Plate 10. Imaging cardiac muscle apoptosis in vivo. Confocal image of TUNEL-positive cardiomyocyte labeling in the mouse heart after aortic banding, with TUNEL-positive nucleus (*red*), sarcomeric actin (*green*), and nuclei counterstained with DAPI (*blue*) (Chapter 22, Fig. 1; *see* discussion on p. 506)

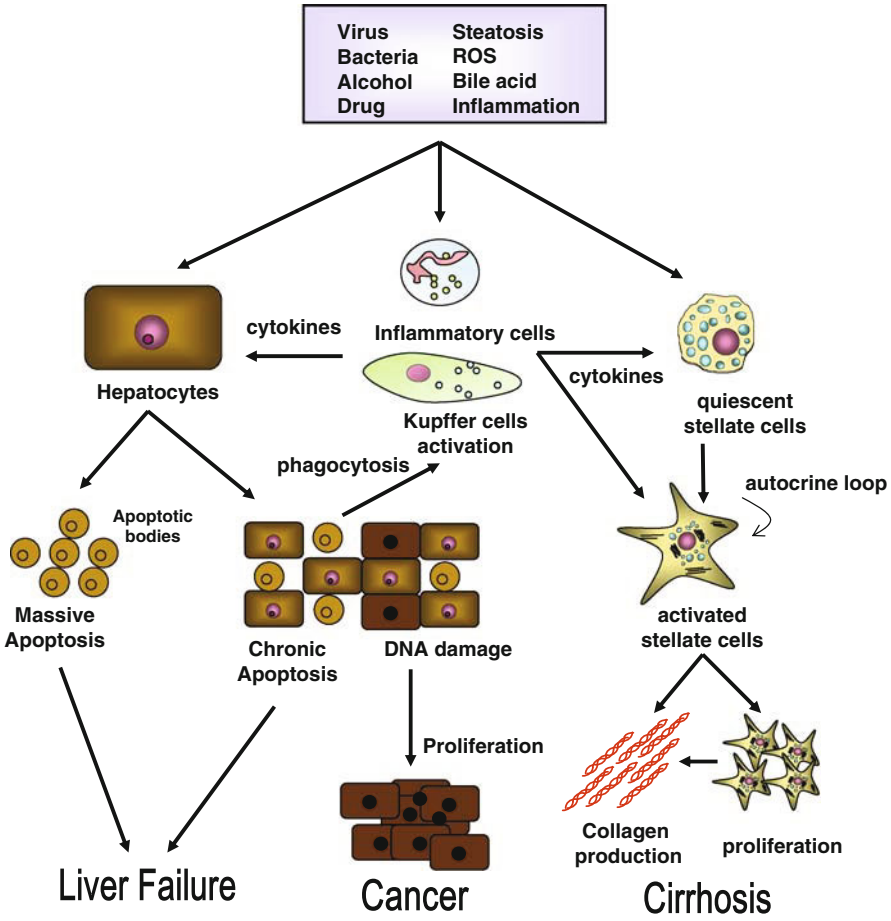


Color Plate 11. Function of Bcl-2 family proteins. Named after the founding member of the family, which was isolated as a gene involved in B-cell lymphoma (hence the name *bcl*), the Bcl-2 family is comprised of well over a dozen proteins, which have been classified into three functional groups. Members of the first group, such as Bcl-2 and Bcl-xL, are characterized by four short, conserved Bcl-2 homology (BH) domains (BH1–BH4). They also possess a C-terminal hydrophobic tail, which localizes the proteins to the outer surface of mitochondria, with the bulk of the protein facing the cytosol. The key feature of group I members is that they all possess antiapoptotic activity and protect cells from death. In contrast, group II consists of Bcl-2 family members with proapoptotic activity. Members of this group, which includes Bax and Bak, have a similar overall structure to group I proteins, containing the hydrophobic tail and all but the most N-terminal, BH4 domain. Group III consists of a large and diverse collection of proteins whose only common feature is the presence of the ~12- to 16-amino-acid BH3 domain. The Bcl-2 family of proteins function primarily to protect or disrupt the integrity of the mitochondrial membrane and control the mitochondrial release of proapoptotic proteins like cytochrome c, AIF, and Smac/DIABLO. Antiapoptotic Bcl-2 members (Bcl-2, Bcl-xL) protect the mitochondrial membrane. In response to environmental cues, these antiapoptotic proteins engage another set of proapoptotic proteins of the Bax subfamily (which includes Bax, Bak), normally loosely residing on the mitochondrial outer membranes or the cytosol. The interaction between Bak and Bax proteins results in oligomerization and insertion into the mitochondrial membrane of the complete complex (Chapter 22, Fig. 2; *see* discussion on p. 508)

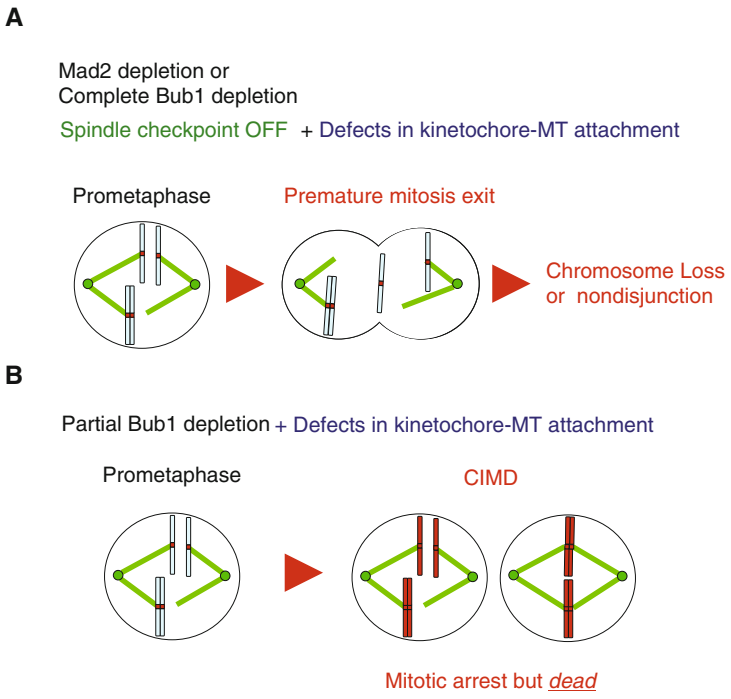


Color Plate 12. Activation of DISC by cigarette smoke. MRC-5 cells at 70% confluence were exposed to 20% CSE in serum-free media. Immunofluorescence images of MRC-5 double-labeled with indicated antibodies (anti-FAS in *blue* and anticaspase-8 in *green*) are shown. The cyan pseudocolor (*arrow, top panel*) indicates a co-localization of Fas and caspase-8. The same images with either the green or blue color removed are shown for clarity (*middle and bottom panels*). Data in this figure are representative of 20–49 cells analyzed for each time point. All panels are the same scale **(**97**)**. [Figure reproduced from Park et al. **(**97**)** with permission from the American Association of Immunologists.]

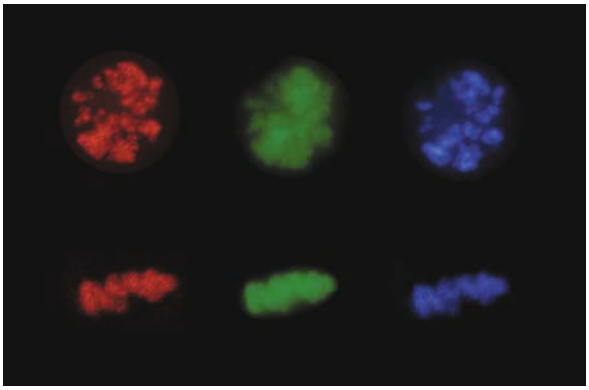
The cartoon illustrates the potential role of protein kinase-c (PKC) isoforms in regulating DISC formation. PKCα displayed an antiapoptotic effect in CSE-treated cells and in chronic cigarette smoke-exposed mice, whereas PKCζ displayed a proapoptotic effect. PKCα potentially inhbits DISC trafficking by activating the PI3K pathway in fibroblasts. PKCζ promoted DISC trafficking by inhibiting the PI3K pathway **(**97**)** (Chapter 23, Fig. 3; *see* discussion on p. 536)



Color Plate 13. Schematic representation of liver diseases. Hepatocyte apoptosis is initiated by various stimuli via direct effects and/or inflammatory responses. Massive hepatocyte apoptosis with impairment of hepatocyte regeneration results in acute liver failure. Chronic hepatocyte apoptosis leads to liver cirrhosis and liver cancer. Kupffer cells engulf apoptotic bodies of hepatocytes and release fibrogenic cytokines, which trigger collagen production by hepatic stellate cells. Chronic hepatocyte apoptosis also stimulates hepatocyte regeneration, and dysregulation of the balance between hepatocyte proliferation and cell death causes hepatocarcinogenesis (Chapter 24, Fig. 1; *see* discussion on p. 547)



Color Plate 14. (**a**) A model that describes how chromosome loss or nondisjunction occurs in spindle checkpoint-defective cells (MAD2-depleted cells or complete BUB1-depleted cells). In spindle checkpoint-mutant cells, the spindle checkpoint is not activated even if there are defects in kinetochore–microtubule attachment. No mitotic delay occurs, which results in the premature exit from mitosis. Thus, there is substantial chromosome loss or nondisjunction, and presumably cell death follows. (**b**) A model that describes the same scenario in partial BUB1-depleted cells. Here, defects in kinetochore–microtubule attachment induce lethal DNA fragmentation (CIMD). Because cells are still arrested in mitosis, the mitotic index remains unchanged. Therefore, the spindle checkpoint appears to be active. (Chapter 28, Fig. 2; *see* discussion on p. 637)



Color Plate 15. CIMD occurs in BUB1-depleted cells in the presence of microtubule inhibitors or 17-AAG. HeLa cells that are BUB1-depleted and 17-AAG-treated exhibit DNA fragmentation (TUNEL-positive; *red*) during mitosis (*top row*, prometaphase; *bottom*, metaphase). Forty-eight hours after HeLa cells were transfected with BUB1 siRNA, they were incubated with 17-AAG (+17AAG, 500 nM) for 24 hours at 37 °C. Fixed samples were stained by using an *in situ* cell death detection system that contained TMR red (TUNEL-signal; *red*), an antiphosphorylated histone H3 mouse monoclonal antibody, and FITC-conjugated secondary antibodies (*green*). DNA was stained with DAPI (*blue*) to visualize (Chapter 28, Fig. 3; *see* discussion on p. 638)