NEW INSIGHTS INTO THE REGULATION OF MITOCHONDRIAL OUTER MEMBRANE PERMEABILIZATION DURING APOPTOSIS

BY

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ABSTRACT

Disruption in normal apoptosis can contribute to the onset of different disease states, including cancer. Additionally, many clinically relevant cancer drugs are effective for their ability to initiate the apoptotic process. Most often this involves the activation the mitochondria-mediated pathway, but the development of specific agents that can activate the receptor-mediated pathway is of increasing interest. Within the existing paradigm of mitochondria-mediated apoptosis, which is activated by intracellular stress, such as DNA damage and cytoskeletal disruption, the pathway is thought to proceed in a linear fashion. Mitochondria outer membrane permeabilization (MOMP) and the release of intermembrane space proteins, such as cytochrome *c*, are early events during mitochondria-mediated apoptotic signaling. In addition, Apaf-1 is a critical component of the mitochondrial pathway and is generally thought to reside downstream of MOMP.

My dissertation investigates the molecular requirements essential for MOMP during stress-induced and receptor-mediated apoptosis. To do so, I used distinct clones of Jurkat T-lymphocytes in which the mitochondria-mediated pathway had been inhibited at three different steps. First, cells stably overexpressed Bcl-2/Bcl-x_L to inhibit the activation of Bak, a key regulator of MOMP. Second, cells were stably depleted of Apaf-1 to inhibit the activation of the initiator death protease caspase-9. Finally, cells were stably overexpressed with full-length X-linked inhibitor of apoptosis protein

(XIAP) to indirectly inhibit activated death proteases caspase-9, -7 and -3. Cells were stably overexpressed with BIR1/BIR2 domains of XIAP to indirectly inhibit activated effector death proteases caspase-3 and -7.

The first aim investigated the molecular requirements necessary for Bak activation. Apaf-1-deficient cells and cells overexpressing full-length XIAP or the BIR1/BIR2 domains of XIAP inhibited mitochondrial apoptotic events. The data obtained suggest that caspase-mediated positive amplification of initial mitochondrial changes can determine the threshold for irreversible activation of the intrinsic apoptotic pathway.

As mentioned previously, a second apoptotic pathway is called the receptor-mediated pathway. In some cell types (type II), the mitochondria-mediated pathway is also required for cell death upon stimulation of the receptor-mediated pathway. In the second aim, I used type II Jurkat clones in which the mitochondrial apoptotic pathway had been inhibited at the different steps to investigate the molecular requirements necessary for Fas-mediated apoptosis. Apaf-1-deficient type II Jurkat cells were sensitive to anti-Fas. Inhibiting downstream caspase activation by overexpression of the BIR1/BIR2 domains of XIAP decreased all anti-Fas-induced apoptotic changes. Combined, my findings strongly suggest that Fas-mediated activation of executioner caspases and induction of apoptosis does not depend on apoptosome-mediated caspase-9 activation in prototypical type II cells.

In the third aim, mitochondrial apoptotic events were examined after prolonged treatment with etoposide (> 6 h). Although Apaf-1-deficient cells were resistant to etoposide, total cellular cytochrome *c* and Smac were decreased after 24 h of incubation. The Apaf-1-deficient cells displayed subtle biochemical markers of autophagy. Interestingly, inhibition of the 26S proteasome by co-treatment of Apaf-1-deficient cells with bortezomib or MG132 led to the robust retention of total cellular cytochrome *c* and Smac. Combined, these data suggest that proteasomal degradation, and, to a lesser extent, autophagy, is responsible for the loss of intracellular cytochrome *c* and Smac in the Apaf-1-deficient cells incubated with etoposide over extended time periods.

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ΔΨ – Mitochondrial membrane potential

^{o}C – Celsius

µg – Microgram

µF – Microfarad

µl – Microliter

AIF – Apoptosis inducing factor

anti-Fas – Agonistic anti-Fas antibody

AIDS – Acquired immune deficiency syndrome

Apaf-1 – Apoptotic protease activating factor 1

Bad – Bcl-2 associated death promoter

Bak – Bcl-2 antagonist/killer 1

Bax – Bcl-2 associated X protein

Bcl-2 – B cell lymphoma 2

Bcl-x_L – Bcl-2 like 1

Bcl-w – Bcl-2 like 2

Bfl-1 – Bcl-2 related protein A1

BH – Bcl-2 homology domain

BH3 – Bcl-2 homology domain 3

BHIP3 – Bcl-2/adenovirus E1B 19 kDa interacting protein 3

Bid – BH3 interacting domain

Bik – Bcl-2 interacting killer

Bim – Bcl-2 interacting mediator of cell death

BIR – Baculovirus IAP repeat

Blk – B lymphoid tyrosine kinase

Bmf – Bcl-2 modifying factor

BMH – Bismaleimidohexane

Bok – Bcl-2 related ovarian killer

CARD – Caspase recruitment domain

CED – Cell death abnormalities

CPS-6 – CED-3 protease suppressor

CO_2 – Carbon dioxide

dATP – 2'-deoxyadenosine 5'-triphosphate

ddH_2O – Double distilled water

DED – Death effector domain

Diablo – Direct IAP binding protein with low pI

DISC – Death inducing signaling complex

DR – Death receptor

DTT – Dithiothreitol

EGL-1 – Egg laying defect

FADD – Fas associated death domain

FBS – Fetal bovine serum

h – Hour

Hrk – Harakiri, Bcl-2 interacting protein

Htr2a – High temperature requirement protein A2

IAP – Inhibitors of apoptosis

IgG – Immunoglobulin G

kDa – Kilodaltons

M – Molar

Mcl-1 – Myeloid cell leukemia-1

min – Minute

ml – Milliliter

MOMP – Mitochondrial outer membrane permeabilization

Puma – p53-upregulated modulator of apoptosis

PBS – Phosphate buffered saline

PBST – Phosphate buffered saline tween

p53 – Protein 53

PI – Propidium iodide

RAIDD – Rip associated ICH-1 homologous protein with death domain

shRNA – Short hairpin RNA

SLE – Systemic lupus erythematosus

Smac – Second mitochondrial activator caspases

SOC – Super optimal broth with catabolite repression

tBid – Truncated Bid

TNF-α – Tumor necrosis factor alpha

TNF-R – Tumor necrosis factor receptor

TRADD – TNF receptor associated death domain

TRAIL – TNF related apoptosis inducing ligand

U – Unit

V – Volume or Volts

W – Weight

WT – Wild-type

WAH-1 – Worm AIF homolog

XIAP – X-linked inhibitor of apoptosis

CHAPTER 1: SIGNIFICANCE AND BACKGROUND

1.1 Significance

Apoptotic cell death is important for normal embryonic development and for tissue homeostasis in the adult organism. Cells can initiate apoptosis for a multitude of reasons including because a cell has sustained irreversible DNA damage, chromosomal instability, oncogene activation, anoikis, kinase inhibition, microtubule perturbation (Letai, 2009; Letai et al., 2002), or because the body's immune system recognizes the cell as infected or abnormal. As a result, any disruption in normal apoptosis can contribute to the onset of different disease states. With diseases such as Parkinson's, AIDS, myocardial infarction, and Type I diabetes, too much cell death has occurred, whereas with diseases such as rheumatoid arthritis, polycythemia vera, and cancer, the body does not have enough cell death, see Figure 1 (Fadeel et al., 1999; Fesik, 2005; Riedl and Shi, 2004; Thompson, 1995).

Cancer is a prevalent chronic disease that touches many Americans in economic and social ways. Economic burdens range from the cost of cancer preventative measures, treatment, and screenings, to the loss of productivity of the cancer patients and their families while they battle the disease. According to the American Cancer Society, an estimated 1,479,350 new

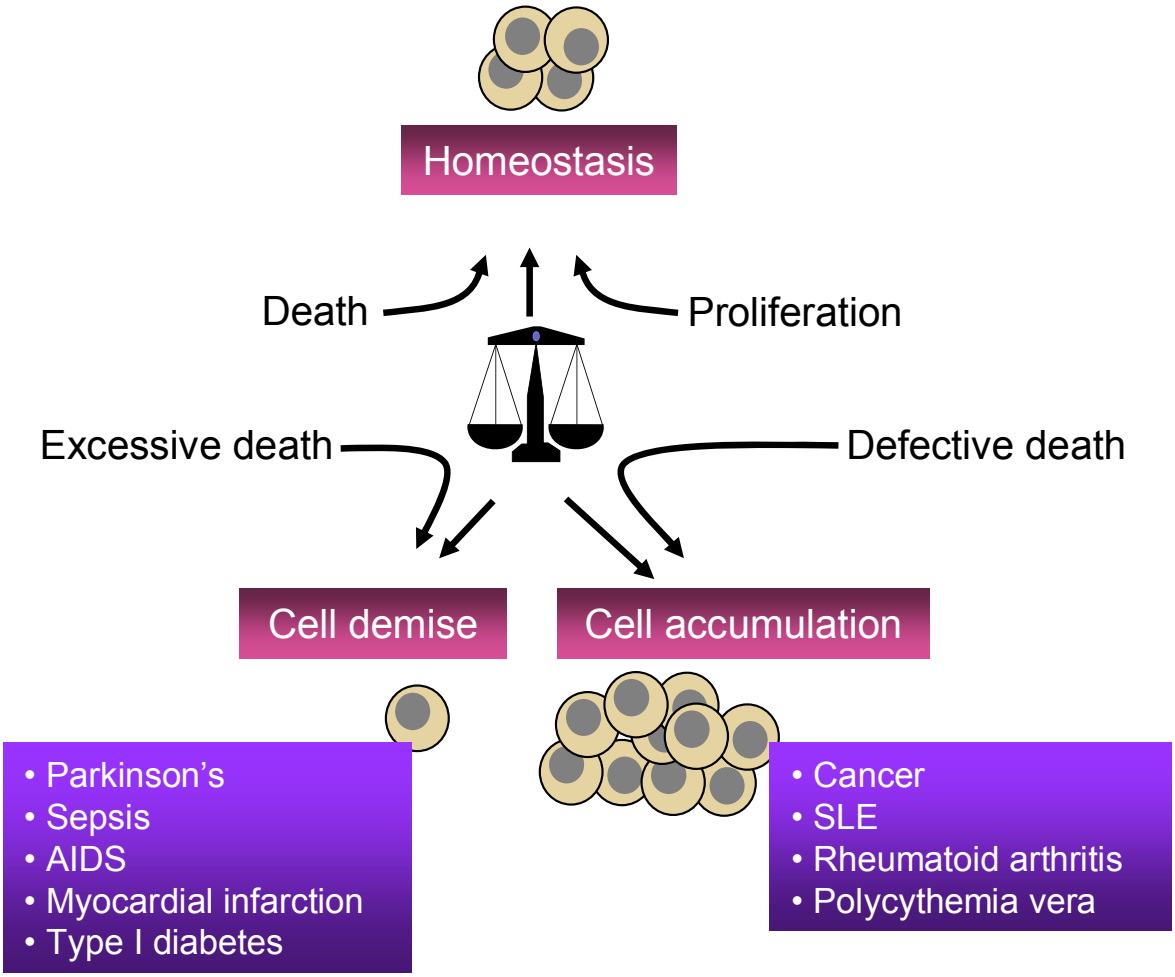

Figure 1. Cell Death in Human Disease.

This schematic illustrates the importance of a balance between the rate of cell death and cell proliferation. When there is too much cell death, it leads to cell demise and has been implicated in many diseases such as Type I diabetes. When there is too little cell death, it can lead to cell accumulation and this has been implicated in diseases such as cancer. *AIDS, acquired immune deficiency syndrome; SLE, systemic lupus erythematosus.* Adapted from (Robertson et al., 2002).

cases of cancer will be diagnosed in the year 2009 and an estimated 1,500 people will die every day of cancer in the United States.

Cancer arises from too much cellular proliferation and/or too little apoptosis. Apoptosis is involved in many aspects of cancer. One mechanism by which the body can evade cancer is for the immune system to recognize an aberrant or precancerous cell and trigger it to undergo apoptosis. During the transformation of a normal cell to a malignant cell, the transforming cell often encounters many pro-apoptotic changes. Such triggers that would normally promote apoptosis induction include chromosomal instability, oncogene activation, and cellular detachment. These triggers need to be overcome and apoptosis needs to be prevented for the cell to become malignant (Green and Evan, 2002; Hanahan and Weinberg, 2000). The malignant cell can overcome apoptosis either by decreasing the expression of pro-apoptotic proteins or increasing the expression of anti-apoptotic proteins (Letai, 2008). In fact, avoidance of apoptosis is one of the six recognized acquired capabilities of cancer cells (Hanahan and Weinberg, 2000). Additionally, many clinically important cancer drugs are effective for their ability to initiate the apoptotic process, most often by activating the mitochondrial apoptotic pathway. Importantly, disruptions in the control of the mitochondrial apoptotic pathway can contribute to the resistance of cancer cells to antineoplastic therapy (Green and Kroemer, 2005; Johnstone et al., 2002).

Currently, the majority of the literature suggests that mitochondria-mediated apoptosis is a linear pathway. Specifically, mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome *c* are widely thought to occur upstream and independently of caspases in an all-or-none manner (Chipuk et al., 2006). However, several lines of recent evidence, including data from our laboratory (Franklin and Robertson, 2007; Shawgo et al., 2008; Shelton et al., 2009), are in conflict with such a rigid view of the mitochondria-mediated apoptotic pathway. The results of my dissertation project have increased our knowledge of the molecular requirements necessary to irreversibly commit a cell to undergo apoptosis in response to genotoxic injury. Specifically, I show that signaling events generally thought to lie downstream of MOMP are, in fact, required to positively amplify cytochrome *c* release in response to genotoxic stress. This contribution is significant because it provides important new basic information about cellular life-death decisions that could be key for the development of more rational approaches to therapy. Such new information of molecular pathways governing apoptosis induced by genotoxic stimuli and the mechanisms cells may use to evade cell death is expected to facilitate a more targeted approach to drug design. Apart from my studies on genotoxic stress-induced apoptosis, I also provide new insights on the molecular requirements necessary for Fas-mediated apoptosis in so-called type II cells.

1.2 Background

Apoptosis was first described in 1972 (Kerr et al., 1972) and the field has had many advances in the past 10-15 years. Apoptosis is a highly conserved mechanism of cell death that occurs in most, if not all, tissue types of multicellular organisms (Figure 2) (Brunelle and Letai, 2009). As mentioned previously, apoptosis is important in various pathological states, as well as during normal cellular processes, such as development, cellular differentiation, and maintaining tissue homeostasis. During development, apoptosis is an important process that is needed to eliminate the excess tissue that is initially produced, for example, the skin between digits (Meier et al., 2000). In order to maintain tissue homeostasis, apoptosis can eliminate older cells when new cells have been produced. The lining of the digestive tract, hair cells, and hematopoietic cells are examples of cell types that have a fast turnover rate and where apoptosis is very important.

There are many regulators of apoptosis (Figure 3). They can be divided up into proteins that (i) prevent apoptosis (anti-apoptotic) and (ii) those that help to promote apoptosis (pro-apoptotic). The anti-apoptotic members can be further subdivided into two groups: (i) those proteins that help to keep the mitochondrial outer membrane intact; and (ii) those that prevent the activation of a family of death proteases known as caspases. Likewise, the pro-apoptotic members can be further subdivided into three

Figure 2. Conservation of Core Cell Death Genes Between *C. elegans* and Humans.

A lot of what we know about mammalian apoptosis is due to the research conducted by Robert Horwitz in the nematode *C. elegans*. He discovered three core death proteins promoting apoptosis: EGL-1, CED-4 and CED-3. The mammalian homologs of these proteins are BH3-only proteins, Apaf-1, and caspases, respectively. He also discovered the anti-apoptotic protein CED-9, whose mammalian homolog is Bcl-2. The main difference between apoptosis in *C. elegans* and humans is that while CED-9 can bind directly to CED-4 to prevent the activation of caspases, the CED-9 homolog Bcl-2 cannot directly bind to the CED-4 homolog Apaf-1. Rather, Bcl-2 prevents the release of pro-apoptotic proteins into cytosol, such as cytochrome *c*, which is required for activation of caspases by Apaf-1. *EGL-1, egg laying defect; CED, cell death abnormalities; WAH-1, worm AIF homolog; CPS-6, CED-3 protease suppressor; BH3, Bcl-2 homology domain 3; Bcl-2, B-cell lymphoma 2; Cyt. c, cytochrome c; Apaf-1, Apoptotic protease activating factor 1; AIF, Apoptosis inducing factor; EndoG, endonuclease G*.

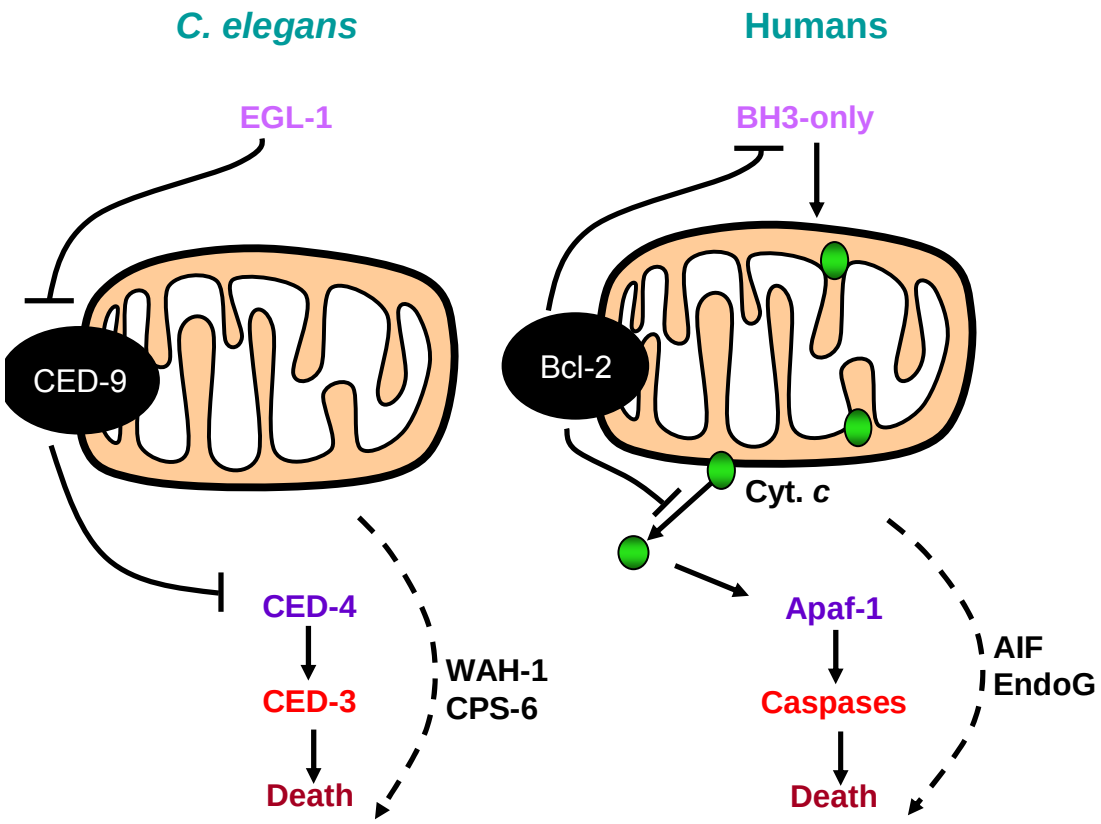
C. elegans
Humans
EGL-1
BH3-only
CED-9
Bcl-2
Cyt. c
CED-4
Apaf-1
WAH-1
CPS-6
AIF
EndoG
CED-3
Caspases
Death
Death

Figure 2.

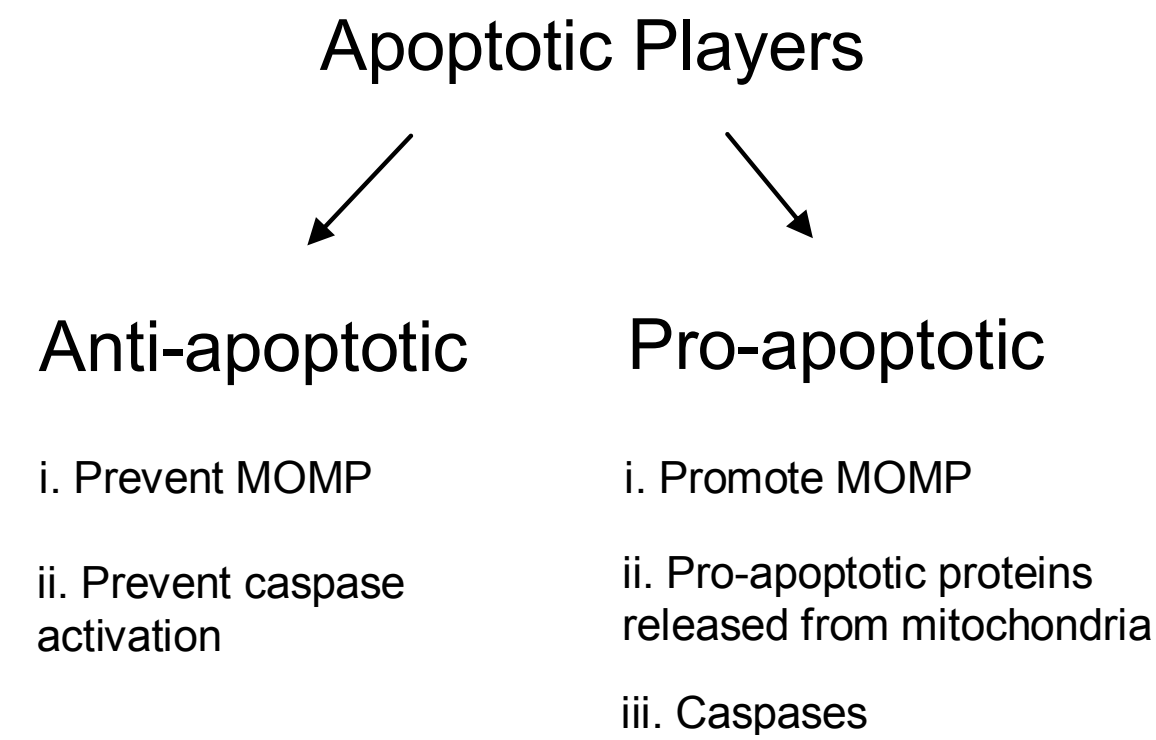

Figure 3. Characterization of Apoptotic Players.

Proteins involved in apoptosis can be divided up into proteins that prevent apoptosis and those that promote apoptosis. Anti-apoptotic proteins can be furthered divided into proteins that prevent mitochondrial outer membrane permeablization (MOMP) (e.g. Bcl-2, Bcl-x_L, and Mcl-1) and those proteins that prevent caspase activation (e.g. inhibitors of apoptosis). Pro-apoptotic proteins can be divided into three categories: (i) Proteins that help MOMP (e.g. Bid, Bim, Bax, and Bak), (ii) Pro-apoptotic proteins that are released from the mitochondria (e.g. cytochrome *c*, Smac, and Omi) and (iii) Caspases.

groups: (i) those that help to permeablize the mitochondrial outer membrane; (ii) pro-apoptotic factors that are released from the mitochondrial intermembrane space; and (iii) caspases. Each of these groups and subgroups will be discussed in greater detail for the remainder of this chapter.

1.2.1 B-Cell Lymphoma 2 (Bcl-2) Family Proteins

One of the regulators of mitochondrial outer membrane integrity is the Bcl-2 family of proteins (Danial and Korsmeyer, 2004). The Bcl-2 family is important in both preventing and promoting MOMP. The founding member of this family of proteins was Bcl-2, which stands for B-cell lymphoma 2. Bcl-2 was first described as an oncogene seen in follicular lymphoma with a (t14:18) chromosomal translocation (Tsujimoto et al., 1984). The translocation put the promoter region of the immunoglobulin heavy chain in front of *Bcl-2* gene to drive an increased expression of Bcl-2 protein. It was determined that increased expression of Bcl-2 did not increase proliferation. Subsequently, it was found to have the ability to prevent cell death (Vaux et al., 1988). The Bcl-2 family of proteins has now grown to contain about 20 members (Jin and El-Deiry, 2005). For a list of well characterized Bcl-2 proteins see Table 1. The Bcl-2 family members share one to four Bcl-2 homology (BH) domains. The BH3 domain, present in all Bcl-2 family members, is important for protein-protein interactions as well as the pro-

Table 1. Characterization of Bcl-2 Family Members.

Anti-Apoptotic	Pro-Apoptotic		
	BH3-Sensitizers (a.k.a. inhibitors/ derepressors)	BH3-Activators (a.k.a. inducers)	Multidomain (a.k.a. BH123)
Bcl-2	Bad	Bid	Bak
Bcl-x_L	Bik	Bim	Bax
Bcl-w	BHIP3	Puma?	Bok
Bfl-1	Bmf		
Mcl-1	Blk		
	Hrk		
	Noxa		
	Puma?		

BH3 – Bcl-2 homology domain 3
Bcl-2 – B-cell lymphoma 2
Bad – Bcl-2 associated death promoter
Bid – BH3 interacting domain
Bak – Bcl-2 antagonist/killer 1
Bcl-x_L – Bcl-2 like 1
Bik – Bcl-2 interacting killer
Bim – Bcl-2 interacting mediator of cell death
Bax – Bcl-2 associated X protein
Bcl-w – Bcl-2 like 2
BHIP3 – Bcl-2/adenovirus E1B 19kDa interacting protein 3
Puma – p53 upregulated modulator of apoptosis
Bok – Bcl-2 related ovarian killer
Bfl-1 – Bcl-2 related protein A1
Bmf – Bcl-2 modifying factor
Mcl-1 – Myeloid cell leukemia sequence 1
Blk – B lymphoid tyrosine kinase
Hrk – Harakiri, Bcl-2 interacting protein

apoptotic effects that some members possess (Cheng et al., 2001; Wei et al., 2001; Zong et al., 2001). The BH4 domain exhibits anti-apoptotic effects, and is only present in the anti-apoptotic proteins. The Bcl-2 family of proteins can be broken down into two separate categories: (i) those proteins that prevent apoptosis and are grouped as anti-apoptotic; and (ii) those proteins that promote cell death and are categorized as pro-apoptotic (Chittenden et al., 1995; Danial and Korsmeyer, 2004).

1.2.2 Bcl-2 Anti-Apoptotic Proteins

The anti-apoptotic Bcl-2 sub-family of proteins that prevents apoptosis includes Bcl-2, Bcl-2-like 1 (Bcl-x_L), Bcl-2-like 2 (Bcl-w), BF-1, A1 and Myeloid cell leukemia-1 (Mcl-1). Anti-apoptotic Bcl-2 family members are primarily located on the mitochondrial outer membrane. They help to preserve the integrity of the mitochondrial outer membrane by preventing permeabilization. One way in which this occurs is that the BH3 domain of an anti-apoptotic Bcl-2 family protein binds to the BH3 domain of the pro-apoptotic Bcl-2 family members to inhibit their pro-apoptotic activity. However, not all anti-apoptotic Bcl-2 family members will bind to all pro-apoptotic Bcl-2 family members. For example, Bcl-2 has preference for binding BH3-interacting-domain (Bid) over Bcl-2-interacting mediator of cell death (Bim), while Mcl-1 prefers to bind to Bcl-2 interacting killer (Bik) and Noxa over binding to Bcl-2-associated death

promoter (Bad) (Certo et al., 2006). One way cancer cells overcome apoptosis is by overexpressing one or more of the Bcl-2 anti-apoptotic family members (Sentman et al., 1991; Strasser et al., 1991).

1.2.3 Bcl-2 Pro-Apoptotic Proteins

The pro-apoptotic Bcl-2 family proteins work together to permeablize the outer mitochondrial membrane. MOMP allows for pro-apoptotic factors (cytochrome *c*, second mitochondrial activator caspases (Smac) and Omi) held within the intermembrane space of the mitochondria to be released into the cytosol (Cartron et al., 2004; Desagher et al., 1999; Kuwana et al., 2005; Kuwana et al., 2002; Luo et al., 1998; Marani et al., 2002) where they function to promote caspase activation. Pro-apoptotic Bcl-2 family members can be further separated into two groups depending on the number of BH domains they contain: (i) proteins with BH domains one through three characterized as mulitdomain; and (ii) proteins with only the third BH domain characterized as BH3-only proteins.

1.2.3.1 Bcl-2 Pro-Apoptotic Proteins – Multidomain

Proteins with BH domains one through three are called multidomain Bcl-2 family members (also known as BH123 proteins). The multidomain Bcl-

2 sub-family has three members: Bcl-2-associated X protein (Bax), Bcl-2-antagonist/killer 1(Bak) and Bcl-2-related ovarian killer (Bok). Bax and Bak are widely distributed throughout different tissue types, while Bok is found only in some reproductive tissues. I will focus on Bax and Bak. Under non-apoptotic conditions, Bax (cytosolic) and Bak (mitochondrial) exist as monomers. However, under apoptotic conditions Bax and Bak will be induced to change their conformation allowing them to homo-oligomerize to form a pore on the outer mitochondrial membrane. It is this pore that allows for the pro-apoptotic factors to be released into the cytosol (Cheng et al., 2001; Wei et al., 2001). It is thought that Bax stays in the cytosol due to its C-terminal membrane anchor that is normally hidden. Once Bax undergoes a conformational change, the C-terminal membrane anchor is exposed to allow for its translocation and homo-oligomerization (Suzuki et al., 2000) (Figure 4). One way that cancer cells overcome apoptosis is through decreasing the expression of Bax and Bak. Both multidomain Bcl-2 family members need to be eliminated before the malignant cell can inhibit apoptosis (Certo et al., 2006).

1.2.3.2 Pro-Apoptotic Proteins – BH3 Only

The other pro-apoptotic Bcl-2 proteins only contain the third BH domain and are typically referred to as BH3-only. Activation of Bax or Bak is

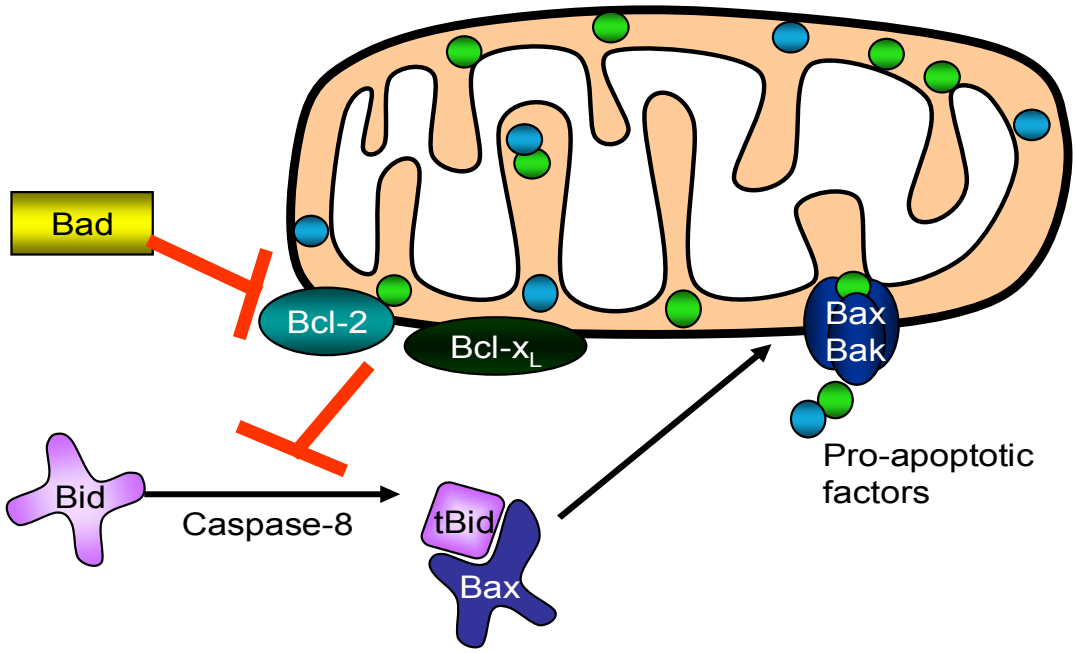

Figure 4. Mechanisms of Regulation of the Bcl-2 Family Members on the Outer Mitochondrial Membrane.

After an internal apoptotic signal a BH3-only-senstizer protein (e.g. Bad) will displace the anti-apoptotic Bcl-2 family members (Bcl-2 or Bcl-x_L) from any activated BH3-only-activator (e.g. Bid) proteins. The BH3-only-activator proteins will then change the conformation of a Bcl-2-multidomain protein (e.g. Bax or Bak). This allows for the homo-oligomerization of the multidomain Bcl-2 family proteins. Mitochondrial intermembrane space proteins (pro-apoptotic proteins) can be released into the cytosol to activate caspases. *Bad, Bcl-2 associated death promoter*; *Bak, Bcl-2 antagonist/killer 1; Bax, Bcl-2 associated X protein; Bcl-2, B-cell lymphoma 2; Bcl-x_L, Bcl-2 like 1; IAP, Inhibitors of apoptosis; Bid, BH3 interacting domain.*

thought to occur by one of two mechanisms. According to the first model, BH3-only proteins are categorized into two groups: (i) activators (also known as inducers); and (ii) sensitizers (also known as inhibitors or derepressors). BH3-only activators that induce multidomain Bcl-2 family members include Bid and Bim (Cartron et al., 2004; Desagher et al., 1999; Kuwana et al., 2005; Kuwana et al., 2002; Luo et al., 1998; Marani et al., 2002). The BH3-only sensitizers are proteins that inhibit the anti-apoptotic proteins and some include Bad, Harakiri, Bcl-2 interacting protein (Hrk), Bcl-2 modifying factor (BMF), and Noxa. Protein 53 (p53)-upregulated modulator of apoptosis (Puma) has been labeled as both an activator and a sensitizer (Letai, 2009). For example, Certo et al. (2006) categorizes Puma as a sensitizer, not an activator, because their studies showed a Puma BH3 domain lacked the ability to activate Bax and/or Bak. However, Kim et al. (2006), demonstrate that full length Puma is equal in its ability to activate Bax as Bim and Bid.

BH3-only activator proteins function to promote apoptosis by changing the conformation of the pro-apoptotic Bcl-2 multidomain family members (Bax/Bak), namely from monomeric to homo-oligomeric. This leads to MOMP induction and the release of pro-apoptotic factors into the cytosol. BH3-only activators have a line of regulation all of their own. Most BH3-only activator proteins are constitutively present in the cytosol in inactive forms. For example, under non-apoptotic conditions Bid is located in the cytosol in an inactivated form (full-length Bid). To be able to induce a conformational

change and thereby activate Bax/Bak, Bid first has to be cleaved to its truncated form (tBid) (Figure 4). While Bim is also located in the cytosol, it is maintained in an inactive state, at least in part, by binding to cytoskeleton (Puthalakath et al., 1999). Bcl-2 anti-apoptotic members can sequester the activated BH3-only activators (Bid and Bim) and prevent them from changing the conformation of Bax/Bak.

BH3-only sensitizer proteins (Bad and Bik) can also help facilitate MOMP. BH3-only sensitizer proteins can boot a BH3-only activator protein off of an anti-apoptotic Bcl-2 family member (Bcl-2, Bcl-x_L). This allows the newly released BH3-only activator proteins (Bid or Bim) to change the confirmation of Bax/Bak (Kim et al., 2006a; Letai et al., 2002). BH3-only sensitizers also have another line of regulation. They either have to be activated or up-regulated. For example, Bad has to be dephosphorylated to become activated (Zha et al., 1996). Bik on the other hand is up-regulated in response to an apoptotic stimulus (Hur et al., 2006). Overall, it is often the relative number of pro-apoptotic Bcl-2 family members compared to the number of anti-apoptotic Bcl-2 family members that either allow MOMP or prevent MOMP from occurring (Brunelle and Letai, 2009).

There is a second model that describes how pro-apoptotic Bcl-2 family of proteins promotes MOMP. This model does not distinguish between BH3-only sensitizers (Bad and Bik) and activators (Bid and Bim) but rather how selective the different BH3-only proteins are for the anti-apoptotic family

members (Bcl-2, Bcl-x_L). For example, Bim and Puma can bind and inhibit all anti-apoptotic proteins but Bad and Noxa can only bind a few (i.e. Noxa can bind Mcl-1 and A1) (Youle and Strasser, 2008). In this model, the BH3-only members do not directly bind Bcl-2 multidomain proteins (Bax and Bak). Under non-apoptotic conditions, Bax and/or Bak are held in their inactive forms by the anti-apoptotic Bcl-2 proteins. Originally it was suggested that BH3-only proteins bind to Bcl-2 anti-apoptotic proteins to change their conformation thus preventing their ability to bind to Bax or Bak (Strasser et al., 2000). One way this model has tried to refute the other model was through the use of binding assays and co-immunoprecipitating studies. They demonstrate through the use of BH3-only peptides that they indirectly activate Bcl-2 multidomain proteins (Bax and Bak) by binding and inhibiting the anti-apoptotic Bcl-2 family proteins and not binding Bax and Bak. They go on to show using full length Bcl-2 that BH3-only proteins (Bid and Bim) do not co-immunoprecipitate with Bax (Chen et al., 2005; Willis et al., 2007). Willis et al. (2007) go on to demonstrate that “Bcl-2 BH3-only activator proteins” (Bid/Bim) double knockout mice can still undergo apoptosis with various apoptotic stimuli. Their data suggest that “Bcl-2 BH3-only activator proteins” are not needed to change the conformation of Bax and/or Bak (Willis et al., 2007). The two conflicting views indicate that the precise biochemical mechanism to activate Bax and Bak is yet to be determined.

1.2.4 Pro-Apoptotic Factors Released From the Mitochondria

As alluded to earlier, once MOMP has occurred, pro-apoptotic factors are released from the intermembrane space of the mitochondria, including cytochrome *c* (Liu et al., 1996), Smac (Du et al., 2000), Omi, endonuclease G (Li et al., 2001), and apoptosis inducing factor (AIF) into the cytosol (Susin et al., 1999). These proteins are thought to move across the outer mitochondrial membrane by going through the pore that is produced by multidomain Bcl-2 family members (Bax or Bak). Cytochrome *c*, Smac and Omi all help to activate caspases while the other proteins promote apoptosis independently of caspases. I will be focusing on some of the pro-apoptotic factors (cytochrome *c*, Smac and Omi). Cytochrome *c* directly activates caspases, whereas Smac and Omi help to activate caspases indirectly (Figure 5).

1.2.4.1 Pro-Apoptotic Factors Released From the Mitochondria – Cytochrome *c*

Cytochrome *c* helps to activate caspases by inducing the formation of an activating platform for the initiator caspase-9 called the apoptosome complex. Once cytochrome *c* is in the cytosol, it will bind the initiator caspase-9's adaptor molecule apoptotic protease activating factor 1 (Apaf-1). Apaf-1 is a 130 kDa protein that contains three domains: (i) caspase

Figure 5. Role of Pro-Apoptotic Proteins (Cytochrome *c*, Smac, and Omi) that Are Released into the Cytosol from the Intermembrane Space of Mitochondria.

Pro-apoptotic factors released from the mitochondria after an apoptotic stimulus include cytochrome *c*, Smac, and Omi. These pro-apoptotic factors help to activate caspases in different ways. Cytochrome *c* helps to activate caspase-9 by allowing for Apaf-1 to homo-oligomerize and form the apoptosome complex. The apoptosome will then mediate caspase-9 activation. Smac helps to activate caspases by displacing inhibitors of apoptosis (IAPs) from activated caspase-3, -7, or -9. Omi will help to activate caspases by causing IAPs to be degraded and released from any activated caspases. *Apaf-1, apoptotic protease activating factor 1; Cyt c, cytochrome c; casp, caspase; IAP, inhibitors of apoptosis; Smac, Second mitochondrial activator caspases.*

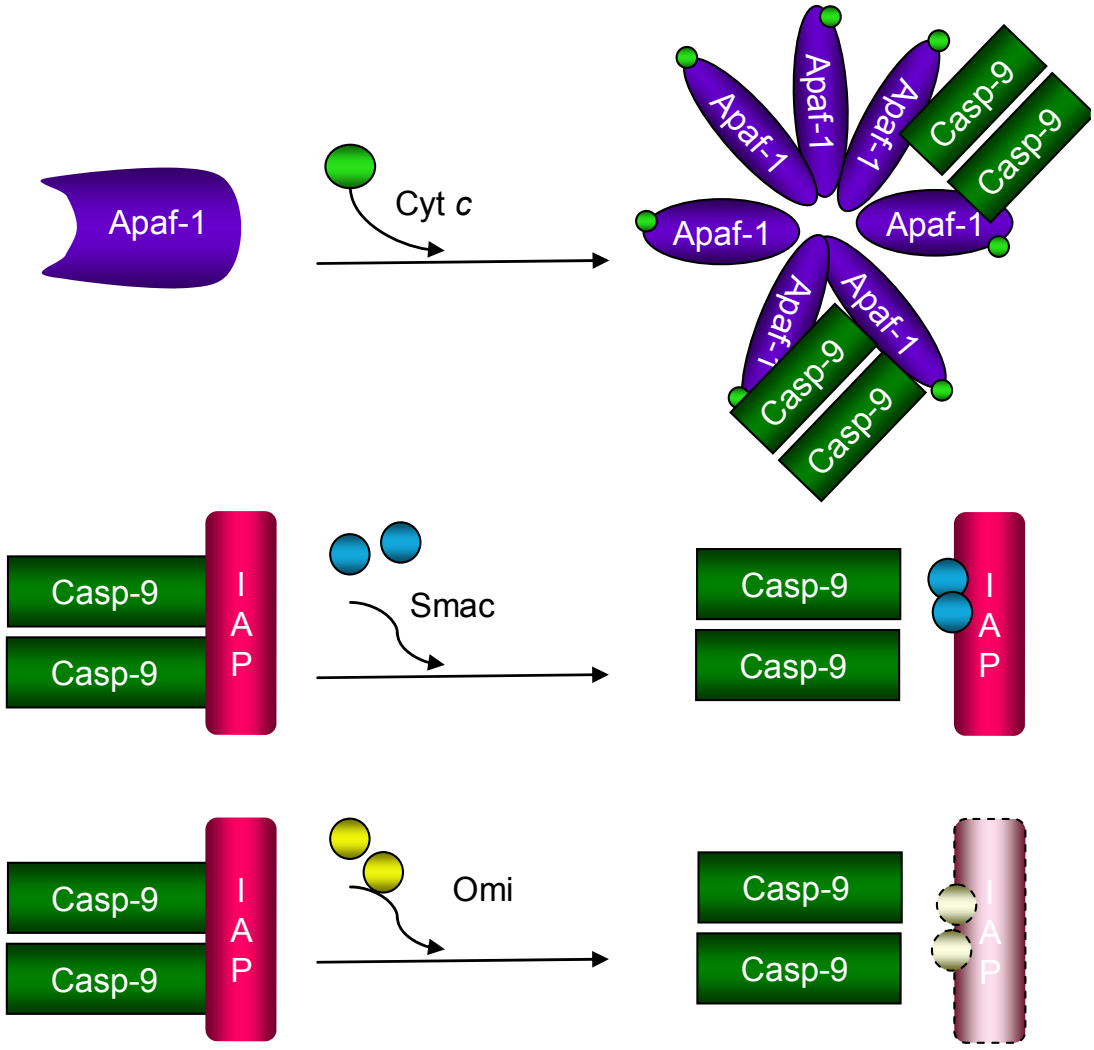
Apaf-1
Cyt c
Apaf-1
Apaf-1
Apaf-1
Apaf-1
Apaf-1
Apaf-1
Casp-9
Casp-9
Casp-9
Casp-9
Casp-9
Casp-9
I
A
P
Smac
Casp-9
Casp-9
I
A
P
Casp-9
Casp-9
I
A
P
Omi
Casp-9
Casp-9
I
A
P

Figure 5.

recruitment domain (CARD) at the N-terminal; (ii) nucleotide binding domain; and (iii) WD-40 repeats at the C-terminal (Zou et al., 1999). Under non-apoptotic conditions, Apaf-1 resides in the cytosol as an inactive monomer and its activation involves heptamerization. It is thought that when Apaf-1 is an inactive monomer, the C-terminal domain covers the N-terminal CARD domain and prevents its heptamerization. When cytochrome *c* is able to bind to the C-terminal domain of Apaf-1, Apaf-1 can change its conformation. Specifically, Apaf-1 can linearize and dATP hydrolysis occurs. Next, dATP is exchanged for the dADP (Kim et al., 2005). Subsequently, Apaf-1 oligomerizes to form a seven (heptamer) spoke wheel known as the apoptosome complex. The apoptosome, in turn, recruits up to seven initiator pro-caspase-9 proteins at one time (Acehan et al., 2002; Srinivasula et al., 1998). Due to the orientation of the apoptosome, the CARD domain of Apaf-1 will be available for interaction with the CARD domain of pro-caspase-9. This permits the autoactivation of pro-caspase-9 (Boatright et al., 2003; Chao et al., 2005; Pop et al., 2006; Rodriguez and Lazebnik, 1999; Shiozaki et al., 2002).

Cytochrome *c* also has a non-apoptotic function. Cytochrome *c* is transcribed by a nuclear gene and then translated in the cytosol as the apocytochrome *c* protein. Apocytochrome *c* then translocates to the mitochondrial intermembrane space where a heme group is covalently attached by cytochrome *c* heme lyase to form holocytochrome *c*. It is the

heme group that allows it to be a redox intermediate and shuttle electrons from complex III to complex IV of the electron transport system to help generate ATP (Ow et al., 2008).

1.2.4.2 Pro-Apoptotic Factors Released From the Mitochondria – Inhibitor of Apoptosis Protein (IAP) Neutralizers

The other two known pro-apoptotic factors released from the mitochondrial intermembrane space that help activate caspases include Smac and Omi. Smac and Omi help to activate caspases indirectly by inhibiting IAPs, most notably X-linked inhibitor of apoptosis (XIAP). Under normal circumstances, IAPs serve a protective role by binding any partially cleaved/active caspase-3, -7, or -9. Preventing caspase full catalytic activation also prevents apoptotic cell death. In this regard, Smac and Omi released into the cytosol can help overcome this inhibition, if necessary (Figure 5).

Smac is also known as direct IAP-binding protein with low pI (Diablo). After MOMP, Smac is released into the cytosol and homo-dimerizes. The dimerization of Smac allows it to bind to IAPs and sequester them away from active caspases (Wu et al., 2000). Specifically, Smac binds XIAP within XIAP's Baculovirus IAP repeat domain known as Baculovirus IAP repeat (BIR) 3. XIAPs will be expanded upon later (Takahashi et al., 1998).

Omi, a serine protease, is also known as high temperature requirement protein A2 (Htr2a). Omi inhibits IAPs slightly differently from Smac, which sequesters IAPs. Omi actually degrades IAPs and thus prevents them from binding to activated caspases (Yang et al., 2003). Omi is also important in degraded of cytoskeleton proteins and cellular substrates that caspases do not cleave (Vande Walle et al., 2007). It has been suggested that it is at the apoptosome with activated caspase-9 where caspase-3 and -7 are activated. It is also at the apoptosome where IAPs bind to inhibit caspase-3, -7, -9 and where Smac and Omi inhibit IAPs (Twiddy et al., 2003).

1.2.5 Caspases

Another major pro-apoptotic player is the caspase family. Caspases (cysteine proteases that cleave after aspartate residues) were first described in 1992 and 1993 by two different groups. One group described the human protease for activated interleukin 1β, caspase-1 (Cerretti et al., 1992; Thornberry et al., 1992), and the other group described the protein product of the cell death abnormalities *(CED) 3* gene (Yuan et al., 1993). Currently, there are 13 known mammalian caspases. Caspase-2, -3, -6, -7, -8, -9, -10 and -12 are involved in apoptosis. However, full length caspase-12 is only found in a small population **of people** since normally it is translated as a truncated protein. Caspase-1, -4, -5, -11, and -14 are most often associated

with activities other than apoptosis, such as inflammation (caspase-4, -5), innate immunity (caspase-1), and skin cell development (caspase-14) (Yi and Yuan, 2009). Caspases are primarily responsible for the biochemical and morphological changes that occur in the cell during apoptosis.

The caspases involved in apoptosis exist in cells as catalytically inactive zymogens known as pro-caspases. Pro-caspases are thought to be activated either through dimerization, allosteric regulation, or cleavage. Apoptotic caspases are a group of proteases that target a range of substrates. These substrates include pro-caspases, structural proteins, and cellular DNA proteins (Cohen, 1997; Jin and El-Deiry, 2005). Caspases have three subunits: (i) a prodomain, (ii) large subunit and (iii) small subunit. Pro-caspases are typically cleaved after their two Asp-*X* sites (Thornberry et al., 1997) to produce a long and short subunit. The large subunit contains the conserved catalytic site QAC*X*G (Q, glutamine; A, alanine; C, cysteine; *X*, any amino acid; G, glycine) (Figure 6). The apoptotic caspases are commonly divided into two groups: initiator caspases and effector caspases. The length of the prodomain generally determines what type of caspase they are.

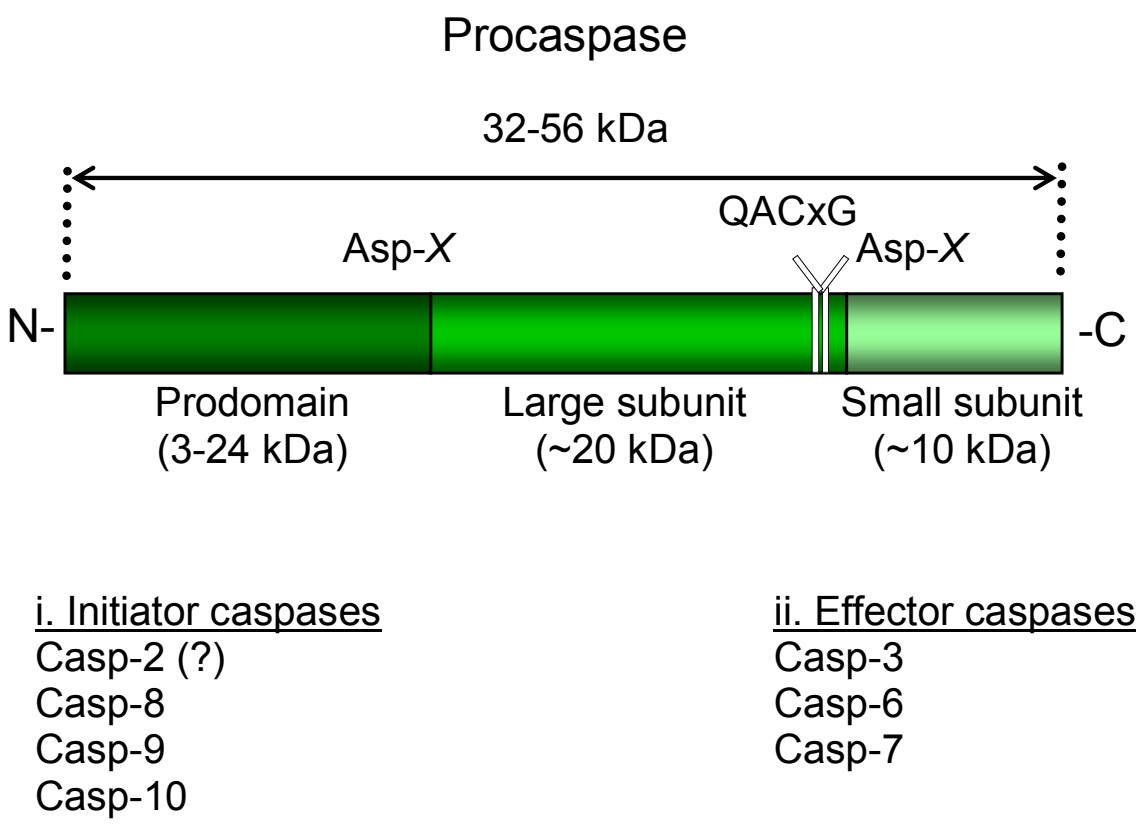

Figure 6. Caspase Classification and Activation.

Caspases are constitutively expressed in the cytosol as inactive aspartate proteases known as procaspases. Caspases can be divided into two groups: (i) Initiator caspases and (ii) Effector caspases. Initiator caspases are activated with the help of adaptor proteins, such as Apaf-1 for caspase-9 and FADD for caspase-8. Effector caspases require proteolysis and then dimerization for activation. Once effector caspases are activated they are able to cleave up to 1,000 cellular substrates to dismantle the cell. *Asp, aspartate; Casp, caspase; Apaf-1, apoptotic protease activating factor 1; FADD, fas associated death domain; X, any amino acid.*

1.2.5.1 Initiator Caspases

The initiator pro-caspase starts the caspase cascade and includes caspases-8, -9, -10 and possibly caspase-2. Initiator caspases have long prodomains that either have a death effector domain (DED) or CARD. Activation of initiator caspases is thought to occur by one of two mechanisms. According to the first model, adaptor proteins will allow for initiator caspases to dimerize with either their DED or CARD domains. The aided dimerization allows for the activation of the initiator caspases (Boatright et al., 2003; Pop et al., 2006). Caspase-8 and -10 are both important for the extrinsic pathway. Their adaptor molecules are fas-associated protein with death domain (FADD) and tumor necrosis factor receptor (TNFR)-associated death domain (TRADD). Pro-caspase-8 (57/55 kDa) can autocleave itself to produce p43/41 fragments. Caspase-9 is important for the intrinsic pathway, and its adaptor molecule is Apaf-1. Pro-caspase-9 (48 kDa) can autocleave itself to generate a p35 fragment. It can also be cleaved by activated caspase-3 to generate a p37 fragment. Caspase-2 has been suggested to be the initiator caspase for heat shock due to its long pro-domain (Tu et al., 2006). Some evidence suggests that caspase-2's adaptor molecule is Rip-associated ICH-1 homologous protein with death domain (RAIDD). Once activated, initiator caspases will cleave and thereby activate effector caspases to cause cell death.

The second model to describe how initiator caspases become activated involves allosteric modification. One argument against the dimerization model is that there are seven spokes in the apoptosome and 3 adaptor/death receptors in the death inducing signaling complex (DISC) (Chao et al., 2005). For example, after apoptosome formation when pro-caspase-9 binds to the apoptosome, pro-caspase-9 undergoes a conformational change to allow for its activation (Chao et al., 2005; Rodriguez and Lazebnik, 1999; Shiozaki et al., 2002). This conformational change has been suggested to change the active site of the initiator caspases to increase their activity (Chao et al., 2005). Recently Malladi *et al.* (2009) demonstrated that pro-caspase-9, has a higher affinity for the apoptosome than does activated caspase-9, suggesting that once activated caspase-9 dissociates from the apoptosome complex to allow another pro-caspase-9 molecule to bind. Secondly, they demonstrate that caspase-3 is cleaved and activated at the apoptosome. They suggest that the amount of pro-caspase-9 present in the cell acts as a molecular clock to determine how long the apoptosome will be formed (Malladi et al., 2009). Even though there are data that support the allosteric activation model, there are currently not enough data to contradict all of the data that support the dimerization model. Chao *et al.* even suggest that both models might exist—allosteric for caspase-9 and dimerization for the other initiator caspases (Chao et al., 2005).

1.2.5.2 Effector Caspases

Effector caspases have short prodomains and include caspase-3, -6, and -7. Effector caspases are usually more abundant than the initiator caspases. Unlike the initiator caspases that require dimerization for activation, effector caspases require cleavage and then dimerization of the cleaved fragments. Some even suggest that effector caspases, specifically caspase-7, exist in their pro-forms as homodimers and cleavage increases their catalytic activity (Chai et al., 2001; Riedl et al., 2001a). The effector caspases will then proteolytically degrade the cellular contents. Caspase-3 has been recognized as the vital effector caspase (Jin and El-Deiry, 2005). Pro-caspase-3 (32 kDa) is cleaved initially by caspase-8 and/or -9 to form a p20 and a p12 fragment. The caspase-3 p20 fragment has only enough catalytic activity to cleave itself into the p19 and then p17 fragment. It is caspase-3's p17/p12 that is the fully active caspase. The p17/p12 dimer can go on to cleave over 1,000 endogenous death substrates (Fuentes-Prior and Salvesen, 2004). It is activity of caspases that is directly or indirectly responsible for the classical morphological and biochemical characteristics of an apoptotic cell such as condensed nuclei, membrane blebbing, DNA laddering, and the exposure of phosphatidylserine on the outer plasma membrane (Brunelle and Letai, 2009; Fuentes-Prior and Salvesen, 2004).

1.2.6 IAPs

As alluded to previously, IAPs are a family of proteins that can prevent the accidental activation of caspases by binding to partially active caspase-3, -7, and -9 to prevent their full catalytic activation. In this way, IAPs can prevent apoptosis by preventing caspase activity. The original IAP was discovered from a baculovirus that infected insect cells. This gene was determined to have the ability to inhibit apoptosis (Crook et al., 1993). Currently, there are eight proteins identified as IAPs, including $cIAP_1$, $cIAP_2$, XIAP, Survivin, and NAIP. Human IAP members share a 70 amino-acid BIR domain that contains the sequence $CX_2CX_{16}HX_6C$ (C, cysteine; H, histidine, *X*, any amino acid). It has been suggested that another way cancer cells have overcome apoptosis is by overexpressing one of the IAPs. IAPs have been observed to be highly expressed in several neoplasms (Srinivasula and Ashwell, 2008).

IAPs can have one or multiple BIR domains (Sun et al., 1999a). IAPs frequently have other functional domains that include RING and CARD domains (Hinds et al., 1999; Sun et al., 1999a; Verdecia et al., 2000). The most studied and understood IAP is XIAP which has three BIR domains and a RING domain (Jin and El-Deiry, 2005). Typically BIR2 and BIR3 are involved in binding to caspases or apoptosis regulatory molecules (i.e. Smac and Omi). It is the region around XIAP's third BIR domain (Takahashi et al., 1998) that inhibits caspase-9 activity by binding to caspase-9's p35 fragment (Chai et al.,

2001; Huang et al., 2001). The linker region around the second BIR region in XIAP inhibits caspase-3 and -7 (Takahashi et al., 1998) (Figure 7). As mentioned before IAPs can be antagonized by mitochondrial intermembrane space proteins (Smac and Omi). Smac and Omi can interact with IAP at their IAP-binding motifs. The BIR2 and BIR3 on the IAP will bind to the IAP-binding motif of Smac or Omi (Shiozaki and Shi, 2004; Srinivasula et al., 2000).

The only known IAP able to bind and inhibit caspase activity is XIAP (Eckelman and Salvesen, 2006). The other IAPs were recently shown to bind only to caspase-3, -7, and -9 but were unable to inhibit their catalytic activity (Eckelman and Salvesen, 2006). There is now persuasive evidence to suggest that IAPs' function is more than just to inhibit apoptosis. IAPs have now been implicated in a role in cell division, morphogenesis, heavy metal homeostasis and MAP kinase signaling (Srinivasula and Ashwell, 2008).

1.2.7 Apoptotic Pathways

After extensive study, it is now known that there are two main apoptotic pathways: (i) the intrinsic or mitochondria-mediated pathway; and (ii) the extrinsic or receptor-mediated pathway (Figure 8).

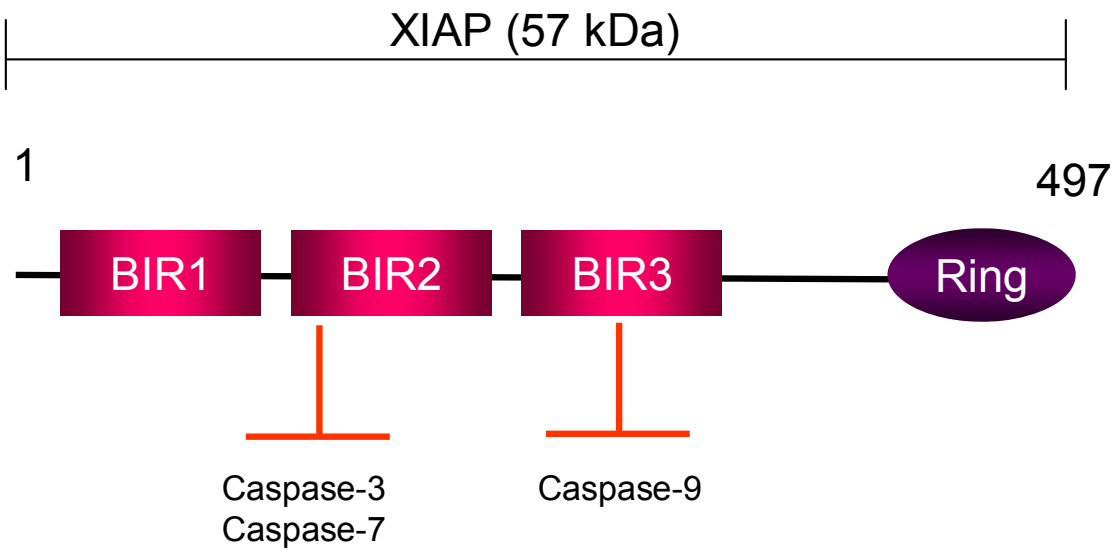

Figure 7. Schematic Representation of XIAP's Domains and How They Inhibit Caspases.

XIAP has three baculoviral inhibitor of apoptosis repeat (BIR) domains. A region of XIAP that includes the BIR2 domain is responsible for binding to and inhibiting activated caspase-3 and caspase-7. The BIR3 domain is responsible for binding to and inhibiting caspase-9.

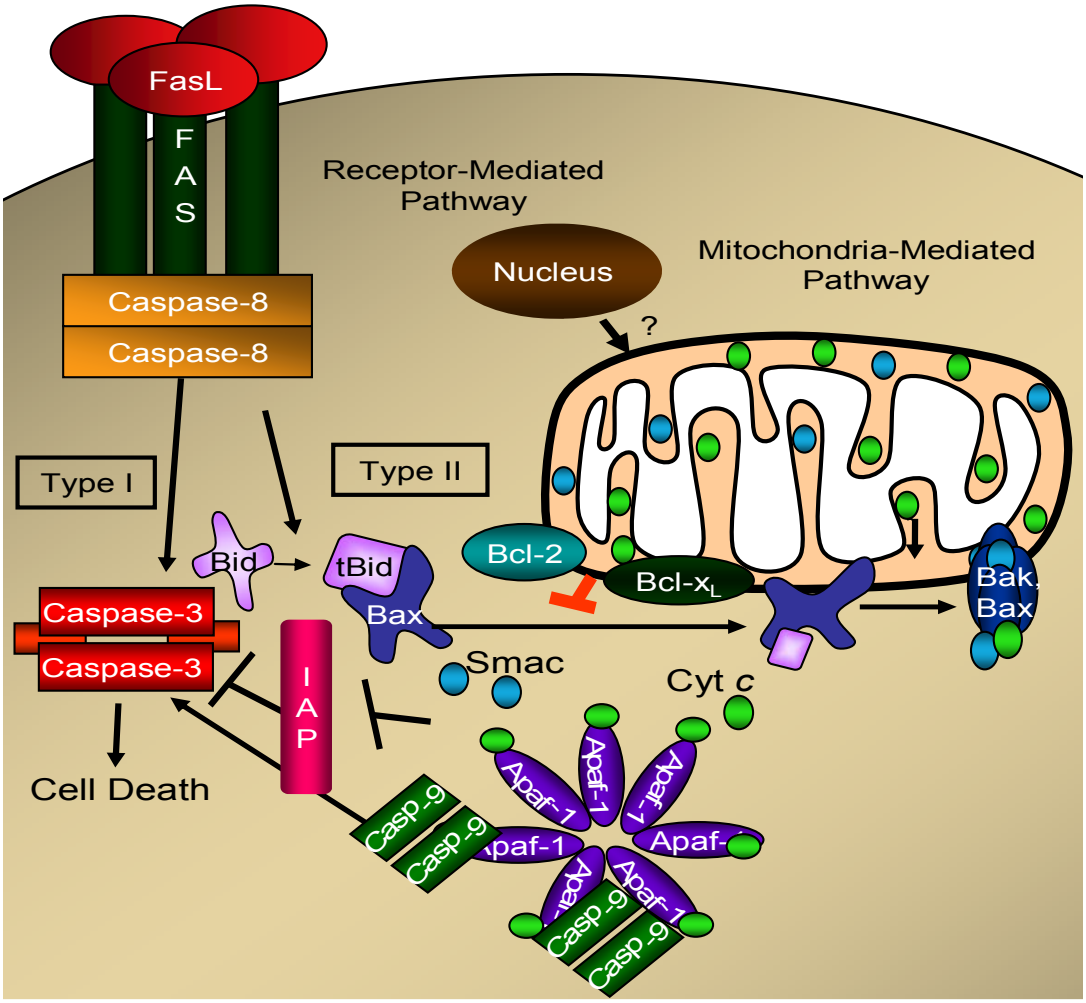

Figure 8. Schematic Representation of the Intrinsic and Extrinsic Apoptotic Pathways.

The intrinsic pathway is also known as the mitochondria-mediated pathway. The extrinsic pathway is also known as the receptor-mediated pathway. *Cyt c, cytochrome c; Casp, caspase; Bid, BH3 interacting domain; tBid, truncated Bid; Apaf-1, apoptotic protease activating factor 1; Bak, Bcl-2 antagonist/killer 1; Bax, Bcl-2-associated X protein; Bcl-2, B cell lymphoma 2; Bcl-x$_L$, Bcl-2 like 1; IAP, Inhibitors of apoptosis.*

1.2.7.1 Apoptotic Pathways – Mitochondria-Mediated

The mitochondria-mediated (intrinsic) pathway is initiated by an intracellular stress signal. Internal stress signals can come from heat shock, growth factor deprivation, oxidative stress, oncogene activation, chromosomal instability, and/or DNA damage. UV radiation or chemicals, such as chemotherapeutic drugs, can cause DNA damage and stimulate the mitochondria-mediated pathway. Even though all mammalian cells can be induced to undergo apoptosis by stimulation of the intrinsic pathway, the sensitivity of mammalian cells differs between cell types. For example, thymocytes are very sensitive to DNA damage, while fibroblasts rarely undergo DNA damage-induced apoptosis (Norbury and Zhivotovsky, 2004).

The mitochondria-mediated pathway is most often depicted as a linear sequence of events. Initially, a Bcl-2 BH3-only family protein (e.g. Bim, Bid, Noxa, or Puma) (Kim et al., 2006a) becomes activated. Currently, the precise molecular link between DNA damage and activation of Bcl-2 BH3-only proteins is unknown. A Bcl-2 BH3-only family member will change the conformation of a Bcl-2 multidomain member (Bak, Bax). Normally, these proteins exist as inactive monomers located in the cytosol (Bax) or associated with the outer mitochondrial membrane (Bak) (Chipuk and Green, 2006; Scorrano and Korsmeyer, 2003). Bax and Bak homo-oligomerize to form a pore on the outer mitochondrial membrane through which the release of pro-apoptotic factors, such as cytochrome *c*, Smac and Omi, occurs. Anti-

apoptotic Bcl-2 family proteins (Bcl-2 and Bcl-x_L) reside on the mitochondrial outer membrane and prevent the cell from undergoing MOMP. They exert their inhibitory function by binding directly to a pro-apoptotic Bcl-2 family member and thereby inhibit the ability of Bax/Bak to form a pore. Cytochrome *c* facilitates the activation of the initiator pro-caspase-9 by promoting the formation of the apoptosome (Apaf-1, cytochrome *c*, dATP). The apoptosome is the activating platform for pro-caspase-9. Smac and Omi help activate caspases by inhibiting IAP (Yang et al., 2003). Once caspase-9 is activated, it can cleave and activate effector pro-caspases, such as caspase-3 or -7. Activated caspase-3 can cleave and/or activate other pro-caspases, such as caspase-2 and -6 to dismantle the cell.

1.2.7.2 Apoptotic Pathways – Receptor-Mediated

The receptor-mediated (extrinsic) pathway is the other main apoptotic pathway. The extrinsic pathway differs from the intrinsic pathway in that an initiator signal comes from outside the cell. Also, extrinsic apoptosis can be either MOMP-dependent or MOMP-independent. Activation of the receptor-mediated pathway is not only important in eliminating infectious cells but it is also very important during development of the immune system by removing self-reactive B or T lymphocytes. The pathway is initiated by a death signal binding to a death receptor. There are three types of death ligand/death

receptor pathways: (i) tumor necrosis factor alpha (TNF-α) binding to tumor necrosis factor receptor 1 (TNF-R1); (ii) Fas ligand (FasL) binding to Fas receptor; and (iii) TNF related apoptosis inducing ligand (TRAIL) binding to its death receptor 4/5 (DR4/5) (Figure 9). I will be focusing on the FasL/Fas pathway due to its implication in the pathogenesis of various malignancies (Jin and El-Deiry, 2005).

The extrinsic pathway begins with an exogenous signal such as a death ligand binding the appropriate death receptor, followed by formation of the death-inducing signaling complex (DISC) (Kischkel et al., 1995). For example, FasL/CD95L binds to Fas/CD95. When a death ligand binds to its death receptor it causes the death receptor to trimerize. Receptor trimerization then recruits the association of an adaptor molecule, such as FADD and TRADD (Chinnaiyan et al., 1995). The adaptor molecule FADD or TRADD, in turn, recruits the initiator pro-caspase-8 proteins to bind (Muzio et al., 1996). Akin to binding to the apoptosome for the activation of pro-caspase-9 molecules, it is believed that binding to the DISC allows pro-caspase-8 proteins to stimulate autoactivation leading to self-cleavage. Recruitment of FADD or TRADD can also recruit initiator pro-caspase-10 proteins to bind to the DISC (Vincenz and Dixit, 1997). Like caspase-9, caspase-8 can also activate effector pro-caspase-3 to initiate cell death.

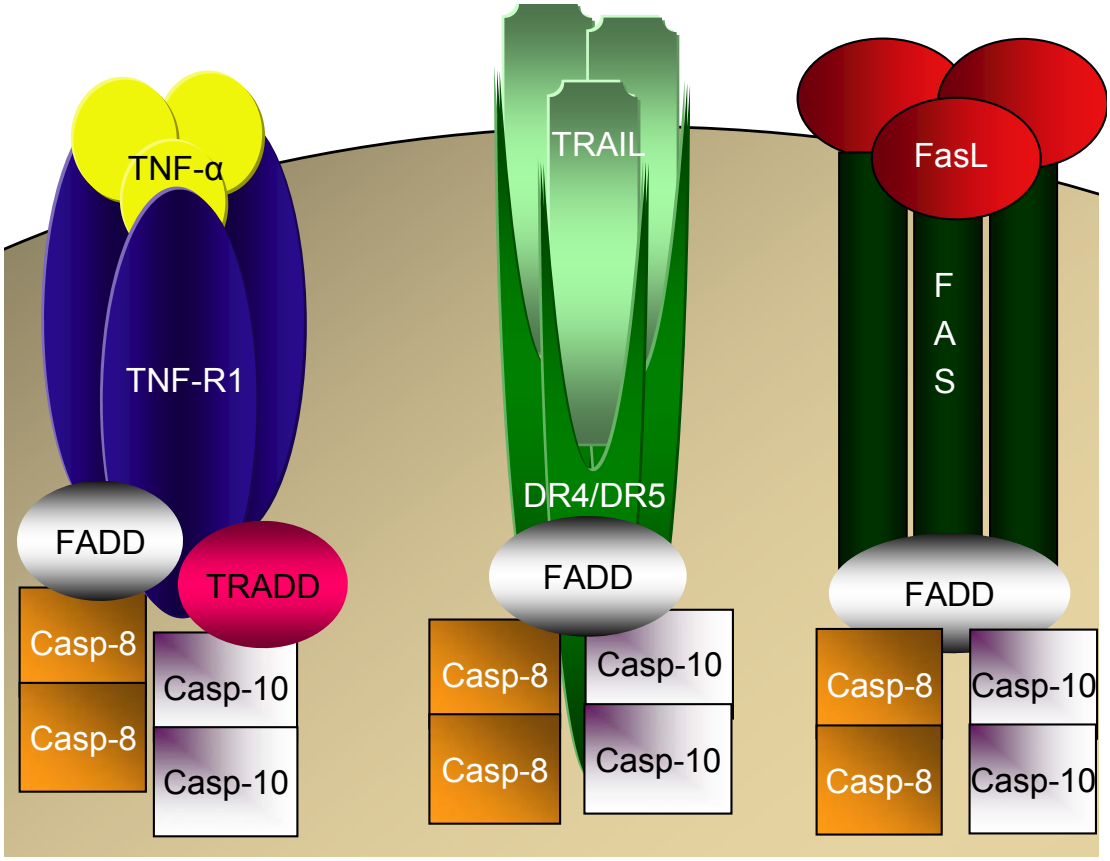

Figure 9. Death-Inducing Signaling Complexes.

To initiate the receptor-mediated apoptotic pathway a death inducing signaling complex (DISC) has to form. DISC is initiated by binding of a death ligand (FasL) to a death receptor (Fas). This allows for trimerization of the death receptor followed by recruitment of an adaptor molecule (e.g. FADD or TRADD), which, in turn, facilitate activation of initiator caspase-8 or caspase-10. *Casp, caspase; DR, death receptor; TNF-α, Tumor necrosis factor alpha; TNF-R, Tumor necrosis factor receptor; TRADD, TNF receptor associated death domain; FADD, fas associated death domain.*

In 1998, Scaffidi and colleagues classified a cell's apoptotic sensitivity to death ligands as either a type I or type II cell. After stimulation of the receptor-mediated pathway by a death ligand, a type I cell relies heavily on caspase-8 activation at the DISC that is sufficient to cleave enough caspase-3 to cause cell death. That initial study identified B lymphoblastoid SKW6.4 and a T cell line H9 as type I cells. In contrast to type I cells, type II cells require the involvement of the mitochondria-mediated pathway after stimulation of a death receptor. Specifically, a type II cell requires the induction of MOMP, due to the insufficient activation of pro-caspase-8 at the DISC. Instead, caspase-8 cleaves the proapoptotic Bcl-2 BH3-only activator family member Bid to yield tBid (Danial and Korsmeyer, 2004; Li et al., 1998). tBid can then relocate to the outer mitochondrial membrane and cause Bax and/or Bak to undergo a conformational change followed by their homo-oligomerization and pore formation (Luo et al., 1998). Pore formation then permits the release of the pro-apoptotic factors (cytochrome *c*, Smac, and Omi). Like in the intrinsic pathway, these pro-apoptotic factors help to activate caspases (Figure 8). Characteristic type II cells include Jurkat T-lymphocytes and leukemic lymphoid CEM cells. The current paradigm states that overexpressing Bcl-2 and Bcl-x_L prevent MOMP. Therefore, overexpression also prevents the initiator pro-caspase-9 from being autoactivated at the apoptosome complex (Scaffidi et al., 1998; Scaffidi et al., 1999) (Figure 10).

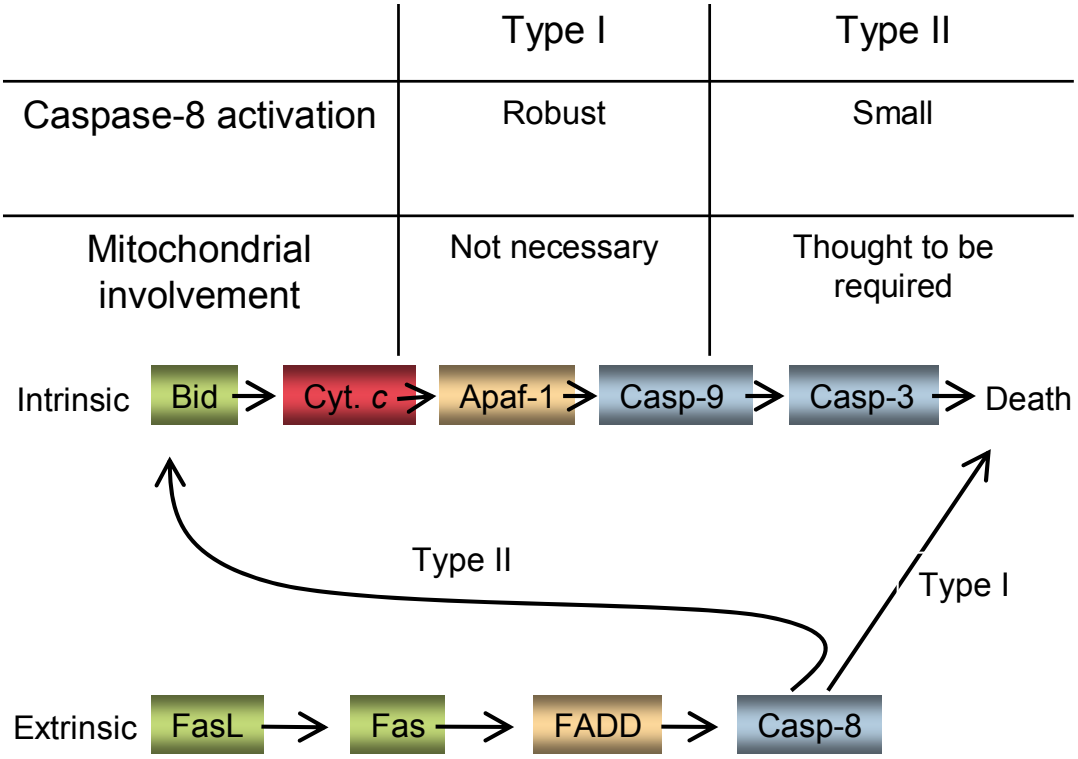

Figure 10. Characterization of Type I vs Type II Cells.

A type I cell has sufficient caspase-8 activation at the DISC to cleave and activate caspase-3 to cause cell death. Also, a type I cell does not require the mitochondria-mediated pathway to undergo receptor-mediated apoptosis. A type II cell does not have sufficient caspase-8 activation at the DISC to activate caspase-3 directly. Instead, a type II cell requires the recruitment of the mitochondria-mediated pathway (via Bid cleavage to tBid) to activate caspase-9. It is widely believed that the activation of caspase-9 drives enough caspase-3 activation to cause cell death. *Casp, caspase; FADD, Fas associated death domain; DISC, death inducing signaling complex. Bid, BH3 interacting domain; Apaf-1, apoptotic protease activating factor 1.*

CHAPTER 2: HYPOTHESIS AND SPECIFIC AIMS

2.1 Hypothesis

As mentioned previously, within the current paradigm of genotoxic stress-induced apoptosis, the mitochondria-mediated apoptotic pathway is thought to proceed in a linear fashion. The pathway is widely thought to begin with the activation of a multidomain pro-apoptotic Bcl-2 protein, such as Bax and Bak. These proteins are induced to form pores in the mitochondrial outer membrane to permeablize it. This results in the release of pro-apoptotic factors that include cytochrome *c* and Smac. These pro-apoptotic factors promote the activation of caspase-9 within the Apaf-1 apoptosome complex. Active caspase-9, in turn, activates caspase-3 and -7 to execute cell death. Apaf-1 is a critical component of the intrinsic pathway that is generally thought to reside *downstream* of MOMP. However, a previous study in our laboratory reported that Apaf-1-deficeint Jurkat T-lymphocytes do not release cytochrome *c* or lose mitochondrial membrane potential in response to the DNA-damaging drug etoposide (Franklin and Robertson, 2007).

In the literature there are also some gaps in the knowledge about the molecular mechanisms of the mitochondria-mediated and receptor-mediated pathways and how these pathways interact. According to the current paradigm, Jurkat T-lymphocytes are labeled as classical type II cells. This means that, upon stimulation of the receptor-mediated pathway, the

mitochondria-mediated pathway must also be engaged to ensure cell demise. A previous study from this laboratory reported that Apaf-1-deficient Jurkat T-lymphocytes were sensitive to anti-Fas-induced apoptosis even though they were insensitive to etoposide-induced apoptosis (Franklin and Robertson, 2007). This was surprising since type II cells are reported to require the mitochondria-meditated pathway but apparently did not need Apaf-1.

Therefore, clarification was needed regarding the molecular requirements necessary for the release of pro-apoptotic factors from the mitochondria during genotoxic stress-induced apoptosis. My *central hypothesis* is that classical mitochondrial events, such as loss of mitochondrial membrane potential and the release of intermembrane space proteins, are mediated by signaling molecules that were once thought to reside strictly downstream of mitochondrial changes, including, but not limited to, activation of pro-caspase-3 and/or pro-caspase-9, neutralization of IAPs, and/or Apaf-1 (Figure 11).

2.2 Specific Aims

Specific aim 1: To determine what classical downstream factors (activated caspase-9, caspase-3 or inhibition of IAPs) are required for MOMP to occur after treatment with DNA-damaging drugs, such as etoposide.

- Jurkat T-lymphocytes in which the intrinsic pathway is inhibited due to the stable knockdown of Apaf-1 or overexpression of full-length XIAP, the BIR1/BIR2 domains of XIAP, or Bcl-2/Bcl-x_L will be used to investigate the molecular requirements necessary for Bak-induced MOMP.

Specific aim 2: To determine why Apaf-1 is dispensable for receptor-mediated death in a classical “type II” Jurkat cell.

- Apaf-1-deficient, Bcl-2/Bcl-x_L-overexpressing, and BIR1/BIR2-expressing Jurkat cells will be used to determine the precise signaling requirements necessary for Fas-mediated apoptosis.

Specific aim 3: To determine the long-term survival potential of Apaf-1-deficient cells.

- Apaf-1-deficient cells, which were shown previously to be resistant to etoposide-induced MOMP induction, will be used to investigate the longer term effects of etoposide incubation on mitochondrial apoptotic events (i.e., mitochondrial membrane potential, release of pro-apoptotic proteins from the mitochondria, and Bak activation).

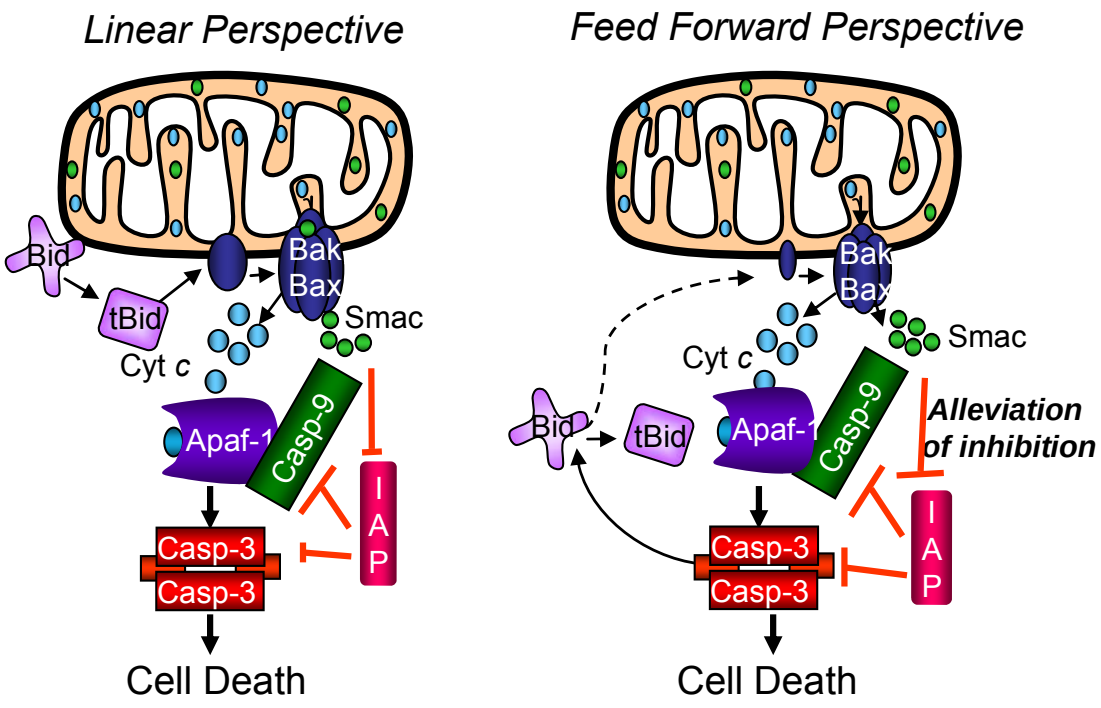

Figure 11. A Comparison Between the Linear and Feed Forward Perspective of How the Mitochondria-Mediated Apoptosis Proceeds.

My hypothesis favors the feed forward perspective. Caspases and mitochondria form an essential amplification circuit that functions as a central control point of genotoxic stress-induced apoptosis. *Cyt c, cytochrome c; Bid, BH3 interacting domain; tBid, truncated Bid; Casp, caspase; IAP, inhibitor of apoptosis proteins; Apaf-1, apoptotic protease activating factor 1; Bak, Bcl-2 antagonist/killer 1; Bax, Bcl-2 associated X protein; Smac, second mitochondrial activator caspases.*

CHAPTER 3: MATERIALS AND METHODS

3.1 Cell Culture

Wild-type Jurkat T-lymphocytes (clone E6.1) were cultured in RPMI 1640 complete medium (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS) (HyClone, Logan, UT), 2% (w/v) glutamine (Invitrogen), 100 U/ml penicillin (Invitrogen) and 100 µg/ml streptomycin (Invitrogen) at 37°C in a humidified 5% CO_2 incubator. Cells were maintained at their optimal growth density of 10^5 - 10^6 cells/ml. For transfected Jurkat cells, 1 mg/ml Geneticin (Invitrogen) was substituted for penicillin and streptomycin. All cells were re-plated in fresh complete nonselective medium prior to all experiments. Cells (5×10^6) were retained in liquid nitrogen for long term storage in 1 ml of cryostorage medium (5% DMSO and RPMI 1640 with appropriate antibiotics). Prior to submersion in liquid nitrogen, cells were cooled to -80°C in a Mr. Frosty (4 h – 1 week). Cryopreserved cells in the 5% DMSO were thawed at 37°C and diluted with 15 ml of appropriate growth medium. Cells were then incubated at room temperature (22°C) for 5 min before centrifugation at 300 x g for 5 min. Cell pellets were resuspended in 10 ml of appropriate medium and allowed to recover for 1 week before experiments were performed. Apoptosis was induced with etoposide (10 µM) (Sigma-Aldrich) or anti-Fas antibody (100 ng/ml) (clone CH-11, MBL International, Woburn, MA). The caspase-3/7

inhibitor Z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH_2F (Z-DEVD-fmk) (Kamiya Biomedical Company, Seattle, WA) was used at a final concentration of 25 μM, and the Smac mimetic (compound 3) was used at a final concentration of 100 nM. Autophagy was inhibited by treating with 3-methlyadenine (3-MA) (5 & 20 mM: Sigma), and the 26S proteasome was inhibited with either bortezomib (10, 20, 40 nM) or MG132 (0.5, 1, 5 μM).

3.2 Short Hairpin RNA (shRNA) Plasmid Production for Apaf-1-Deficient Cells

To stably decrease the expression of Apaf-1 in wild-type Jurkat cells, a shRNA plasmid was created. First, a 60-mer gene-specific targeting insert corresponding to nucleotides 875-893 (5'-AGGCAGATTTGCCAGAACA-3') downstream of the transcription start site of the *APAF-1* gene was designed. Two oligos (anti-sense and sense) were purchased from Integrated DNA Technologies (Coralville, IA). Each oligonucleotide included the 19 base pair (bp) target in both sense and antisense directions separated by a 9 bp non-complementary spacer sequence (5'-TTCAAGAGA-3'). Oligos were resuspended in nuclease-free ddH_2O (10 μg/μl) and annealed to produce the 60-mer. The annealing reaction was set up as follows: 10 μg sense oligo, 10 μg antisense oligo and 48 μl annealing buffer (100 mM NaCl, 50 mM Hepes, pH 7.4). An Eppendorf Mastercycler was used to anneal the oligos for 4 min at 94°C, 4 min at 85°C, 4 min at 82°C, 4 min at 80°C, 4 min at 78°C, 4 min at

75°C, 10 min at 70°C, 4 min at 50°C, 20 min at 37°C, 20 min at 22°C, 20 min at 10°C, then held at 4°C and stored at -20°C.

Next, the pSUPER.neo vector (Oligoengine, Seattle, WA) was linearized for cloning of the 60-mer (annealed oligos). The pSUPER.neo vector (1 µg) was incubated for 1 h at 37°C water bath with 10 U XhoI (Invitrogen) and 1X REact® 2 buffer (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl) (Invitrogen). The vector was then incubated for 2 h at 37°C with 10 U Bgl II (Invitrogen) and 1X REact® 3 (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 100 mM NaCl) (Invitrogen). Restriction enzymes were then heat inactivated by incubating them for 10 min at 65°C. Linearized vector (pSUPER.neo) was purified on a 1% agarose gel to remove the 221 bp fragment and undigested plasmid away from the 4478 bp linearized fragment. QIAEX II agarose gel extraction kit (Qiagen, Valencia, CA) was used to extract DNA from agarose gel.

Next, the 60-mer was ligated into the linearized pSUPER.neo vector. Linearized pSUEPER.neo vector (300 ng) was incubated with 400 - 600 ng of annealed oligos, 1X T4 DNA ligase buffer (250 mM Tris-HCL pH7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM Dithiothreitol (DTT), 25% (w/v) polyethylene glycol-8000) and 1 U T4 DNA ligase (Invitrogen) for 1.5 h at 22°C, and then 4°C overnight. The Bgl II site should be destroyed after ligation of the 60-mer into the vector. Not all linearized vector will have the 60-mer ligated into it. The linearized vector will ligate back on itself. To optimize the chance of

transforming only the vectors with the 60-mer insert digest the vector again with 5 U Bgl II for 1 hr at 37°C.

Subsequently, the shRNA plasmid (10 ng) was incubated with 25 µl TOP10 *Escherichia coli* cells (Invitrogen) for 45 sec at 42°C. Heat shocked bacteria were then incubated on ice for 10 min before adding 250 µl of super optimal broth with catabolite repression (SOC; 2% trypotone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) and incubated for 1 h at 37°C in a shaking incubator 220-250 RPM. Bacteria were then plated out on LB ampicillin plates (0.1 mg/ml) overnight at 37°C. Five to ten individual colonies were extracted and incubated with 3 ml of LB-ampicillin broth (0.1 mg/ml) at 37°C in a shaking incubator (220-250 RPM) for 12 - 16 h. Cultures were spun down at 8,000 x g for 5 min. Pellets were subjected to Qiagen miniprep kit to isolate plasmid DNA. Plasmid DNA was quantified using the BioMini DNA/RNA/Protein Analyzer (Shimadzu).

Next, the plasmid was digested and run on an agarose gel to ensure that the plasmid contains the 60-mer insert. Specifically, the plasmid (1 µg) was incubated with 10 U XhoI (Invitrogen), 10 U EcoRI, and 1X REact® 2 buffer (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl) (Invitrogen) for 1 h in a 37 ^{o}C water bath. 100 ng of digested plasmid and 100 ng of undigested plasmid DNA were run on a 1.5% agarose gel. Vector containing the insert will run slightly higher (287 bp versus 249 bp) on the gel, allowing for the detection of which plasmid has the 60-mer insert. One or two plasmids

containing the 60-mer insert was/were submitted for sequencing at University of Kansas Medical Center's DNA Sequencing Core. Once plasmid sequence was verified, the plasmid was transformed again into TOP10 *E. coli* cells and subjected to Qiagen Endofree Midiprep.

3.3 Transfections

Wild-type Jurkat T-lymphocytes (10^7) were washed with 1X sterile warm phosphate buffered saline (PBS) and pelleted by centrifugation at 300 x g for 5 min. Pellets were resuspended in 800 µl RPMI and incubated with 20 µg of plasmid DNA (pSFFV-Bcl-2, pSFFV-Bcl-x_L, pSFFV-neo, pSUPER-Apaf-1, pSUPER-neo, pcDNA3-XIAP, pcDNA3-XIAP-BIR1/BIR2, or pcDNA3-neo). Cells were electroporated using a Bio-Rad Gene Pulser Xcell system (0.4 cm cuvette, 300 V and 950 µF). Cells were then allowed to recover in RPMI 1640 complete growth medium for 48 h at 37°C in a humidified 5% CO_2 incubator. Selection of transfected cells was performed in the presence of 1 mg/ml Geneticin for several weeks (3-4 weeks). Transfected cells were then serially diluted to 8 cells/ml and 125 µl/well were plated out in a 96-well plate. Wells were observed every few days to ensure the presence of only 1 cell was initially added to each well. Single-cell clones of Bcl-2- or Bcl-x_L-overexpressing cells, Apaf-1-silenced cells, and cells overexpressing full-length XIAP or the BIR1/BIR2 domains of XIAP were produced. The extent of

protein overexpression or knockdown was determined by Western blot analysis.

3.4 Flow Cytometry Measurements for Cell Death, Mitochondrial Membrane Potential (ΔΨ), and Bak Activation

Phosphatidylserine (PS) exposure on the outer leaflet of the plasma membrane was detected by staining with Annexin V-FITC (BD PharMingen) and disruption in the plasma membrane was detected by staining DNA with the non-cell permeable dye propidium iodide (PI). In brief, 10^6 cells were pelleted by centrifuging at 300 x g for 5 min, following etoposide or anti-Fas treatment and washed with cold PBS. Cells were washed with PBS and centrifuged at 300 x g for 5 min. Pellets were resuspended in 500 µl of binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) containing 1.5 µg annexin V-FITC and 5 µl PI. Cells were then analyzed by flow cytometry with the BD LSRII and BD FACS Diva v6.1.2 software.

For ΔΨ determination, cells were stained with $DiIC_1(5)$ (Invitrogen), which is a cell-permeable dye that accumulates in mitochondria with active membrane potentials to produce bright far-red fluorescence. Specifically, the fluorrochrome emits a red light at 658 nM when excited with a 633 nM laser. The intensity of the fluorescence decreases as ΔΨ diminishes. Briefly, cells (10^6) were pelleted by centrifugation at 300 x g for 5 min at the end of the

experiment. Cells were then washed once in PBS, and resuspended in 1 ml of warm PBS. Next, 5 µl of $DiIC_1(5)$ (10 µM) were added to the cells and incubated in a humidified 5% CO_2 incubator at 37°C for 15 min. Cells were pelleted by centrifugation at 300 x g for 5 min and resuspended in 500 µl of cold PBS. Cells were then analyzed by flow cytometry with BD LSRII and BD FACS Diva v6.1.2 software.

For detection of activated Bak by flow cytometry, cells (10^6) were washed in PBS, fixed in 400 µl of 0.25% paraformaldehyde for 5 min, subsequently washed two times with 1% fetal bovine serum in PBS, and incubated in 50 µl of staining buffer (1% fetal bovine serum and 100 µg/ml digitonin in PBS) with a conformation-specific mouse monoclonal antibody against Bak (1:30; AM03, Calbiochem) for 30 min at room temperature (22°C). Then, cells were washed and resuspended in 50 µl of staining buffer containing 0.25 µg of Alexa Fluor 488-labeled chicken anti-mouse antibody for 30 min in the dark. Cells were washed again and analyzed by flow cytometry. Analysis and histogram overlays were performed using FlowJo software (Tree Star, Ashland, OR).

3.5 Western Blotting

Cells (5 x 10^6) were centrifuged at 300 x g for 5 min. Pelleted cells were then resuspended and lysed in 200 µl of ice-cold lysis buffer (10 mM

Tris/HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 0.1% Nonidet P-40) supplemented with a mixture of protease inhibitors (Complete Mini EDTA-Free, Roche Applied Science). Resuspended pellets were incubated on ice for 10 min before protein concentrations were determined using the bicinchoninic acid assay (Pierce). BSA standards (0 µg, 1 µg, 2 µg, 4 µg, 8 µg, 16 µg, 32 µg, 64 µg) were used to determine sample protein concentrations. Then protein was mixed with loading 1X sample buffer (50 mM Tris HCl pH 6.8, 2% SDS, 6% glycerol, 1% β-mercaptoethanol, 0.004 % bromophenol blue) before being incubated for 5 min at 100°C. Protein (30 – 40 µg) was loaded on a polyacrylamide gel (8 - 15%). When blotting for Apaf-1 an 8% polyacrylamide gel was used, and when blotting for caspases a 15% gel was used. Polyacrylamide gels were run for ~ 40 min at 200 volts in a Mini-PROTEAN® 3 Cell (BioRad, Hercules, CA) in electrode buffer (25 mM Tris, 192 mM glycine, 1% SDS). Next, proteins were transferred to nitrocellulose membrane in a BioRad transfer apparatus for 1 h 15 min at 100 volts using the Trans-Blot® Electrophoretic Transfer Cell (BioRad) with SDS-PAGE Gel buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3). Nitrocellulose membranes were incubated in 5% non-fat dry milk/PBS for 1 h at 22°C with gentle shaking. Nitrocellulose membranes were rinsed in PBS before being incubated with primary antibody overnight.

The primary antibodies were diluted in 1% BSA in PBS. Nitrocellulose membranes were incubated with primary antibody overnight at 4°C with

gentle shaking. Antibodies and dilutions used were mouse 1:500 anti-Apaf-1 (clone 94408, R&D Systems, Minneapolis, MN), 1:1,000 rabbit anti-Bax (Cell Signaling Technology, Danvers, MA), 1:1,000 rabbit anti-Bak NT (Millipore), 1:500 mouse anti-Bcl-2 (DakoCytomation, Glostrup, Denmark), 1:1,000 rabbit anti-Bcl-x_L (clone 54H6, Cell Signaling), 1:5,000 mouse anti-β-actin (clone AC-15, Sigma), 1:1,000 rabbit anti-Bid (Cell Signaling), 1:1,000 rabbit anti-caspase-3 (clone 8G10, Cell Signaling), 1:1,000 rabbit anti-caspase-6 (Cell Signaling), 1:1,000 rabbit anti-caspase-7 (Cell Signaling), 1:1,000 mouse anti-caspase-8 (clone 1C12, Cell Signaling), 1:1,000 rabbit anti-caspase-9 (Cell Signaling), 1:1,000 mouse anti-cytochrome *c* (clone 7H8.2C12, Pharmingen), 1:5,000 rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (Trevigen, Gaithersburg, MD), 1:500 mouse anti-Myc (clone 9E10, Santa Cruz Biotechnology), 1:1,000 mouse anti-Smac/DIABLO (Cell Signaling), and 1:1,000 mouse anti-XIAP (clone 28, BD Transduction Laboratories).

Nitrocellulose membrane was washed with 1X PBS for 10 min, 1X PBST for 10 min, and 1X PBS for 10 min all at room temperature (22°C) with gentle shaking to remove excess primary antibody. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature (22°C) with gentle shaking. Secondary antibodies were diluted to 1:10,000 in 2.5% non-fat dry milk in PBS. Secondary antibodies used included goat anti-mouse IgG, (H+L) and goat anti-rabbit IgG (Thermo Scientific, Pierce Biotechnology). Next, nitrocellulose membranes were

washed with 1X PBS for 10 min, 1X PBST for 10 min and 1X PBS for 10 min all at room temperature (22°C) with gentle shaking. Afterward, the nitrocellulose membrane was patted dry and incubated with 3 ml of 50:50 ECL Detection Reagent 1:ECL Detection Reagent 2 (Amersham, Buckinghamshire, UK) for 1-2 min before developing using classic autoradiography film BX (MidSci, St. Louis, Missouri).

3.6 Subcellular Fractionation

Subcellular fractionation was used to determine whether mitochondria released pro-apoptotic factors (cytochrome *c* and Smac) into the cytosol. Cells (10^6) were centrifuged at 300 x g for 5 min at room temperature (22°C) and washed in cold PBS. Cells were then centrifuged again at 300 x g for 5 min at room temperature (22°C). Cells were resuspended in 50 µl of buffer (140 mM mannitol, 46 mM sucrose, 50 mM KCl, 1 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EGTA, 5 mM Tris, pH 7.4) supplemented with a mixture of protease inhibitors (Complete Mini-EDTA Free), and permeabilized with 3 µg of digitonin (Sigma) on ice for 10 min. Plasma membrane permeabilization was monitored by trypan blue staining. Cell suspensions were centrifuged at 12,000 x g for 10 min at 4°C to separate the cytosol (supernatant) from the mitochondria (pellet). Supernatant and pellet fractions were subjected to Western blot analysis.

3.7 Determination of Bak Oligomerization

Bak oligomerization can be visualized by Western Blot analysis after incubating with a cross-linking agent (Wei et al., 2000). For detection of Bak oligomerization, cells (2.5 x 10^6) were harvested after treatment with drug by centrifugation at 300 x g for 5 min at room temperature (22°C). Cells were then washed in cold PBS and centrifuged for 5 min at 300 x g at room temperature (22°C). Cells were then resuspended in 200 µl of 100 mM EDTA/PBS and incubated with the cross-linking agent bismaleimidohexane (BMH) (1 mM). After the addition of BMH, tubes were mixed by inverting 2-3X before incubation for 30 min at room temperature (22°C) while rocking. The reaction was quenched with 100 mM DTT for 15 min at room temperature (22°C) while rocking. The cell suspension was then centrifuged at 500 x g for 5 min and processed for Western blot analysis. Protein concentration of cell lysate is unable to be determined due to the BMH, therefore approximately 2 X 10^6 cells were loaded onto a 12% polyacrylamide gel.

3.8 Scanning Electron Microscopy

Cells (10^6) were washed in PBS and pelleted. Pellet was fixed in fixative (2% gluteraldehyde in 0.1 M sodium cacodylate, pH 7.4) for 20 min. Cells were washed twice (0.1 M sodium cacodylate, pH 7.4) before being

post-fixed for 1 h (1% osium tetroxide buffer in 0.1 M sodium cacodylate, pH 7.4). Cells were washed three times with dH_2O and dehydrated in a series of ethanol washes as follows: 30%, 70%, 80%, 95%, 100%, 100% for 10 min each. Next, cells were incubated in propylene oxide for 10 min. Cell pellets were then infiltrated overnight in 1:1 ratio of propylene oxide:embed 812 resin medium mixture (Electron Microscopy Sciences, Inc). Cell pellets were then incubated for 1 h in Embed 812 resin mixture. Subsequently, cells were placed in flat embedding molds with fresh resin and cured overnight in 60^{o}C oven. Sections were cut (80 nm) using a Diatome diamond knife on a Leica UCT ultramicrotome. Sections were then stained with 1% uranyl acetate and Sato's modified lead citrate. Lastly, sections were viewed and photographed using a J.E.O.L. 100CXII Transition Electron Microscopy at 80 KV.

CHAPTER 4: CASPASE-MEDIATED BAK ACTIVATION AND CYTOCHROME *C* RELEASE DURING INTRINSIC APOPTOTIC CELL DEATH IN JURKAT CELLS

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4.1 Abstract

MOMP and the release of intermembrane space proteins, such as cytochrome *c*, are early events during intrinsic (mitochondria-mediated) apoptotic signaling. Although this process is generally accepted to require the activation of Bak or Bax, the underlying mechanism responsible for their activation during true intrinsic apoptosis is not well understood. In the current study, we investigated the molecular requirements necessary for Bak activation using distinct clones of Bax-deficient Jurkat T-lymphocytes in which the intrinsic pathway had been inhibited. Cells stably overexpressing Bcl-2/Bcl-x_L or stably depleted of Apaf-1 were equally resistant to apoptosis induced by the DNA-damaging anticancer drug etoposide as determined by phosphatidylserine externalization and caspase activation. Strikingly, characterization of mitochondrial apoptotic events in all three drug-resistant cell lines revealed that, without exception, resistance to apoptosis was associated with an absence of Bak activation, cytochrome *c* release, and mitochondrial membrane depolarization. Furthermore, we found that

etoposide-induced apoptosis and mitochondrial events were inhibited in cells stably overexpressing either full-length XIAP or the BIR1/BIR2 domains of XIAP. Combined, our findings suggest that caspase-mediated positive amplification of initial mitochondrial changes can determine the threshold for irreversible activation of the intrinsic apoptotic pathway.

4.2 Introduction

Apoptosis is a gene-regulated form of cell death that is critical for normal development and tissue homeostasis. Disruptions in the control of apoptosis can contribute to the onset of various pathological states including cancer, where avoidance of apoptosis confers a survival advantage to tumorigenic cells. Apoptosis is mediated by a family of cysteine proteases that cleave after aspartate residues (caspases) and can be activated by two distinct signaling pathways.

The intrinsic (mitochondria-mediated) pathway is activated by cytotoxic stressors, such as DNA damage, γ-radiation, growth factor withdrawal, and heat. Such stimuli are known to cause MOMP and stimulate the release of cytochrome *c*, Smac, (also known as DIABLO), and Omi (also known as HtrA2) into the cytosol, where they work together to activate the initiator pro-caspase-9 within the apoptotic protease-activating factor-1 (Apaf-1) apoptosome complex (Li et al., 1997) . Once activated, caspase-9 activates

effector pro-caspase-3 or -7, which, in turn, can cleave various protein substrates, leading to the morphological and biochemical features of apoptosis.

The process of MOMP is generally thought to require the activation of a multidomain Bcl-2 family protein, notably Bax or Bak (Chipuk and Green, 2006; Ow et al., 2008). Cells deficient in either Bax or Bak display relatively minor defects in apoptosis, whereas doubly deficient cells are often found to be highly resistant to mitochondria-mediated apoptosis induced by a variety of stimuli (Lindsten et al., 2000; Wei et al., 2001). Normally, these proteins exist as inactive monomers either in the cytosol (Bax) or associated with the mitochondria (Bak). Activation of Bax and Bak coincides with their homo-oligomerization, which is thought to occur by one of two mechanisms. One model argues for the existence of “sensitizer” Bcl-2 homology 3 (BH3)-only proteins, such as Noxa and Bad, that can displace “activator” BH3-only Bcl-2 family proteins, such as Bim, truncated Bid (tBid), and Puma, from a prosurvival protein, such as Bcl-2 or Bcl-x_L, to activate Bak or Bax (Kim et al., 2006a). The second model indicates that BH3-only proteins bind and neutralize prosurvival Bcl-2 proteins that normally keep Bak or Bax inactive (Chen et al., 2005; Willis et al., 2007) . A well characterized example of Bak or Bax activation being induced by a BH3-only protein is during receptor-mediated apoptosis in so-called type II cells. In this case, death receptor ligation activates caspase-8, which, in turn, cleaves the cytosolic BH3-only

protein Bid to tBid (Li et al., 1998; Luo et al., 1998), resulting in the direct or indirect activation of Bak or Bax. A similar emergent understanding of a mechanism responsible for the activation of Bak/Bax during true mitochondria-mediated apoptosis is currently lacking.

In this regard, the aim of the current study was to investigate the signaling requirements for MOMP during intrinsic apoptosis, as well as to help define the elusive threshold a cell must cross in order to be irreversibly committed to undergo this form of cell death. We have engineered several clones of Bax-deficient Jurkat cells in which the intrinsic pathway is inhibited due to the stable overexpression of Bcl-2 or Bcl-x_L, the stable knockdown of Apaf-1, or the overexpression of full-length XIAP or the baculoviral inhibitor of apoptosis protein repeat 1 and 2 (BIR1/BIR2) domains of XIAP. The results indicated that all five of the gene-manipulated Jurkat cell lines were similarly resistant to etoposide-induced apoptosis. As anticipated, Bcl-2/Bcl-x_L-overexpressing cells were refractory to all etoposide induced mitochondrial events. Strikingly, however, Apaf-1-silenced cells and cells overexpressing full-length XIAP or the BIR1/BIR2 domains of XIAP were also found to be impaired in their ability to activate Bak, release cytochrome *c*, or lose mitochondrial membrane potential ($\Delta\Psi$) in response to etoposide.

4.3 Results and Discussion

4.3.1 Blockade of the Intrinsic Pathway by Depleting Apaf-1 or Overexpressing Bcl-2/Bcl-x_L Completely Inhibits Etoposide-Induced Apoptosis in Jurkat Cells

The largest body of evidence indicates that MOMP and the release of intermembrane space proteins, such as cytochrome *c*, involves the activation of multidomain pro-apoptotic Bcl-2 family proteins Bak and Bax (Chipuk et al., 2006; Ow et al., 2008). Although it is clear that active caspase-8 within the extrinsic apoptotic pathway can activate Bak or Bax by cleaving the BH3- only protein Bid to tBid (Garrido et al., 2006; Li et al., 1998; Luo et al., 1998), a similar prominent role for one or more caspases in modulating MOMP during true intrinsic apoptosis is not appreciated. In some instances, incubation of cells with the topoisomerase II inhibitor etoposide was found to induce caspase-8-mediated cleavage of Bid by stimulating an up-regulation of Fas ligand that, in turn, activated the Fas receptor (Friesen et al., 1996; Kasibhatla et al., 1998; Petak et al., 2000) . However, other studies, including our own unpublished results, have shown that caspase-8-deficient cells are sensitive to etoposide-induced apoptosis (Juo et al., 1998). Other evidence has suggested that caspase-2 may be able to activate Bax by first cleaving Bid in response to stress (Bonzon et al., 2006; Gogvadze et al., 2006; Guo et al.,

2002; Upton et al., 2008), although it was also shown that Bid is a relatively poor substrate for caspase-2 (Bonzon et al., 2006; Guo et al., 2002). In addition, we recently demonstrated that Apaf-1 is required for etoposide-induced activation of all caspases, suggesting that most, if not all, caspase activation occurs downstream of apoptosome formation (Franklin and Robertson, 2007).

To investigate the molecular requirements for etoposide-induced MOMP, we initially used the same Apaf-1-deficient Jurkat cells described previously (Figure 12*A*, *lane 5*) (Franklin and Robertson, 2007), as well as cells in which the intrinsic pathway had been inhibited due to the overexpression of either Bcl-x_L or Bcl-2 (*lanes 2* and *3*). As illustrated in Figure 12*A*, manipulating the level of expression of Apaf-1, Bcl-2, or Bcl-x_L did not result in any alteration in the expression of the other two proteins. According to Figure 12*B*, control-transfected Jurkat cells were induced to undergo apoptosis (~30%), as determined by annexin V-FITC labeling of externalized phosphatidylserine on the plasma membrane, when incubated in the presence of a clinically relevant concentration of etoposide (10 μM) for 6 h. By comparison, Jurkat cells in which either Apaf-1 expression had been silenced or an anti-apoptotic Bcl-2 family protein had been overexpressed were totally protected from etoposide-induced apoptosis (Figure 12*B*). In agreement with these findings, Western blot analysis of cell lysates obtained at 6 h post-etoposide treatment revealed that proteolytic processing of pro-

Figure 12. Inhibiting the Intrinsic Pathway by Bcl-2/Bcl-x_L Overexpression or Apaf-1 Knockdown Blocks Etoposide-Induced Apoptosis in Jurkat Cells.

A, wild-type, control-transfected, Bcl-x_L- and Bcl-2-overexpressing, and Apaf-1-deficient Jurkat clones were harvested and lysed for Western blotting. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a loading control. *B*, cells (10^6 /ml) were cultured with DMSO or 10 μM etoposide for 6 h and processed for cell death determination by flow cytometric analysis of annexin V-FITC and propidium iodide staining. Quadrants are defined as live (*lower left*), early apoptotic (*lower right*), late apoptotic (*upper right*), and necrotic (*upper left*). *Numbers* refer to the percentage of cells in each quadrant. *C*, duplicate aliquots of cells in *B,* were harvested and lysed for Western blotting. *shRNA, short hairpin RNA; Casp, caspase.*

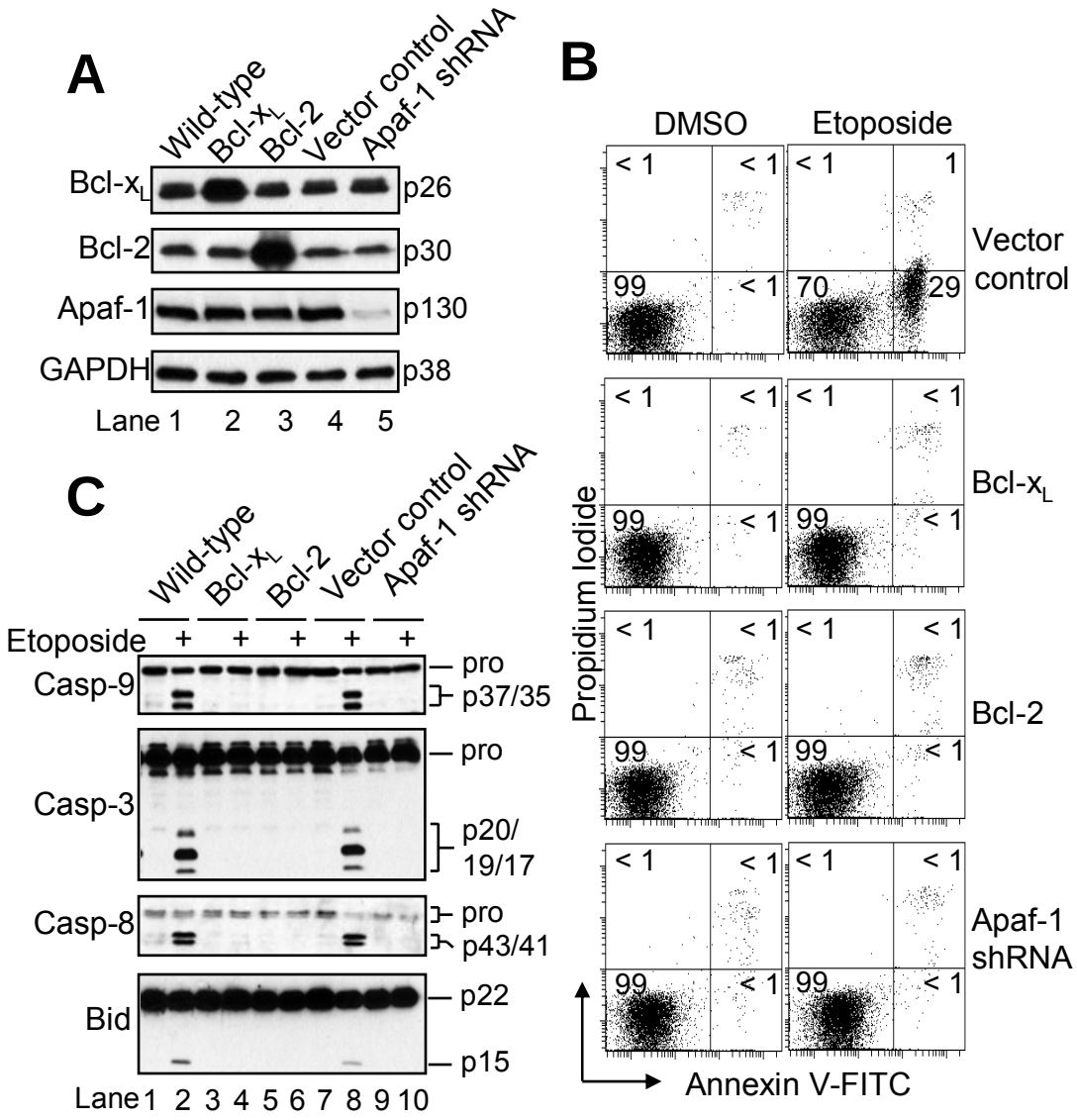
A
Wild-type
Bcl-xL
Bcl-2
Vector control
Apaf-1 shRNA
Bcl-xL
p26
Bcl-2
p30
Apaf-1
p130
GAPDH
p38
Lane 1 2 3 4 5
B
DMSO
Etoposide
Vector control
Bcl-xL
Bcl-2
Apaf-1 shRNA
Propidium Iodide
Annexin V-FITC
C
Wild-type
Bcl-xL
Bcl-2
Vector control
Apaf-1 shRNA
Etoposide
Casp-9
pro
p37/35
Casp-3
pro
p20/
19/17
Casp-8
pro
p43/41
Bid
p22
p15
Lane 1 2 3 4 5 6 7 8 9 10

Figure 12.

caspase-8, -9, and -3 occurred in wild-type and control-transfected cells, but not in any of the three gene-manipulated clones (Figure 12*C*). In addition, cleavage of Bid to tBid was observed only in etoposide-treated wild type and vector control cells, suggesting that Bid cleavage occurs downstream of the apoptosome. Taken together, these data clearly demonstrate that silencing Apaf-1 or overexpressing Bcl-2/Bcl-x_L results in a total blockade of the intrinsic pathway in Jurkat cells.

4.3.2 Bak Activation Is Impaired in Apaf-1-Deficient Cells Treated with Etoposide

In many settings, cytochrome *c* release is a rapid, complete, and irreversible event that represents the "point of no return" within the intrinsic apoptotic pathway (Goldstein et al., 2000). In addition, it is generally accepted that caspase-9 activation requires cytochrome *c*-mediated oligomerization of Apaf-1 and that cytochrome *c* release occurs independently of downstream caspase activity (Bossy-Wetzel et al., 1998; Li et al., 1997; Susin et al., 1999). In this regard, cells deficient in Apaf-1, caspase-9, or executioner caspases-3 and -7 would be expected to undergo MOMP and release intermembrane space proteins with the same kinetics as wild-type cells. However, some evidence is at odds with such a view of the intrinsic pathway. For instance, it has been shown that pro-caspases can be activated

by a cytochrome *c*- and apoptosome-independent, but Apaf-1-dependent, mechanism in thymocytes exposed to γ-radiation (Hao et al., 2005). Also, Lakhani *et al.* (2006) demonstrated that caspase-3 and -7 are key mediators of Bax-directed mitochondrial apoptotic events in response to ultraviolet radiation.

Having demonstrated that Jurkat cells in which either an anti-apoptotic Bcl-2 family protein had been overexpressed or Apaf-1 had been knocked down, were equally impaired in their ability to activate caspases, we next evaluated the different cells for changes in ΔΨ. As illustrated in Figure 13*A*, a partial loss of ΔΨ in response to 10 μM etoposide was detected in control-transfected Jurkat cells, which activated caspases (Figure 12*C*), whereas Apaf-1-deficient and Bcl-2/Bcl-x_L-overexpressing cells did not lose ΔΨ. These data are consistent with evidence indicating that the loss of ΔΨ during apoptotic cell death requires caspase mediated proteolytic inactivation of the respiratory complex I subunit NDUSF1 (Ricci et al., 2004). Because a drop in ΔΨ is often associated with the release of mitochondrial intermembrane space proteins into the cytosol, we next investigated whether the release of cytochrome *c* and Smac had occurred in the different cell types. Consistent with the membrane potential results, etoposide treatment stimulated a strong release of cytochrome *c* and Smac only in vector control cells (Figure 13*B*, *lane 6 versus 5*). By comparison, cells overexpressing either Bcl-2 or Bcl-x_L failed to release cytochrome *c* or Smac in response to etoposide (Figure 13*B*,

Figure 13. Apaf-1 is Essential for MOMP Induction and Bak Activation in Response to Etoposide.

A, control transfected, Bcl-x_L- and Bcl-2 overexpressing, and Apaf-1-deficient Jurkat clones (10^6/ml) were cultured in the presence or absence of DMSO or 10 μM etoposide for 6 h, harvested, and processed for mitochondrial membrane potential (ΔΨ) determination by flow cytometry. Reduced $DiIC_1(5)$ fluorescence is indicative of a loss of ΔΨ, and *numbers* refer to the percentage of cells that underwent a dissipation of ΔΨ. *B*, duplicate aliquots of cells in *A* were harvested and processed for subcellular fractionation. Supernatant (*s*) and pellet (*p*) fractions were analyzed by Western blotting. *C*, whole-cell lysates of Jurkat (E6.1) and MCF-7 (positive control for Bax expression) cells were separated by SDS-PAGE and Western-blotted. *D* and *E*, wildtype, control-transfected, Bcl-x_L- and Bcl-2-overexpressing, and Apaf-1-deficient Jurkat clones (10^6/ml) were cultured in the absence or presence of DMSO or 10 μM etoposide for 6 h and processed for determination of Bak oligomerization by Western blotting (*D*) or Bak activation by flow cytometric analysis (*E*). *Numbers* in *E* refer to the percentage increase in Bak-associated fluorescence between etoposide- and DMSO-treated samples. *MOMP, mitochondrial outer membrane permeablization; shRNA, short hairpin RNA; BMH, bismaleimidohexane; Cyt c, cytochrome c*

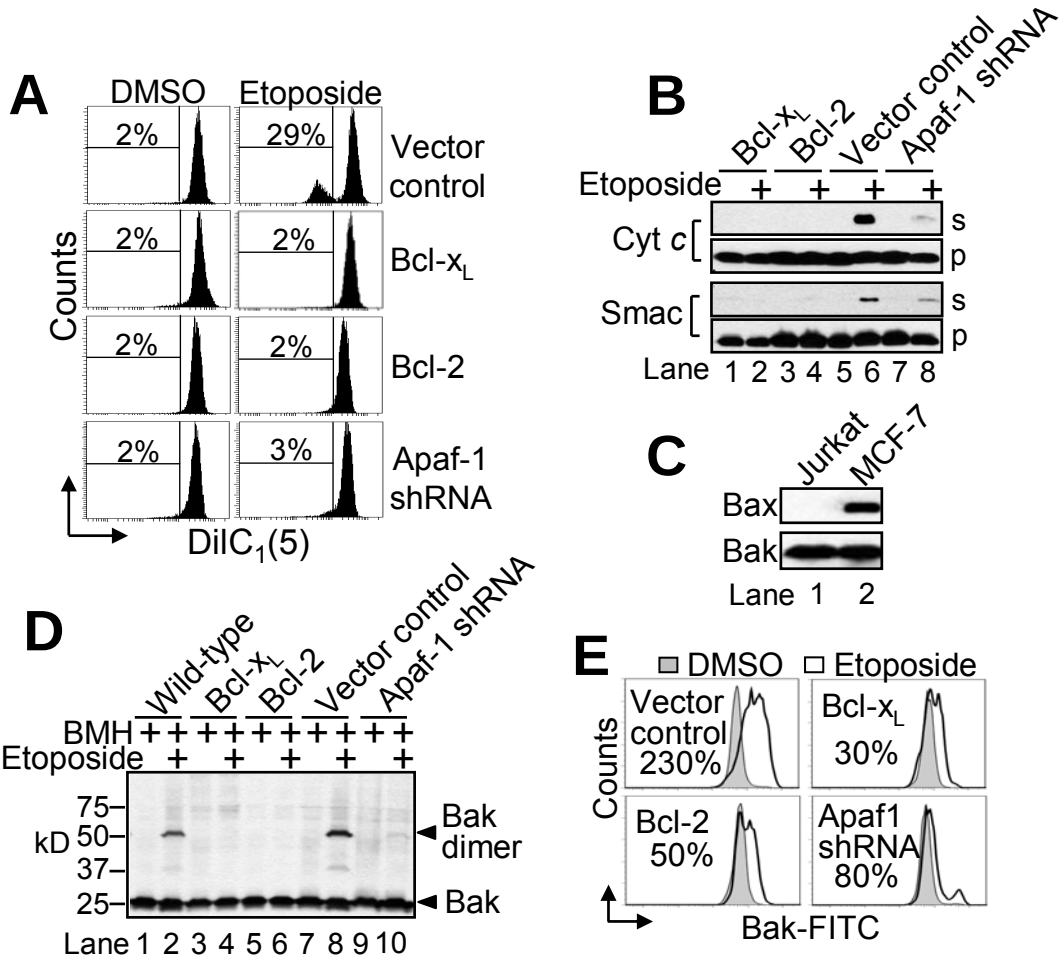
A
DMSO
Etoposide
2%
29%
Vector control
Bcl-xL
Bcl-2
Apaf-1 shRNA
Counts
DilC1(5)
B
Bcl-xL
Bcl-2
Vector control
Apaf-1 shRNA
Etoposide
Cyt c
Smac
s
p
Lane 1 2 3 4 5 6 7 8
C
Jurkat
MCF-7
Bax
Bak
Lane 1 2
D
Wild-type
Bcl-xL
Bcl-2
Vector control
Apaf-1 shRNA
BMH
Etoposide
75–
kD 50–
37–
25–
Bak dimer
Bak
Lane 1 2 3 4 5 6 7 8 9 10
E
DMSO
Etoposide
Vector control 230%
Bcl-xL 30%
Bcl-2 50%
Apaf1 shRNA 80%
Counts
Bak-FITC

Figure 13.

lanes 1–4), and Apaf-1-deficient cells released only a trace of each protein (*lane 8 versus 7*).

Numerous studies from other laboratories have shown that the induction of MOMP is tightly controlled by pro- and anti-apoptotic members of the Bcl-2 family of proteins (Chipuk and Green, 2006; Scorrano and Korsmeyer, 2003). In fact, it is often the ratio of pro- to anti-apoptotic proteins that determines the sensitivity of a cell to a given intrinsic apoptotic stimulus. Induction of MOMP by either of the multidomain pro-apoptotic proteins Bax and Bak is thought to require the prior activation of a BH3-only Bcl-2 family protein (Letai, 2008). The anti-apoptotic proteins Bcl-2 and Bcl-x_L prevent MOMP by directly binding Bax/Bak or by preventing their activation by inhibiting BH3-only proteins (Youle and Strasser, 2008). As mentioned earlier, Bax is largely cytosolic in healthy cells, whereas Bak resides on the outer mitochondrial membrane. Both proteins are monomeric in their inactive state, and activation coincides with their homo-oligomerization to form protein-permeable pores in the outer mitochondrial membrane.

To investigate the extent to which differences in the release of cytochrome *c* and Smac corresponded to differences in the activation of Bax or Bak, we first evaluated the different cell clones for the expression of Bax and Bak. Interestingly, Western blot results revealed that Jurkat (E6.1) cells express Bak, but not Bax, protein (Figure 13*C*, *lane 1*). Thus, to evaluate changes in Bak under the different conditions, we first investigated whether

Bak underwent oligomerization using the crosslinking agent BMH. As illustrated in Figure 13*D*, cross-linked Bak complexes consistent with dimers were observed in wild-type (*lane 2*) and control-transfected (*lane 8*) Jurkat cells treated with 10 μM etoposide for 6 h. These findings were in agreement with results obtained using an active conformation-specific monoclonal Bak antibody and flow cytometric analysis, where Bak activation amounts to a shift to the right in the resulting histogram (Figure 13*E*). Consistent with previous reports, etoposide-induced oligomerization (Figure 12*D*) and activation (Figure 13*E*) of Bak were blocked in cells overexpressing Bcl-2 or Bcl-x_L, perhaps because of their reported ability to sequester activator BH3-only proteins (Kim et al., 2006a) or to bind Bak directly (Chen et al., 2005; Willis et al., 2007). Significantly, Bak oligomerization and activation were also strongly inhibited in Apaf-1-deficient Jurkat cells (Figure 13*D*, *lane 10 versus 9*, and 13*E*), which likely accounts for the lack of cytochrome *c* and Smac release in these cells following etoposide treatment (Figure 13*B*, *lane 8 versus 7*). Combined, these data raise the distinct possibility that signaling events, which are widely believed to be post-mitochondrial, are essential for Bak-mediated cytochrome *c* release and dissipation of $\Delta\Psi$ during true intrinsic apoptotic cell death.

4.3.3 Overexpression of Full-Length XIAP or the BIR1/BIR2 Domains of XIAP Inhibits Etoposide-Induced Apoptosis

Because Bak activation and MOMP were strongly inhibited in Apaf-1-deficient cells, we were next interested to determine whether this was due to a direct role of Apaf-1 in mediating MOMP or whether the absence of Bak activation and the release of intermembrane space proteins were attributable to the lack of etoposide-induced caspase activation. Because of the reported problems with the selectivity of “specific” peptide based caspase inhibitors (Berger et al., 2006; McStay et al., 2008), we developed a genetic strategy to inhibit caspases residing downstream of apoptosome formation. The approach was to modulate the expression of XIAP.

XIAP is a member of the inhibitor of apoptosis protein family that potently inhibits apoptotic cell death by specifically targeting caspase-9, -3, and/or -7 (Deveraux et al., 1997). Recent work has described the regions of XIAP that are responsible for its caspase inhibitory properties (Scott et al., 2005; Shiozaki et al., 2003). Specifically, a region of XIAP that includes the BIR2 domain inhibits caspase-3 and -7, whereas the BIR3 domain inhibits caspase-9. In a cell dying by apoptosis, the anti-apoptotic activity of XIAP can be inhibited by two mitochondrial intermembrane space proteins, namely Smac (Du et al., 2000; Verhagen et al., 2000) and Omi (Hegde et al., 2002;

Suzuki et al., 2001; Verhagen et al., 2002) that are released into the cytosol, along with cytochrome *c*, following the induction of MOMP.

For our studies, we generated Jurkat cells that overexpressed either full-length XIAP (Figure 14*A*, *left panel*) or the BIR1/BIR2 domains of XIAP (*right panel*). We hypothesized that, if full-length XIAP inhibited Bak activation and MOMP, then cells expressing only the BIR1/BIR2 domains would shed light on whether this was largely a caspase-9- or caspase-3/7-mediated effect. The primary justification for expressing BIR1/BIR2, instead of BIR2 alone, is that XIAP-BIR1/BIR2 has been shown to express better in mammalian cells than XIAP-BIR2 alone (Scott et al., 2005). As illustrated in Figure 14*B*, vector control cells underwent apoptosis (~48%) when incubated in the presence of 10 μM etoposide for 6 h. By comparison, cells overexpressing full-length XIAP or the BIR1/BIR2 domains underwent ~8 and ~13% apoptosis, respectively. Western blot analysis of cell lysates generated after 6 h of etoposide incubation revealed that extensive cleavage of caspase-9, -3, and -7 had occurred, as well as the partial disappearance of the precursor form of caspase-6, only in control-transfected cells (Figure 14*C*). Overall, these findings demonstrate that cells overexpressing either XIAP or the BIR1/BIR2 domains of XIAP are similarly protected from etoposide-induced apoptosis.

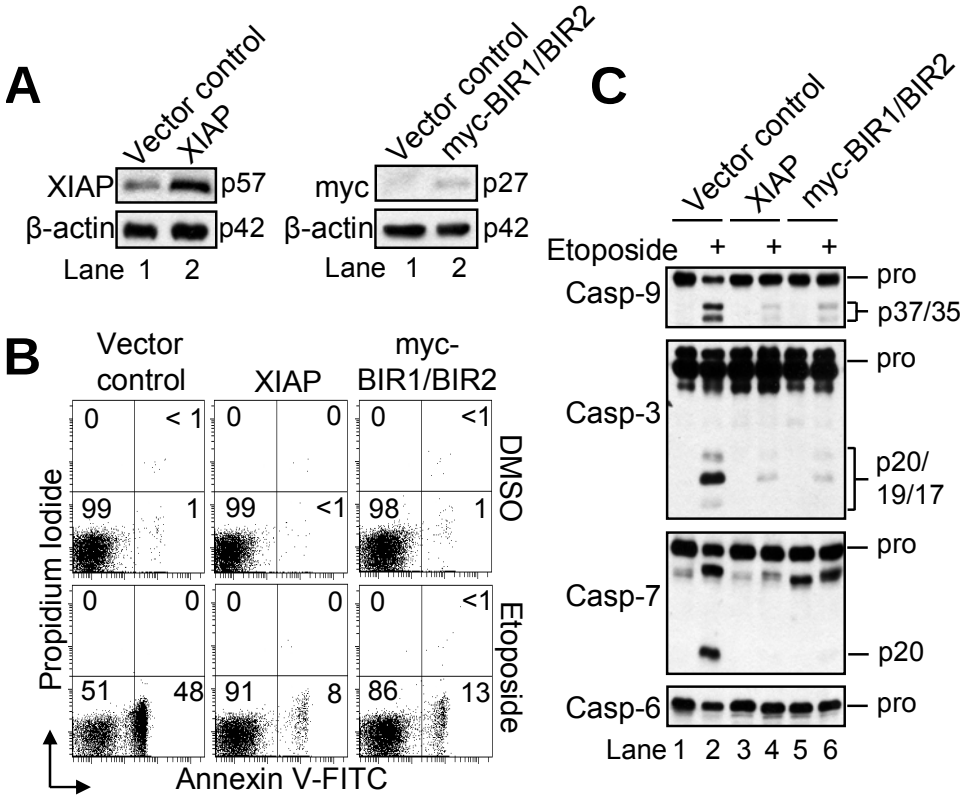

Figure 14. Overexpression of XIAP or Myc-BIR1/BIR2 Inhibits Etoposide-Induced Apoptosis.

A, control transfected, XIAP-overexpressing, and Myc-BIR1/BIR2-overexpressing Jurkat clones were harvested and lysed for Western blotting.-Actin was used as a loading control. *B*, cells (10^6/ml) were cultured with DMSO or 10 μM etoposide for 6 h and processed for cell death determination by flow cytometric analysis of annexin V-FITC and propidium iodide staining. Quadrants are defined as live (*lower left*), early apoptotic (*lower right*), late apoptotic (*upper right*), and necrotic (*upper left*). *Numbers* refer to the percentage of cells in each quadrant. *C*, duplicate aliquots of cells in *B* were harvested and lysed for Western blotting. *Casp*, caspase.

4.3.4 Activation of Bak and Induction of MOMP are Dependent on Downstream Caspase Activation

Once we had demonstrated that cells expressing either of the two XIAP constructs were similarly resistant to etoposide-induced apoptosis, the next step was to determine the extent to which this involved an inhibition of mitochondrial apoptotic events. As illustrated in Figure 15*A*, slightly more than half of the etoposide-treated vector control cells experienced a loss of ΔΨ, whereas XIAP- and BIR1/BIR2-overexpressing cells underwent a more modest drop in ΔΨ of ~10 and 11%, respectively. In agreement with the ΔΨ findings, vector control cells were triggered to undergo etoposide-induced MOMP as assessed by Western blot analysis of cytochrome *c* and Smac release into the cytosol (Figure 15*B*, *lane 2 versus 1*). By comparison, XIAP overexpressors, and to a slightly lesser extent BIR1/BIR2-expressing cells, were refractory to etoposide-induced cytochrome *c* and Smac release (Figure 15*B*, *lanes 3*–*6*). Finally, we were interested to know whether the inhibition of cytochrome *c* and Smac release in the XIAP- and BIR1/BIR2-overexpressing cells coincided with a similar inhibition of Bak activation in cells incubated with etoposide. As shown in Figure 15*C*, the results indicated that incubation of vector control cells with 10 μM etoposide for 6 h induced large-scale Bak oligomerization (*lane 2*). By comparison, etoposide-treated XIAP-overexpressing cells failed to activate Bak (Figure 15*C*, *lane 4*), and only a

Figure 15. Downstream Caspases Play an Integral Role in Bak Activation and MOMP Induction.

A, control-transfected, XIAP-overexpressing, and Myc-BIR1/BIR2-expressing Jurkat clones (10^6/ml) were cultured in the presence or absence of DMSO or 10 μM etoposide for 6 h, harvested, and processed for determination by flow cytometry. Reduced $DiIC_1(5)$ fluorescence is indicative of a loss of, and *numbers* refer to the percentage of cells that underwent a dissipation of ΔΨ. *B*, duplicate aliquots of cells in *A* were harvested and processed for subcellular fractionation. Supernatant (*s*) and pellet (*p*) fractions were analyzed by Western blotting. *C*, cells were cultured as described in *A* and processed for determination of Bak oligomerization by Western blotting. *ΔΨ, mitochondrial membrane potential; BMH, bismaleimidohexane; Cyt c,* cytochrome *c*; MOMP, mitochondrial outer membrane permeabilization.

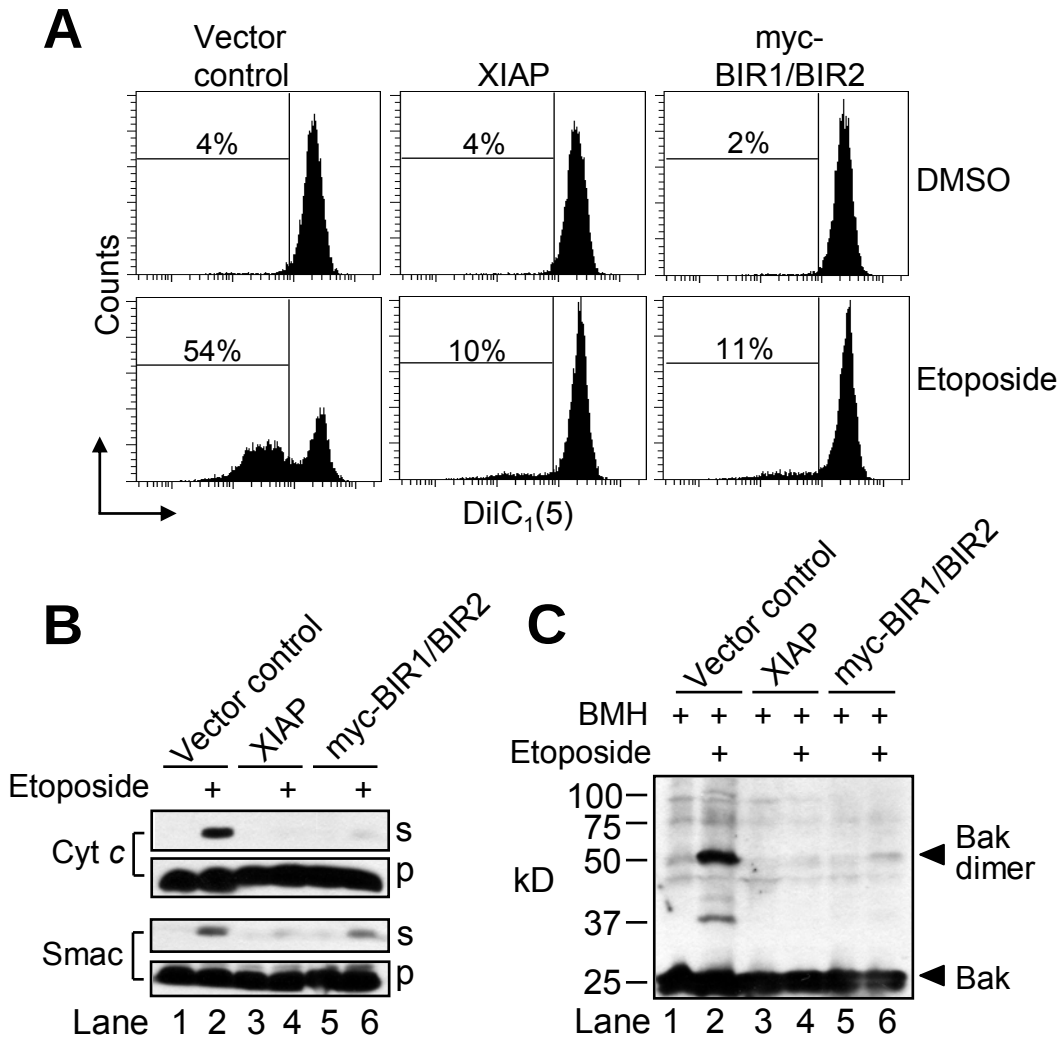
A
Vector control
XIAP
myc-BIR1/BIR2
4%
4%
2%
DMSO
54%
10%
11%
Etoposide
Counts
DiIC1(5)
B
Vector control
XIAP
myc-BIR1/BIR2
Etoposide + + +
Cyt c
s
p
Smac
s
p
Lane 1 2 3 4 5 6
C
Vector control
XIAP
myc-BIR1/BIR2
BMH + + + + + +
Etoposide + + +
kD
100
75
50
37
25
Bak dimer
Bak
Lane 1 2 3 4 5 6

Figure 15.

trace amount of active Bak was detected in BIR1/BIR2-expressing cells (*lane 6*). Taken together, these findings strongly suggest that downstream caspases play an essential role in the activation of Bak and in the induction of MOMP during etoposide-induced apoptosis. They also imply that the reason etoposide-induced mitochondrial apoptotic events were inhibited in Apaf-1-deficient cells is due to the fact that caspases were not activated, rather than any direct role for Apaf-1 in mediating Bak activation or MOMP.

4.4 Concluding Remarks

Ever since Wang and colleagues (Liu et al., 1996) made the seminal observation that the induction of the apoptotic program in cell-free extracts requires cytochrome *c* (and dATP), a major effort has been made to characterize the molecular mechanisms responsible for the regulation of the intrinsic (mitochondria-mediated) apoptotic pathway. A more complete understanding of how this pathway is regulated has ensued, and it is now widely accepted that various cytotoxic stressors kill cells by activating the intrinsic pathway.

As mentioned previously, one of the earliest events during true intrinsic apoptosis is the activation of Bax and/or Bak (Wei et al., 2001). Under unstressed conditions, Bax and Bak can be found in the cytosol and on the outer mitochondrial membrane, respectively, as monomers (Youle and

Strasser, 2008). Activation of Bax and Bak is marked by their homo-oligomerization to form pores in the outer mitochondrial membrane through which intermembrane space proteins, including cytochrome *c*, can be released into the cytosol to promote apoptosome-dependent activation of caspases. Although caspase-8-mediated cleavage of Bid to tBid is known to play an important role in the activation of Bak or Bax during receptor-mediated apoptosis in type II cells, the underlying mechanism responsible for the activation of these multidomain pro-apoptotic proteins within the intrinsic apoptotic pathway is not well understood. In cells with normal p53, genotoxic anticancer drugs have been suggested to activate Bax/Bak because of a transcriptional up-regulation of Puma (Jeffers et al., 2003; Villunger et al., 2003) . However, because cancer cells, including Jurkat (E6.1), are often defective in their p53 signaling pathway, a general non-redundant role for Puma up-regulation seems unlikely. In fact, most evidence in the literature indicates that stress-induced activation of Bak and Bax requires the presence of either Bid or Bim, irrespective of p53 status (Letai, 2008). However, insight into the precise mechanism(s) responsible for the activation of these two BH3-only proteins in response to genotoxic injury is currently lacking.

A common view of the mitochondrial apoptotic pathway is that cytochrome *c* release is an all-or-none phenomenon in that, once a single mitochondrion in an “apoptosing” cell has released its cytochrome *c*, all mitochondria in that cell will release all of their cytochrome *c* within about 10

min (Chipuk et al., 2006; Goldstein et al., 2005; Goldstein et al., 2000; Waterhouse et al., 2001). Two additional viewpoints are (i) pro-caspase-9 activation occurs in a cytochrome *c*-dependent manner involving the formation of an Apaf-1 apoptosome complex, and (ii) the release of cytochrome *c* and other intermembrane space proteins from mitochondria occurs by a mechanism that is independent of downstream caspases (Bossy-Wetzel et al., 1998; Hao et al., 2005; Li et al., 1997; Sun et al., 1999b; Waterhouse et al., 2001; Wilkinson et al., 2004). However, our observations, as well as other lines of evidence, do not support a strictly *linear* view of the mitochondrial apoptotic pathway. As mentioned previously, a cytochrome *c*- and apoptosome-independent, but Apaf-1-dependent, mechanism leading to caspase activation has been described (Hao et al., 2005). Also, there is evidence that caspase-3 and -7 can be key mediators of Bax-directed mitochondrial apoptotic events in response to UV radiation (Lakhani et al., 2006). Furthermore, our results demonstrate that downstream caspases are required for Bak activation and MOMP induction in response to the genotoxic drug etoposide in Jurkat cells.

In summary, our findings support a model of the intrinsic pathway in which caspase-mediated positive amplification of initial mitochondrial events can function as an essential part of cell death and, as such, may determine whether a given cell dies in response to stress. Furthermore, it is tempting to speculate that at least one reason a single biologically active apoptosome

complex is around 1 MDa in size (Cain et al., 2000; Zou et al., 1999) and consists of seven Apaf-1 and cytochrome *c* molecules coassembling to form a wheel-like complex with seven spokes (Acehan et al., 2002) is to prevent the unintentional activation of caspase-9. In other words, it seems plausible that, in instances in which a weak apoptotic signal induces the release of miniscule amounts of cytochrome *c* or if mitochondria leak trace amounts of cytochrome *c* during normal turnover of these organelles, a cell would not necessarily die unless enough cytochrome *c* had been released to support sufficient apoptosome-mediated activation of caspase-9 molecules. Even then, it is conceivable that activation of caspase-9 might not always signal the end of the road for a cell if the level of activity that ensues is not sufficient to activate enough executioner caspase-3 or -7 molecules to kill a cell outright and/or promote the positive amplification of initial mitochondrial apoptotic events.

CHAPTER 5: CASPASE-9 ACTIVATION BY THE APOPTOSOME IS NOT REQUIRED FOR FAS-MEDIATED APOPTOSIS IN TYPE II JURKAT CELLS

5.1 Abstract

Activation of executioner caspases during receptor-mediated apoptosis in type II cells requires the engagement of the mitochondrial apoptotic pathway. Although it is well established that recruitment of mitochondria in this context involves the cleavage of Bid to tBid, the precise post-mitochondrial signaling responsible for executioner caspase activation is less clear. Here, we used distinct clones of type II Jurkat T-lymphocytes in which the mitochondrial apoptotic pathway had been inhibited to investigate the molecular requirements necessary for Fas-induced apoptosis. Cells overexpressing either Bcl-2 or Bcl-x_L were protected from apoptosis induced by agonistic anti-Fas antibody. By comparison, Apaf-1-deficient Jurkat cells were sensitive to anti-Fas, exhibiting Bid cleavage, Bak activation, the release of cytochrome *c* and Smac, and activation of executioner caspase-3. Inhibiting downstream caspase activation with the pharmacological inhibitor Z-DEVD-fmk or by expressing the BIR1/BIR2 domains of XIAP decreased all anti-Fas-induced apoptotic changes. Additionally, pretreatment of Bcl-x_L-overexpressing cells with a Smac mimetic sensitized these cells to Fas-

induced apoptosis. Combined, our findings strongly suggest that Fas-mediated activation of executioner caspases and induction of apoptosis do not depend on apoptosome-mediated caspase-9 activation in prototypical type II cells.

5.2 Introduction

Apoptosis is an active form of cell death that is executed by a family of cysteine proteases that cleave intracellular substrates after aspartate residues (caspases). Because apoptosis is important for cell removal during development and tissue homeostasis, disruptions in the regulation of apoptosis can play a role in the onset of numerous pathologies, including neurodegenerative disorders, autoimmunity, and cancer (Fadeel et al., 1999).

Apoptosis can occur through two distinct signaling pathways. One is the extrinsic (receptor-mediated) pathway in which binding of a death receptor (*e.g.* TNF-R1 and Fas) by a cognate ligand (*e.g.* TNFα and FasL) or an agonistic antibody (*e.g.* CH-11) causes the recruitment of adaptor proteins (*e.g.* TRADD and FADD) and initiator pro-caspase-8 molecules to the cytosolic side of the receptor to form the death-inducing signaling complex (DISC) (Kischkel et al., 1995). Within the DISC, pro-caspase-8 molecules are activated by dimerization and subsequently undergo autoprocessing (Boatright et al., 2003; Pop et al., 2007). In most cell types (type I), active

caspase-8 activates the executioner caspase-3, which, in turn, is responsible for many of the morphological and biochemical manifestations of apoptosis. In other cell types (type II), the amount of caspase-8 that is activated within the DISC is low and insufficient to directly activate downstream executioner pro-caspases, including caspase-3 and -7 (Scaffidi et al., 1998). Instead, active caspase-8 in type II cells is known to cleave the cytosolic BH3-only protein Bid to tBid (Li et al., 1998; Luo et al., 1998; Shelton et al., 2009). In turn, tBid can activate a multidomain Bcl-2 family protein (*i.e.* Bax and Bak) that stimulates MOMP and the release of intermembrane space proteins into the cytosol (Kim et al., 2006b; Wei et al., 2001). In this regard, it is now widely accepted that the mitochondrial apoptotic pathway plays an important role during receptor-mediated death in type II cells (Li et al., 2002; Samraj et al., 2006; Scaffidi et al., 1998; Scaffidi et al., 1999; Schmitz et al., 1999; Sun et al., 2002; Wilkinson et al., 2004), although conflicting results exist regarding the precise molecular signaling requirements. Some evidence obtained using a reconstituted *in vitro* cell-free system showed that recombinant Smac was sufficient to activate caspase-3 activity by inhibiting XIAP (Sun et al., 2002). A different study using type II lymphoid cells demonstrated that overexpression of XIAP could inhibit Fas-induced apoptosis in wild-type cells, and knocking down XIAP expression could sensitize Bcl-x_L-transfected cells to receptor-mediated cell killing (Wilkinson et al., 2004). Although the results of these two studies suggested that Smac-

dependent inhibition of XIAP was the foremost consequence of MOMP during extrinsic apoptosis in type II cells, neither one of the studies used an experimental system in which a component of the apoptosome was missing. Interestingly, a more recent study reported that a caspase-9-deficient clone of type II lymphoid cells was highly resistant to Fas-induced caspase activation, which, in contrast to the two earlier studies, implicated an essential role for cytochrome *c*-dependent apoptosome formation and the activation of caspase-9 during Fas-induced apoptosis (Samraj et al., 2006).

In this regard, the aim of this study was to clarify the signaling requirements necessary for Fas-mediated apoptosis in prototypical type II cells. Because each of the aforementioned studies was performed using Jurkat cells, we also used clones of this cell line in which the intrinsic pathway had been inhibited due to either stable overexpression of Bcl-2/Bcl-x_L, the stable depletion of Apaf-1, or the stable expression of the baculoviral inhibitor of apoptosis protein repeat 1 and 2 (BIR1/BIR2) domains of XIAP (Shawgo et al., 2008). The data provided direct evidence that apoptosome-mediated caspase-9 activation is not required for extrinsic apoptosis induced by anti-Fas (CH-11). Instead, our findings suggested that the inhibition of XIAP was a better determinant of the susceptibility of type II cells to anti-Fas-induced apoptosis.

5.3 Results and Discussion

5.3.1 Bcl-2 or Bcl-x_L Overexpression, but Not Apaf-1 Knockdown, Confers Resistance to Fas-Induced Apoptosis

Previously, we demonstrated that Jurkat cells, in which either an anti-apoptotic Bcl-2 family protein had been overexpressed or Apaf-1 had been knocked down, were entirely resistant to mitochondria-mediated apoptosis (Shawgo et al., 2008). Here, we were interested in the susceptibility of the different cells to receptor-mediated killing induced by agonist anti-Fas antibody (CH-11). As shown in Figure 16*A*, only control-transfected and Apaf-1-deficient cells underwent Fas-induced apoptosis. Specifically, after treatment with a concentration of 100 ng/ml of anti-Fas, vector control and Apaf-1-silenced cells displayed ~38% and ~25% apoptosis, respectively, at 6 h. By comparison, when the Bcl-2- and Bcl-x_L-overexpressing cells were incubated under the same conditions they exhibited ~2% and ~4% apoptosis, respectively. In agreement with these findings, Western blot analysis of cell lysates revealed that proteolytic processing of caspase-8 was significantly more pronounced in wild-type, vector control, and Apaf-1-deficient cells as compared to Bcl-2- and Bcl-x_L-overexpressing cells (Figure 16*B*). That is, more of both the cleaved intermediate (p43/p41) and active (p18) fragments of caspase-8 were evident after incubation with 100 ng/ml of anti-Fas for 6 h.

Figure 16. Bcl-2 or Bcl-x_L Overexpression, But not Apaf-1 Knockdown, Inhibits Apoptosis in Response to Agonistic anti-Fas Antibody (CH-11). (A) Control-transfected, Bcl-x_L- and Bcl-2-overexpressing, and Apaf-1-deficient Jurkat clones (10^6/ml) were cultured in the presence or absence of 100 ng/ml anti-Fas for 6 h, harvested, and processed for cell death determination by flow cytometric analysis of annexin V-FITC and propidium iodide (PI) staining. Quadrants are defined as: live (lower left); early apoptotic (lower right); late apoptotic (upper right); and necrotic (upper left). *Numbers* refer to the percentage of cells in each quadrant. (B) Duplicate aliquots of cells were harvested and lysed for Western blotting. (C) Bcl-x_L- and Bcl-2-overexpressing cells were cultured in the presence of 100 ng/ml anti-Fas for 24 h, harvested, and processed for cell death determination as described in (A). *shRNA, short hairpin RNA; Casp, caspase; PI, propidium iodide.*

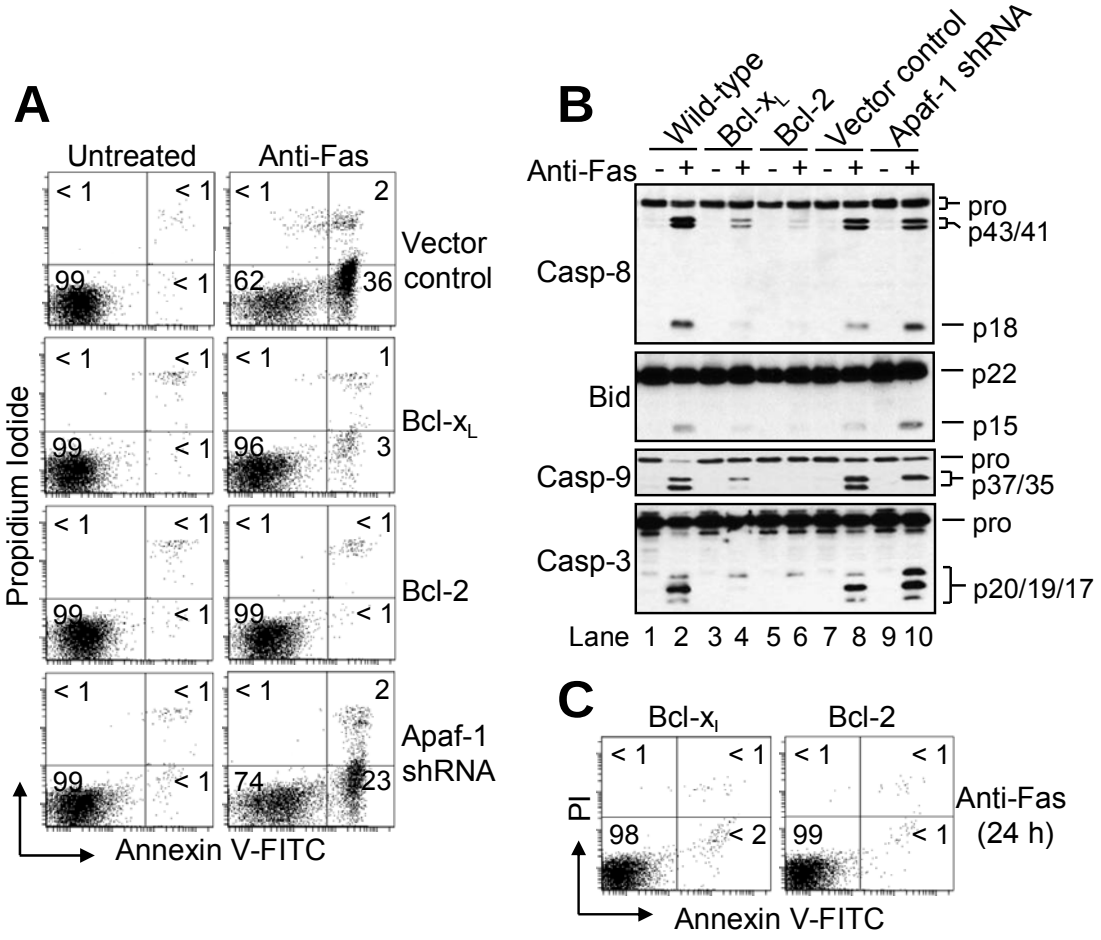
A
Untreated
Anti-Fas
< 1
< 1
< 1
2
99
< 1
62
36
Vector control
< 1
< 1
< 1
1
99
< 1
96
3
Bcl-xL
< 1
< 1
< 1
< 1
99
< 1
99
< 1
Bcl-2
< 1
< 1
< 1
2
99
< 1
74
23
Apaf-1 shRNA
Propidium Iodide
Annexin V-FITC
B
Wild-type
Bcl-xL
Bcl-2
Vector control
Apaf-1 shRNA
Anti-Fas - + - + - + - + - +
Casp-8
pro
p43/41
p18
Bid
p22
p15
Casp-9
pro
p37/35
Casp-3
pro
p20/19/17
Lane 1 2 3 4 5 6 7 8 9 10
C
Bcl-xl
Bcl-2
< 1
< 1
< 1
< 1
98
< 2
99
< 1
Anti-Fas
(24 h)
PI
Annexin V-FITC

Figure 16.

Similarly, cleavage of Bid to tBid and the processing of caspase-3 to its fully active p19/p17 fragments occurred in the wild-type, vector control, and Apaf-1-deficient cells (Figure 16*B*, *lanes 2*, *8*, and *10*). By comparison, only the p20 caspase-3 intermediate fragment was observed in the Bcl-x_L- and Bcl-2-overexpressing cells (Figure 17*B*, *lanes 4* and *6*) suggesting that normal caspase-3-mediated autocatalytic removal of the N-terminal prodomain had been impaired (Han et al., 1997; Liu et al., 2005), presumably due to inhibition by an IAP (Riedl et al., 2001b). Cleavage of caspase-9 occurred in four out of the five cell lines, although producing two fragments (p37/p35) in wild-type and control-transfected cells, and only one fragment (p37) in Apaf-1-deficient and Bcl-x_L-overexpressing cells (Figure 16*B*, *lanes 2* and *8 versus lanes 4* and *10*). This is important because apoptosome-mediated activation and self-cleavage of caspase-9 has been reported to occur after Asp^{315} yielding a p35 fragment, whereas the appearance of the catalytically inactive p37 fragment (Denault et al., 2007) is most often linked to caspase-3-mediated cleavage of caspase-9 after Asp^{330} (Srinivasula et al., 1998). The small amount of p37 caspase-9 that was produced in the anti-Fas-treated Bcl-x_L-overexpressing cells could be due to a trace amount of autocatalytic processing of p20 caspase-3 to p19 caspase-3 that had occurred (Figure 16*B*, *lane 4*). Nevertheless, as shown in Figure 16*C*, both Bcl-2- and Bcl-x_L-overexpressing cells were refractory to receptor-mediated apoptosis even after 24 h of incubation with anti-Fas. Combined, Bcl-2- and Bcl-x_L-overexpressing cells

are highly resistant to apoptosis induced by anti-Fas antibody, whereas cells with impaired apoptosome function and thus caspase-9 activation remain highly susceptible to this form of cell killing.

5.3.2 Sensitivity of Apaf-1-Deficient Jurkat Cells to anti-Fas Treatment is Associated with Extensive Mitochondrial Apoptotic Changes

As mentioned previously, Fas-mediated apoptotic signaling in type I cells is generally thought to occur by active caspase-8 directly cleaving and activating caspase-3, whereas recruitment of the intrinsic (mitochondria-mediated) pathway is widely thought to be essential for Fas-mediated apoptosis in type II cells. Because our different gene-manipulated Jurkat cell clones with impaired intrinsic pro-apoptotic signaling were so markedly dissimilar in their sensitivity to apoptosis induced by anti-Fas, we sought to investigate the extent to which this might be due to differences in mitochondrial events. Thus, we first evaluated the different cells for changes in $\Delta\psi$. Vector control and Apaf-1-deficient cells underwent a drop in $\Delta\psi$ when incubated in the presence of anti-Fas for 6 h (Figure 17*A*), and it is worth noting that Apaf-1-deficient cells were resistant to $\Delta\psi$ loss when incubated in the presence of etoposide (Franklin and Robertson, 2007). In contrast, overexpression of either Bcl-x_L or Bcl-2 rendered cells totally

Figure 17. Fas-Induced Apoptosis in Apaf-1-Deficient Cells is Accompanied by Extensive Mitochondrial Events.

(A) Control-transfected, Bcl-x_L- and Bcl-2-overexpressing, and Apaf-1-deficient Jurkat clones (10^6/ml) were cultured in the absence or presence of 100 ng/ml anti-Fas for 6 h and processed for mitochondrial membrane potential ($\Delta\Psi$) determination by flow cytometry. Reduced $DiIC_1(5)$ fluorescence is indicative of a loss of $\Delta\Psi$, and *number*s refer to the percentage of cells that underwent a dissipation of $\Delta\Psi$. (B) Duplicate aliquots of cells were harvested and processed for subcellular fractionation. Supernatant (s) and pellet (p) fractions were analyzed by Western blotting. (C and D) Wild-type, control-transfected, Bcl-x_L- and Bcl-2-overexpressing, and Apaf-1-deficient Jurkat clones (10^6/ml) were cultured in the absence or presence 100 ng/ml anti-Fas for 6 h and processed for determination of Bak oligomerization by Western blotting (C) or Bak activation by flow cytometric analysis (D). In panel D histograms show Bak-associated fluorescence in vehicle-treated (filled histograms) and apoptotic stimulus-treated (open histograms) cells after 6 h, and *numbers* refer to the percentage increase in mean fluorescence intensity. *shRNA, short hairpin RNA; BMH, bismaleimidohexane; Cyt c, cytochrome c.*

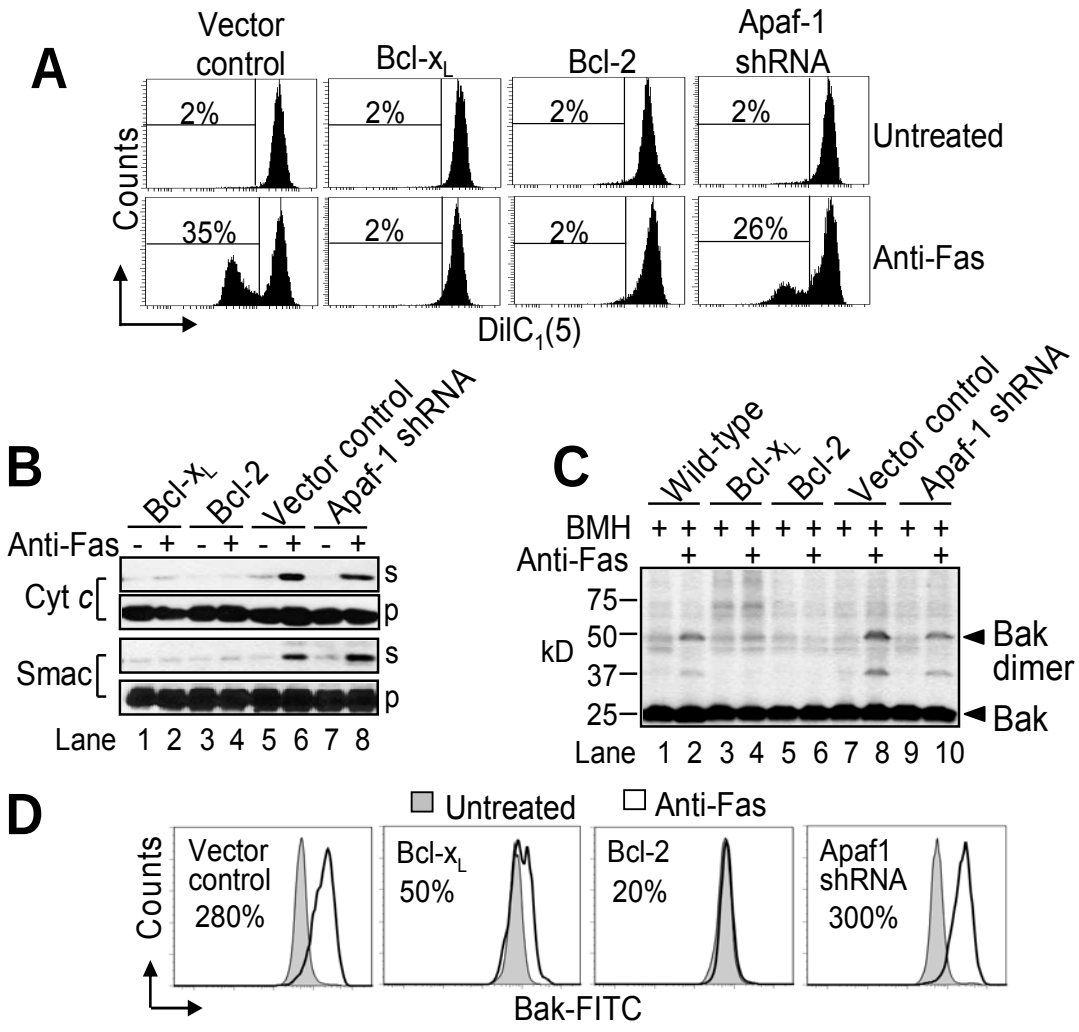
A
Vector control
Bcl-xL
Bcl-2
Apaf-1 shRNA
2%
35%
26%
Untreated
Anti-Fas
Counts
DilC1(5)
B
Bcl-xL
Bcl-2
Vector control
Apaf-1 shRNA
Anti-Fas
Cyt c
Smac
s
p
Lane 1 2 3 4 5 6 7 8
C
Wild-type
Bcl-xL
Bcl-2
Vector control
Apaf-1 shRNA
BMH
Anti-Fas
kD
75
50
37
25
Bak dimer
Bak
Lane 1 2 3 4 5 6 7 8 9 10
D
Untreated
Anti-Fas
Vector control 280%
Bcl-xL 50%
Bcl-2 20%
Apaf1 shRNA 300%
Counts
Bak-FITC

Figure 17.

refractory to any loss of Δψ induced by anti-Fas. Because a drop in Δψ is often associated with the release of mitochondrial intermembrane space proteins into the cytosol, we next investigated whether the release of cytochrome *c* and Smac had occurred in the different cell types. Treatment with anti-Fas led to a pronounced release of cytochrome *c* and Smac from vector control and Apaf-1-deficient cells (Figure 17*B*) consistent with the Δψ data.

To investigate the extent to which differences in the release of cytochrome *c* and Smac corresponded to differences in the activation of Bak (E6.1 cells do not express Bax (Shawgo et al., 2008; Shelton et al., 2009)) we used two different techniques. We first investigated whether Bak underwent oligomerization using the cross-linking agent BMH. As illustrated in Figure 17*C*, cross-linked Bak protein complexes were observed in wild-type and control-transfected Jurkat cells treated with 100 ng/ml of anti-Fas for 6 h. In line with the mitochondrial apoptotic changes that were observed (Figure 17*A* and *B*), dimerization and activation of Bak were detected in wild-type, vector control, and Apaf-1-deficient Jurkat cells, whereas cells overexpressing Bcl-2 or Bcl-x_L were resistant to changes in Bak status (Figure 17*C*). These findings were in agreement with results obtained using an active conformation-specific monoclonal Bak antibody and flow cytometric analysis, where Bak activation amounts to a shift to the right in the resulting histogram (Figure 17*D*). Specifically, vector control and Apaf-1-deficient cells exhibited

large increases in Bak-associated fluorescence of 280 and 300%, respectively, whereas Bcl-x_L and Bcl-2 cells displayed more modest increases of 50 and 20%, respectively. Collectively, these findings suggest that the sensitivity of cells with Apaf-1-deficient cells to Fas-induced apoptosis is due, at least in part, to that fact that these cells undergo MOMP.

5.3.3 Caspase-3/7 (DEVDase) Activity Mediates the Majority of Fas-Mediated Apoptotic Events in Apaf-1-Deficient Cells

As mentioned, stimulation of the Fas receptor with its corresponding ligand or an agonistic antibody leads to formation of the DISC and activation of caspase-8 by dimerization (Boatright et al., 2003). Because of existing evidence indicating that the amount of caspase-8 that is activated at the DISC in type II cells is too low to efficiently cleave and activate caspase-3 (Scaffidi et al., 1999), it is widely believed that caspase-8 cleaves Bid to tBid, which, in turn, recruits the intrinsic (mitochondria-mediated) apoptotic pathway by activating Bax or Bak. In this regard, it is thought that type II cells rely heavily on the intrinsic pathway for receptor-mediated apoptosis (Samraj et al., 2006; Scaffidi et al., 1998; Scaffidi et al., 1999; Schmitz et al., 1999). However, such a model would not explain why the Apaf-1-deficient cells, which cannot activate caspase-9 (p35) in response to intrinsic (Franklin and Robertson, 2007; Shawgo et al., 2008) or extrinsic (Figure 16*B*, *lane 9 versus 10*) stimuli,

are sensitive to Fas-mediated apoptosis, exhibiting Bak activation (Figure 17*C* and *D*), induction of MOMP (Figure 17*B*), and the robust cleavage of caspase-8 to its intermediate (p43/p41) and active (p18) fragments (Figure 16*B*, *lane 9 versus 10*).

Because caspase-3 was also cleaved to its fully active p19/p17 fragments in cells lacking Apaf-1 (Figure 16*B*, *lane 9 versus 10*), we investigated the potential importance of caspase-3/7 in producing the various apoptotic changes in anti-Fas-treated Apaf-1-deficient cells. To that end, we incubated cells with the caspase inhibitor Z-DEVD-fmk (25 μM) for 1 h prior to treatment with anti-Fas (100 ng/ml) for 6 h. As illustrated, Z-DEVD-fmk pre-treatment prevented Fas-induced autocatalytic cleavage of caspase-3 to its fully active p19/p17 fragments, whereas it was not able to eliminate the generation of the p20 caspase-3 intermediate fragment by caspase-8 (Figure 18*A*, *lanes 2 versus 3*, *5 versus 6*, and *8 versus 9*). Pre-treatment of cells with Z-DEVD-fmk dramatically reduced the appearance of the p43/p41 fragments of caspase-8 suggesting that most of the Fas-mediated cleavage of pro-caspase-8 occurs downstream of caspase-3/7 activation (Figure 18*A*). In addition, Z-DEVD-fmk markedly inhibited the appearance of tBid (Figure 18*A*), arguing strongly that the majority of Fas-induced Bid cleavage occurs downstream, or independently, of the low level of caspase-8 activity that occurs initially during receptor-mediated apoptosis in type II Jurkat cells. Z-DEVD-fmk also inhibited the processing of caspase-9 (Figure 18*A*).

Figure 18. Requirement of Caspase-3/7 (DEVDase) Activity for Fas-Induced Apoptotic Changes in Apaf-1-Deficient Cells.

(A) Wild-type, control-transfected, and Apaf-1-deficient Jurkat cells (10^6/ml), pre-incubated with or without 25 μM Z-DEVD-fmk for 1 h, were incubated with 100 ng/ml anti-Fas for 6 h, harvested and lysed for Western blotting. (B-E) Duplicate aliquots of cells were harvested and processed for cell death determination by flow cytometric analysis of annexin V-FITC and propidium iodide staining (B), Bak oligomerization and activation (C), determination by flow cytometric analysis of $DiIC_1(5)$ associated fluorescence (D), and subcellular fractionation followed by Western blotting of supernatant (s) and pellet (p) fractions (E). In panel (B), quadrants are defined as: live (lower left), early apoptotic (lower right), late apoptotic (upper right), and necrotic (upper left). *Numbers* refer to the percentage of cells in each quadrant. In panel (D), *numbers* refer to the percentage of cells that underwent a dissipation of ΔΨ. *shRNA, short hairpin RNA; Casp, caspase; BMH, bismaleimidohexane; Z-DEVD-fmk; Z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH2F; Cyt c, cytochrome c; ΔΨ, mitochondrial membrane potential.*

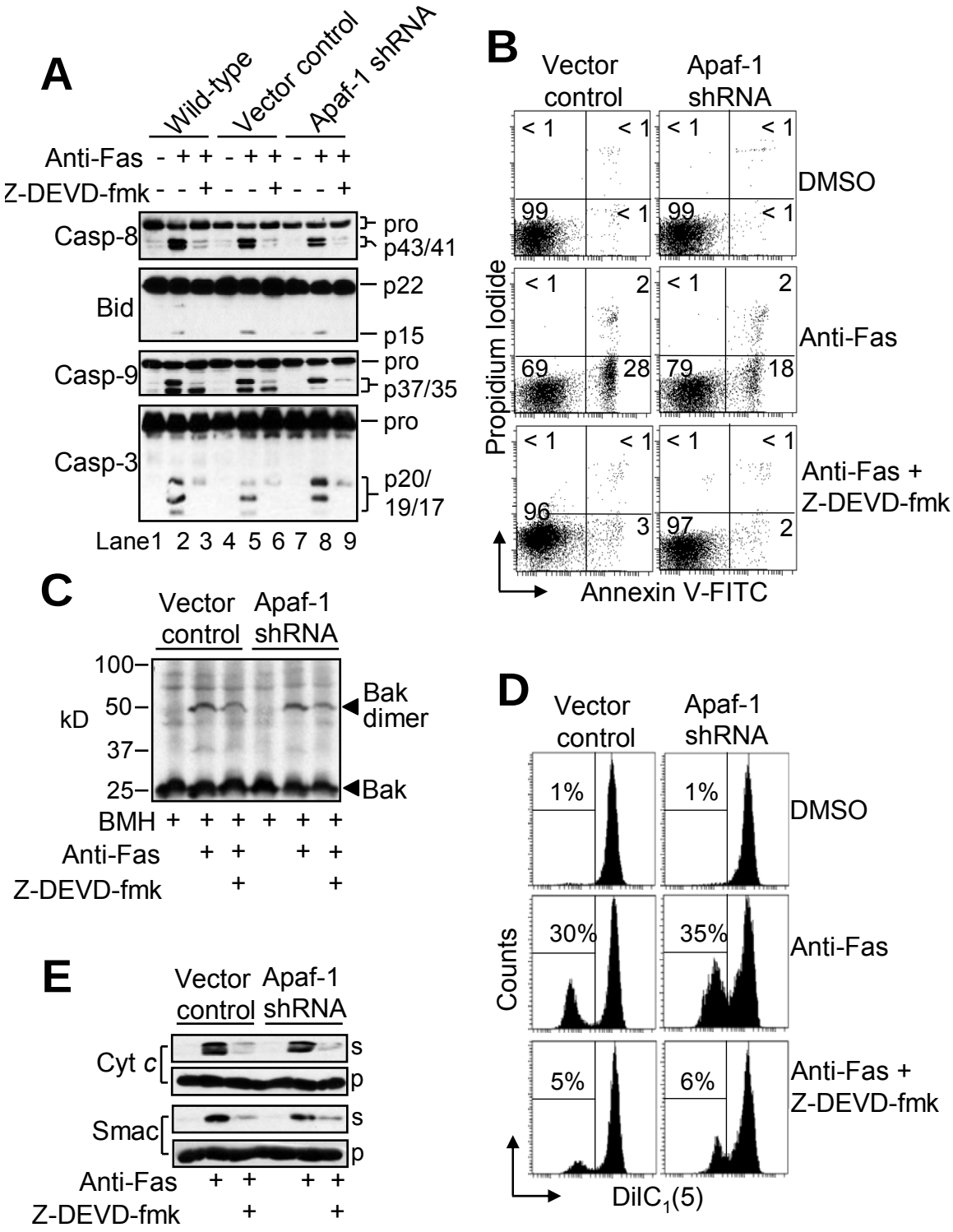
A
Wild-type
Vector control
Apaf-1 shRNA
Anti-Fas - + + - + + - + +
Z-DEVD-fmk - - + - - + - - +
Casp-8
pro
p43/41
Bid
p22
p15
Casp-9
pro
p37/35
Casp-3
pro
p20/
19/17
Lane 1 2 3 4 5 6 7 8 9
B
Vector control
Apaf-1 shRNA
DMSO
Anti-Fas
Anti-Fas +
Z-DEVD-fmk
Propidium Iodide
Annexin V-FITC
< 1
99
2
69
28
79
18
96
3
97
C
Vector control
Apaf-1 shRNA
kD
100
50
37
25
Bak dimer
Bak
BMH + + + + + +
Anti-Fas + + + +
Z-DEVD-fmk + +
D
Vector control
Apaf-1 shRNA
DMSO
Anti-Fas
Anti-Fas +
Z-DEVD-fmk
Counts
1%
30%
35%
5%
6%
DilC1(5)
E
Vector control
Apaf-1 shRNA
Cyt c
s
p
Smac
Anti-Fas + + + +
Z-DEVD-fmk + +

Figure 18.

Specifically, the appearance of the p37 fragment of caspase-9 was largely prevented by pre-treatment with Z-DEVD-fmk. As mentioned previously, the p37 fragment of caspase-9, which lacks proteolytic activity (Denault et al., 2007), is generated by caspase-3-mediated proteolysis, whereas the p35 fragment of caspase-9 is produced by apoptosome-mediated dimerization and autocatalysis (Srinivasula et al., 1998). As anticipated, Z-DEVD-fmk also blocked Fas-induced apoptosis as determined by annexin V-FITC fluorescence (Figure 18*B*).

We next examined the effect of Z-DEVD-fmk on Fas-induced mitochondrial apoptotic events. As shown, pre-treatment of cells with Z-DEVD-fmk partially inhibited Bak oligomerization (Figure 18*C*), the loss of $\Delta\psi$ (Figure 18*D*), and the release of cytochrome *c* and Smac (Figure 18*E*) induced by anti-Fas. Taken together, these data suggest that many of the observable apoptotic changes induced by anti-Fas treatment are mediated by caspase-3/7 (DEVDase) activity. In addition, these findings support a model of receptor-mediated apoptosis in which the intrinsic pathway *per se* is not required for apoptosis to occur in type II Jurkat cells.

5.3.4 Expression of the BIR1/BIR2 Domains of XIAP Inhibits Fas-Induced Apoptosis

Because Z-DEVD-fmk pre-treatment inhibited to a similar extent Fas-induced apoptotic changes in wild-type, vector control, and Apaf-1-deficient cells, and because of concerns about the specificity of so-called specific peptide-based caspase inhibitors, including Z-DEVD-fmk (McStay et al., 2008), we next took a genetic approach to inhibiting downstream caspases. Specifically, we examined the sensitivity of Jurkat cells engineered to stably express the BIR1/BIR2 domains of XIAP (Shawgo et al., 2008) to Fas-induced apoptosis. XIAP belongs to the inhibitor of apoptosis protein family (Deveraux et al., 1997) and a region of this protein that includes the BIR2 domain is known to inhibit caspase-3 and -7 (Chai et al., 2001; Riedl et al., 2001b; Scott et al., 2005).

As illustrated in Figure 19*A*, vector control cells underwent apoptosis (~40%) when incubated with anti-Fas (100 ng/ml) for 6 h, whereas the BIR1/BIR2 transfectants exhibited significantly less apoptosis (~14%). In agreement with these findings, Western blot analysis of cell lysates generated at 6 h after incubation with anti-Fas showed that extensive proteolytic processing of caspase-8, -9, -3, and -7 had occurred in vector control cells, whereas caspase processing in cells expressing the BIR1/BIR2 domains of XIAP was markedly reduced (Figure 19*B*).

Figure 19. BIR1/BIR2-Expressing Jurkat cells are Resistant to Early and Late Fas-Induced Apoptotic Changes.

(A) Control-transfected and Myc-BIR1/BIR2-overexpressing Jurkat cells were incubated in the presence or absence of 100 ng anti-Fas for 6 h and processed for cell death determination by flow cytometric analysis of annexin-V-FITC and propidium iodide staining. Quadrants are defined as live (lower left), early apoptotic (lower right), late apoptotic (upper right), and necrotic (upper left). *Numbers* refer to the percentage of cells in each quadrant. (B) Duplicate aliquots of cells in (A) were harvested and lysed for Western blotting (B). (C) Duplicate aliquots of cells in (A) were processed for mitochondrial membrane potential ($\Delta\Psi$) determination by flow cytometry. Reduced $DiIC_1(5)$ fluorescence is indicative of loss of $\Delta\Psi$, and *numbers* refer to the percentage of cells that underwent a dissipation of $\Delta\Psi$. (D) Duplicate aliquots of cells in A were processed for subcellular fractionation followed by Western blotting of supernatant (s) and pellet (p) fractions. (E) Duplicate aliquots of cells in A were processed for Bak oligomerization by Western Blotting. *Casp, caspase; Cyt c, cytochrome c; BMH, bismaleimidohexane.*

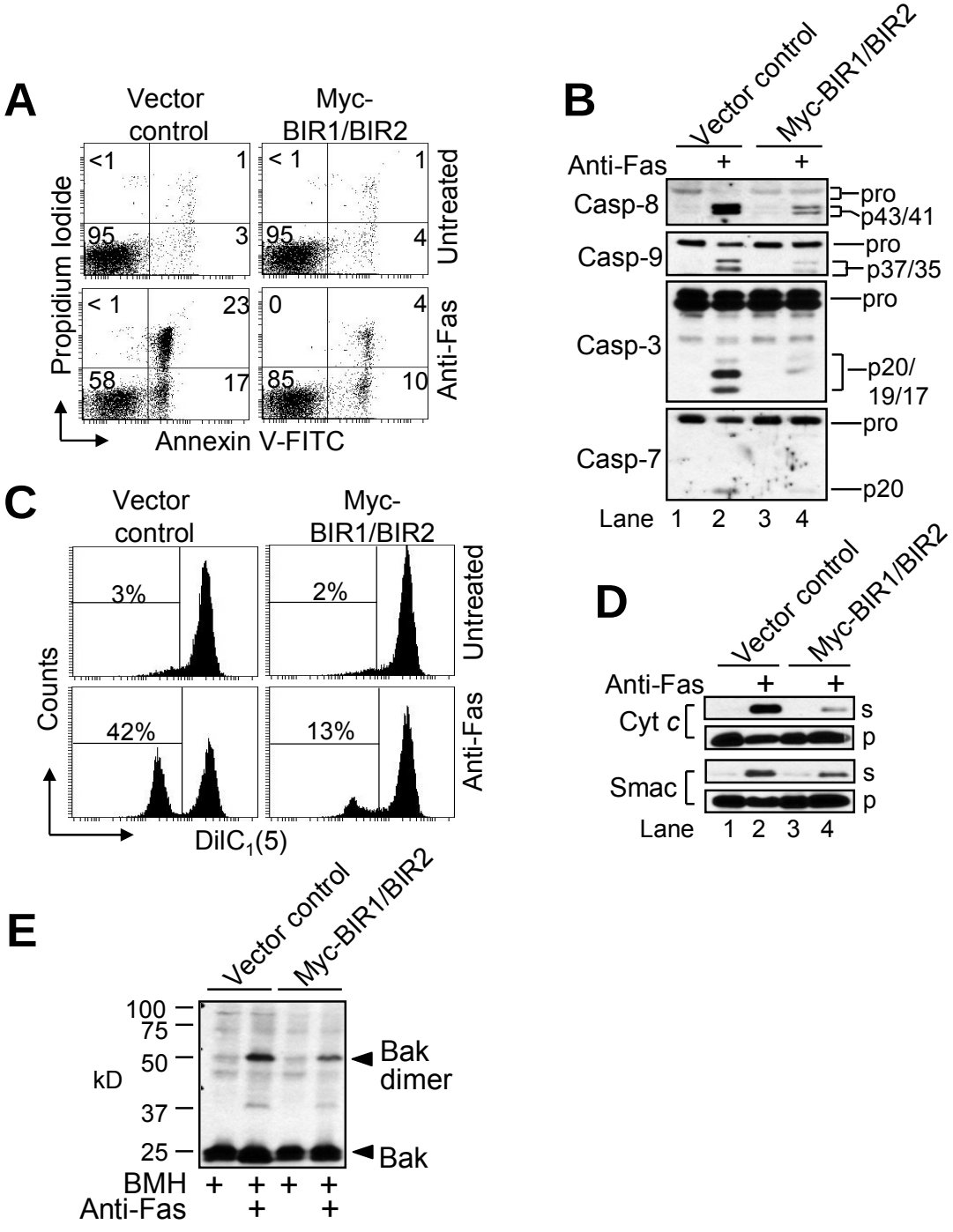
A
Vector control
Myc-BIR1/BIR2
Propidium Iodide
Annexin V-FITC
Untreated
Anti-Fas
<1
1
95
3
< 1
1
95
4
< 1
23
58
17
0
4
85
10
B
Vector control
Myc-BIR1/BIR2
Anti-Fas
Casp-8
pro
p43/41
Casp-9
pro
p37/35
Casp-3
pro
p20/19/17
Casp-7
pro
p20
Lane 1 2 3 4
C
Vector control
Myc-BIR1/BIR2
Counts
3%
2%
42%
13%
Untreated
Anti-Fas
DiIC1(5)
D
Vector control
Myc-BIR1/BIR2
Anti-Fas
Cyt c
s
p
Smac
s
p
Lane 1 2 3 4
E
Vector control
Myc-BIR1/BIR2
kD
100
75
50
37
25
Bak dimer
Bak
BMH + + + +
Anti-Fas + +

Figure 19.

Next, we determined whether Fas-induced mitochondrial events were altered in BIR1/BIR2-expressing cells. As shown in Figure 19*C*, treatment of cells with anti-Fas for 6 h resulted in nearly half (~42%) of the control-transfected cells displaying a loss of Δψ, while cells expressing the BIR1/BIR2 domains of XIAP underwent a more modest drop in Δψ of ~12%. Similar findings were obtained with respect to MOMP induction as measured by Western blot analysis of cytochrome *c* and Smac release into the cytosol (Figure 19*D*, *lane 2 versus 1* and *lane 4 versus 3*). Finally, we were interested to investigate whether the maintenance of Δψ and reduced MOMP in the BIR1/BIR2-expressing cells corresponded with decreased Bak activation. As shown in Figure 19*E*, Fas-induced Bak activation was attenuated in cells expressing the BIR1/BIR2 domains of XIAP. Overall, these findings support and extend the results presented in Figure 18 and offer genetic evidence that executioner caspases play an important role in eliciting MOMP in response to anti-Fas in type II cells.

5.3.5 A Smac Mimetic Partially Sensitizes Bcl-x_L-Overexpressing Cells to Fas-Induced Apoptosis

Because the p20 fragment of caspase-3 was detected uniformly in (i) anti-Fas-treated Apaf-1-deficient cells that had been pre-incubated with Z-DEVD-fmk (Figure 18*A*), (ii) cells made to express the BIR1/BIR2 domains of

XIAP (Figure 19*B*), and (iii) Bcl-x_L-overexpressing cells incubated with anti-Fas alone (Figure 16*B*), we speculated that the reason p20 caspase-3 was not able to undergo autocatalysis to its p19/p17 fragments in cells overexpressing Bcl-x_L might be due to persistent inhibition of its enzyme activity by an IAP, such as XIAP, as reported previously *in vitro* (Sun et al., 2002). Smac, which is a protein that is normally sequestered in the mitochondrial intermembrane space, is released together with cytochrome *c* in response to various apoptotic stimuli (Du et al., 2000; Verhagen et al., 2000) and binds to and inhibits XIAP by targeting XIAP's second baculoviral inhibitory repeat (BIR) domain (Chai et al., 2000; Srinivasula et al., 2001; Wu et al., 2000). In this respect, Smac is considered to be a derepressor of caspase inhibition by XIAP, and several cell-permeable Smac mimetics have been designed that can potentiate pro-apoptotic stimuli (Li et al., 2004; Park et al., 2005; Sun et al., 2004; Wu et al., 2003) as well as induce apoptosis independently (Petersen et al., 2007).

To test the extent to which Smac could sensitize Bcl-x_L-overexpressing cells to anti-Fas, we obtained a Smac mimetic (compound 3) that was shown previously to bind XIAP (also cellular IAP 1(cIAP1) and cIAP2) and act synergistically with TRAIL or TNFα to induce apoptosis in cancer cell lines that are normally resistant to these two receptor stimuli (Li et al., 2004). In agreement with findings from that study, incubation of cells with 100 nM compound 3 for up to 16 h did not induce apoptosis (data not shown) or

proteolytic processing of caspase-8 (Figure 20*B*). However, when Bcl-x_L-overexpressing cells were pre-treated with 100 nM compound 3 for 4 h and subsequently incubated with anti-Fas (100 ng/ml) for 12 h, ~11% of cells underwent apoptosis compared to ~25% of control-transfected cells incubated with anti-Fas alone (Figure 20*A*). Likewise, when used in combination with anti-Fas, compound 3 caused enhanced proteolytic cleavage of caspase-8 as well as the generation of some of the p19/p17 fully active fragments of caspase-3 in Bcl-x_L-overexpressing cells (Figure 20*B*). Alternatively, it could be that the Smac mimetic can partially sensitize Bcl-x_L-overexpressing cells to anti-Fas through a caspase-8-dependent mechanism that involves TNF-α autocrine signaling (Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007). However, this seems unlikely since incubation of cells with the Smac mimetic alone did not cause caspase proteolysis or an induction of apoptosis. Combined, these findings suggest that Smac release is important for receptor-mediated activation of executioner caspases and death in Jurkat cells insofar as Smac may be needed to alleviate caspase inhibition by an IAP.

5.4 Concluding Remarks

Type II cells are distinguished from type I cells by their reliance on the intrinsic (mitochondria-mediated) apoptotic pathway for

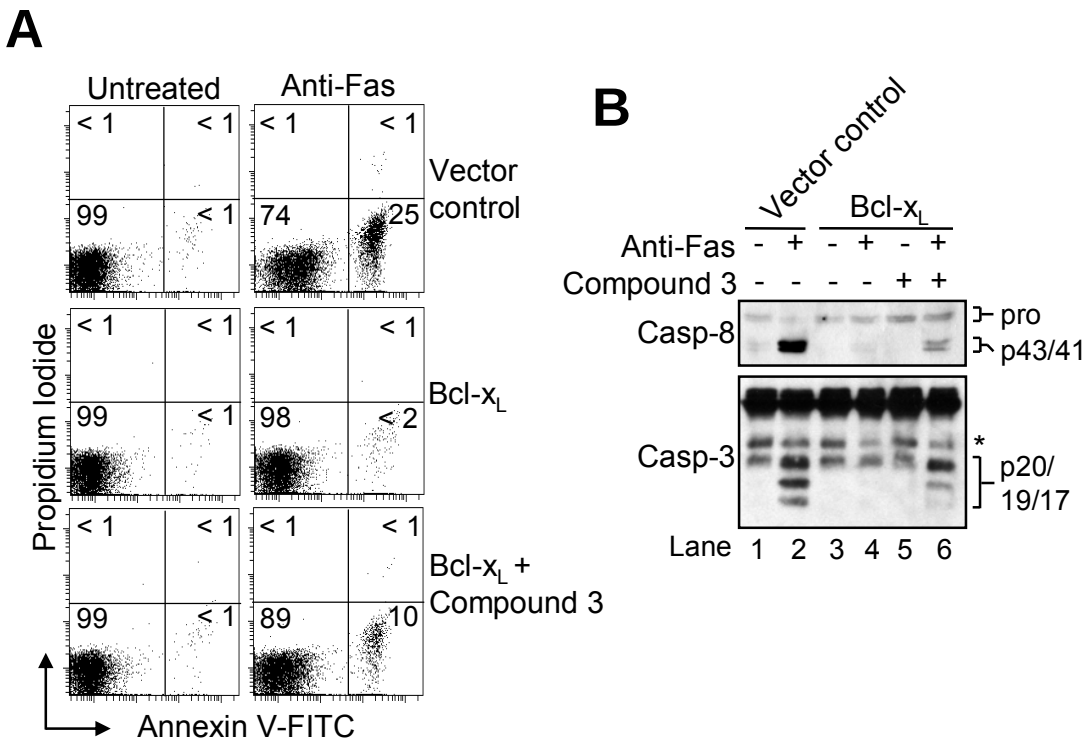

Figure 20. A Smac Mimetic (Compound 3) Synergizes with anti-Fas Antibody to Induce Apoptosis in Bcl-x_L-Overexpressing Cells.

(A) Control-transfected and Bcl-x_L-overexpressing cells (10^6/ml) were incubated in the absence or presence of 100 nM Smac mimetic (compound 3) for 4 h and/or anti-Fas for 12 h, harvested, and processed for cell death determination by flow cytometric analysis of annexin V-FITC and propidium iodide staining. Quadrants are defined as: live (lower left), early apoptotic (lower right), late apoptotic (upper right), and necrotic (upper left). *Numbers* refer to the percentage of cells in each quadrant. (B) Duplicate aliquots were harvested and lysed for Western blotting. Asterisk denotes nonspecific band. *Casp, caspase.*

apoptosis induced by a death ligand (*e.g.* FasL or TNFα) or an agonistic antibody (*e.g.* CH-11). The proposed need for the intrinsic pathway in type II cells centers around the fact that formation of the DISC is impaired in these cells and that the low level of active caspase-8 that is produced within the DISC is sufficient only to cleave the BH3-only protein Bid. In turn, tBid activates a multidomain Bcl-2 protein, Bax or Bak, leading to the recruitment of the mitochondrial pathway. The strongest evidence supporting the existence of a two-pathway receptor-mediated model of apoptosis is that overexpressing a suppressor of the intrinsic pathway, notably Bcl-2 or Bcl-x_L, protects against apoptosis in some cell types (type II) but not others (type I) (Scaffidi et al., 1998; Scaffidi et al., 1999; Schmitz et al., 1999). However, recent studies examining the type I/II paradigm in lymphoid cells have produced conflicting results (Samraj et al., 2006; Sun et al., 2002; Wilkinson et al., 2004). The primary point of contention is the extent to which mitochondrial involvement is truly necessary for receptor-mediated executioner caspase activation and apoptosis in type II cells.

Our findings, using apoptosome-deficient cells incapable of activating caspase-9, provide direct evidence that the mitochondrial apoptotic pathway *per se* is not essential for Fas-induced apoptosis in type II cells. In particular, we have demonstrated that Apaf-1-deficient Jurkat cells, which are equally resistant to etoposide-induced apoptosis as Jurkat cells overexpressing Bcl-2 or Bcl-x_L (Shawgo et al., 2008), are highly susceptible to receptor-mediated

apoptosis. Although the sensitivity of Apaf-1-deficient cells to anti-Fas treatment was associated with extensive cleavage of Bid and induction of MOMP, the resulting release of intermembrane space proteins failed to activate caspase-9. Additionally, our findings indicated that Fas-induced cleavage of Bid depends to a large extent on DEVDase activity and/or occurs downstream of caspase-3/7 activation. However, it is possible that the initial cleavage of Bid by caspase-8 occurs at a level that is not detectable by Western blotting but is nevertheless sufficient to cause Bak-mediated MOMP in cells other than those overexpressing Bcl-2 or Bcl-x_L. Indeed, estimates have indicated that miniscule amounts of tBid are all that is needed to induce Bax/Bak-dependent cytochrome *c* release in some settings (Zha et al., 2000). In this way, it could be that the portion of Bid that appears to be cleaved downstream of caspase-3/7 activation functions to positively amplify MOMP in response to Fas and seal a cell's fate (Shelton et al., 2009).

Because cytochrome *c* release following MOMP appeared to be dispensable for Fas-induced apoptosis in Apaf-1-deficient cells, since it did not lead to the formation of a functioning apoptosome, we hypothesized that Smac release might function in this context to promote executioner caspase activity by inhibiting an IAP, as reported previously for XIAP using *in vitro* cell-free systems (Sun et al., 2002). Indeed, we found that a cell-permeable Smac mimetic was able to partially sensitize Bcl-x_L-overexpressing cells to Fas-induced apoptosis. This finding, in part, might help reconcile our results

with those of Samraj *et al.* (Samraj et al., 2006). In that study, the authors reported that a caspase-9-deficient clone of Jurkat cells they had identified was strongly resistant to Fas-mediated apoptosis, despite undergoing MOMP as indicated by cytochrome *c* release. However, no mention of whether Smac was released together with cytochrome *c* was included, and some evidence suggests the release of these proteins can be differentially regulated (Adrain et al., 2001; Kandasamy et al., 2003). In this regard, it would be of particular interest to know whether a Smac mimetic could sensitize those caspase-9-deficient cells to Fas-induced apoptosis. In our case, the fact that the Smac mimetic-induced sensitization of Bcl-x_L-overexpressing cells to anti-Fas was not more pronounced suggests that the release of other mitochondrial factors, such as Omi, may contribute significantly toward alleviating the suppressive effects of IAPs. Alternatively, it is possible that Bcl-x_L can exert anti-apoptotic functions in the cell that are separate from its established ability to inhibit MOMP. Overall, these findings support a model of Fas-mediated apoptosis in type II cells in which the main function of the intrinsic pathway is to inhibit IAP-mediated inhibition of caspase-3/7, rather than to activate caspase-9 and/or executioner caspases directly.

CHAPTER 6: APAF-1 DEFICIENCY PROMOTES THE ELIMINATION OF CYTOCHROME *C* AND SMAC BY PROTEASOMAL DEGRADATION, AND TO A LESSER EXTENT AUTOPHAGY, IN RESPONSE TO GENOTOXIC STRESS

6.1 Abstract

Within the existing paradigm of genotoxic stress-induced apoptosis, the intrinsic apoptotic pathway is thought to proceed in a linear fashion. Apaf-1 is a critical component of the intrinsic pathway that is generally thought to reside downstream of MOMP. However, our laboratory reported previously that Apaf-1-deficient Jurkat T-lymphocytes do not release cytochrome *c* or activate Bak following a short incubation (6 h) with the DNA-damaging drug etoposide. In the current study, we show that the same Apaf-1-deficient cells were resistant to etoposide-induced apoptosis up to 24 h. Surprisingly, total cellular cytochrome *c* and Smac were markedly decreased after 24 h of incubation. A closer examination of the cellular response revealed that etoposide-treated Apaf-1-deficient cells displayed subtle biochemical markers of autophagy and co-incubating cells with an autophagy inhibitor, 3-methyladenine, partially retained total cellular cytochrome *c* and Smac. At the same time, etoposide-treated Apaf-1-deficient cells underwent some MOMP as evidenced by Bak activation and trace cytochrome *c* release beginning at

8 h. Interestingly, inhibition of the 26S proteasome by pretreatment of Apaf-1-deficient cells with bortezomib or MG132 led to the robust retention of total cellular cytochrome *c* and Smac. Combined, these data suggest that proteasomal degradation, and to a lesser extent autophagy, are responsible for the loss of intracellular cytochrome *c* and Smac in the Apaf-1-deficient cells incubated with etoposide over extended time periods.

6.2 Introduction

Mitochondria-mediated apoptosis (intrinsic pathway) is a form of cell death that is critical for eliminating cells with irreparable DNA damage (Chipuk et al., 2006; Letai, 2009; Letai et al., 2002) Disruptions in control of the mitochondrial apoptotic pathway can facilitate tumorigenesis as well as contribute to the resistance of cancer cells to antineoplastic therapy (Green and Kroemer, 2005; Johnstone et al., 2002). In addition, many cancer chemotherapy drugs, including etoposide, are effective against malignancies due to their ability to induce intrinsic apoptosis.

Within the current model of genotoxic stress-induced apoptosis, the intrinsic apoptotic pathway progresses in a linear fashion (Chipuk et al., 2006; Goldstein et al., 2005; Goldstein et al., 2000; Waterhouse et al., 2001). This pathway is generally thought to begin with the homo-oligomerization and activation of a multidomain pro-apoptotic Bcl-2 protein (i.e. Bax and/or Bak) (Chipuk and Green, 2006; Ow et al., 2008), which depends on the prior

activation of a BH3-only protein, such as tBid, Bim, Puma, Noxa, and Bad. Monomeric, inactive Bax resides in the cytosol and has to move to the mitochondrial outer membrane, whereas monomeric, inactive Bak is normally loosely associated with the outer mitochondrial membrane (Cheng et al., 2001; Chipuk et al., 2006; Scorrano and Korsmeyer, 2003; Wei et al., 2001). Homo-oligomerization and activation of Bax and/or Bak allows the formation of pores in the mitochondrial outer membrane (Chipuk et al., 2006; Ow et al., 2008), which, in turn, permits the release of pro-apoptotic factors (e.g. cytochrome *c* and Smac) into the cytosol (Cartron et al., 2004; Chipuk et al., 2006; Desagher et al., 1999; Kuwana et al., 2005; Kuwana et al., 2002; Luo et al., 1998; Marani et al., 2002; Scorrano and Korsmeyer, 2003). Anti-apoptotic Bcl-2 family proteins, such as Bcl-2 and Bcl-x_L, reside on the mitochondrial outer membrane and prevent the cell from undergoing MOMP by binding and inhibiting Bax/Bak directly (Chen et al., 2005; Willis et al., 2007) or by sequestering a BH3-only protein (Kim et al., 2006a). MOMP is generally thought to be the "point of no return" within the intrinsic pathway, meaning that the cells are unable to recover from MOMP (Green and Kroemer, 2004; Kroemer and Reed, 2000). Once released from the mitochondria, these pro-apoptotic factors promote the activation of caspases. When cytochrome *c* is released into the cytosol it will bind Apaf-1 (Zou et al., 1999). Subsequently, Apaf-1 can alter its conformation and then linearize to allow the binding of dATP (Kim et al., 2005). Next, Apaf-1 olgerimerizes to form a seven-spoked

wheel apoptosome complex that, in turn, recruits and promotes the activation of initiator pro-caspase-9 protein (Acehan et al., 2002; Boatright et al., 2003; Chao et al., 2005; Pop et al., 2006; Rodriguez and Lazebnik, 1999; Shiozaki et al., 2002; Srinivasula et al., 1998). Active caspase-9, in turn, activates caspase-3/7 to execute cell death (Fuentes-Prior and Salvesen, 2004). Often, inhibitors of apoptosis (IAPs) bind to and inhibit partially activated caspase-3, -7, and -9 (Chai et al., 2001; Huang et al., 2001; Takahashi et al., 1998). Smac helps to activate caspase-3, -7, and -9 by alleviating any inhibition IAPs have on caspase-3, -7, and -9 (Shiozaki et al., 2003; Srinivasula et al., 2000).

Apaf-1 is a critical component of the intrinsic pathway that, in most settings, is thought to function strictly *downstream* of MOMP. However, our laboratory reported that Apaf-1-deficient Jurkat T-lymphocytes do not release cytochrome *c* or lose $\Delta\Psi$ in response to short-term incubation with the DNA-damaging drug etoposide (Franklin and Robertson, 2007; Shawgo et al., 2008). These events are considered to be *upstream* of Apaf-1 involvement. The aim of this study was to determine the effects of prolonged incubation with etoposide (> 6 h) on Apaf-1-deficient cells. Specifically, which, if any, mitochondrial apoptotic events occurred? The results indicated that total cellular cytochrome *c* and Smac disappear from cells deficient in Apaf-1 after prolonged (24 h) treatment with etoposide. Although some evidence suggested that the depletion of cytochrome *c* and Smac could be due, in part, to autophagic removal of mitochondria, other findings indicated that a larger

percentage of their disappearance was due to proteolytic degradation by the 26S proteasome.

6.3 Results and Discussion

6.3.1 All Gene-Manipulated Cells are Resistant to Etoposide Up to 24 h

Recently, our lab published that mitochondria-mediated apoptotic events such as release of cytochrome *c* (Franklin and Robertson, 2007) and Bak activation are inhibited in Apaf-1-deficient cells after treatment with etoposide for 6 h (Shawgo et al., 2008). We also suggest that "downstream" events in the intrinsic pathway are essential for MOMP after treatment with etoposide. Specifically, effector caspases and Bid form an important feed forward amplification loop to fully activate the intrinsic pathway (Shelton et al., 2009). According to the model, a small amount of pro-apoptotic factors are released into the cytosol initially in response to DNA damage. The stimulus remains sublethal as long as it does not lead to sufficient apoptosome-dependent activation of caspase-9 to activate caspase-3/7. In turn, caspase-3/7 can cleave Bid to tBid to activate a feed forward amplification loop to seal a cell's fate.

Here, we were interested in determining whether these cells were resistant to etoposide over longer durations of treatment or if apoptosis

induction was just delayed. If apoptosis were merely delayed, it would suggest that the feed-forward loop between the mitochondria and downstream caspase activation functions as an amplification loop. If, on the other hand, the cells were resistant to DNA-damaging drugs at later time-points, it would suggest that the feed-forward loop is essential.

To investigate these two possibilities, we first determined whether Apaf-1-deficient cells, as well as cells in which the intrinsic pathway had been blocked due to Bcl-2 or Bcl-x_L overexpression, were resistant to apoptosis induced by etoposide at time-points longer than 6 h. Cells were incubated with etoposide for 0, 4, 8, 16 or 24 h. The time-course was halted at 24 h because this represents the doubling time for Jurkat cells. After treatment, cells were harvested for flow cytometry. Specifically, we probed for externalized phosphatidylserine on the plasma membrane and cell membrane permeabilization by co-staining with annexin V-FITC and propidium iodide. Compared to the vector control cells that initially had 1% of the cells that were apoptotic to over 61% of the cells over the time course of treatment (Figure 21*A top row*), the Bcl-x_L-and Bcl-2-overexpressing cells were resistant to treatment with etoposide up to 24 h (3% and 3%, respectively; Figure *21A, 2nd and 3rd rows*). Similarly, only 6% of the Apaf-1-deficient cells underwent apoptosis at the longest time-point of 24 h (Figure 21*A, bottom row*). These data suggest that apoptosis is not merely delayed, but actually inhibited, in the Apaf-1-deficient cells. These data also

Figure 21. Inhibition of the Intrinsic Pathway by Bcl-2/Bcl-x_L Overexpression or Apaf-1 Depletion Protects Against Etoposide-Induced Apoptosis up to 24 h.

A, vector control, Bcl-x_L-, and Bcl-2-overexpressing, and Apaf-1-deficient Jurkat clones (5 x 10^5/ml) were cultured with DMSO or etoposide (10 μM) for up to 24 h and processed for cell death determination by flow cytometric analysis of annexin V-FITC and propidium iodide staining. Quadrants are defined as: live (*lower left*); early apoptotic (*lower right*); late apoptotic (*upper right*); and necrotic (*upper left*). *Numbers* refer to the percentage of cells in each quadrant. *B*, duplicate aliquots of cells in *A* were harvested and lysed for Western blot analysis. *shRNA, short hairpin RNA; Casp, caspase; pro, procaspase*.

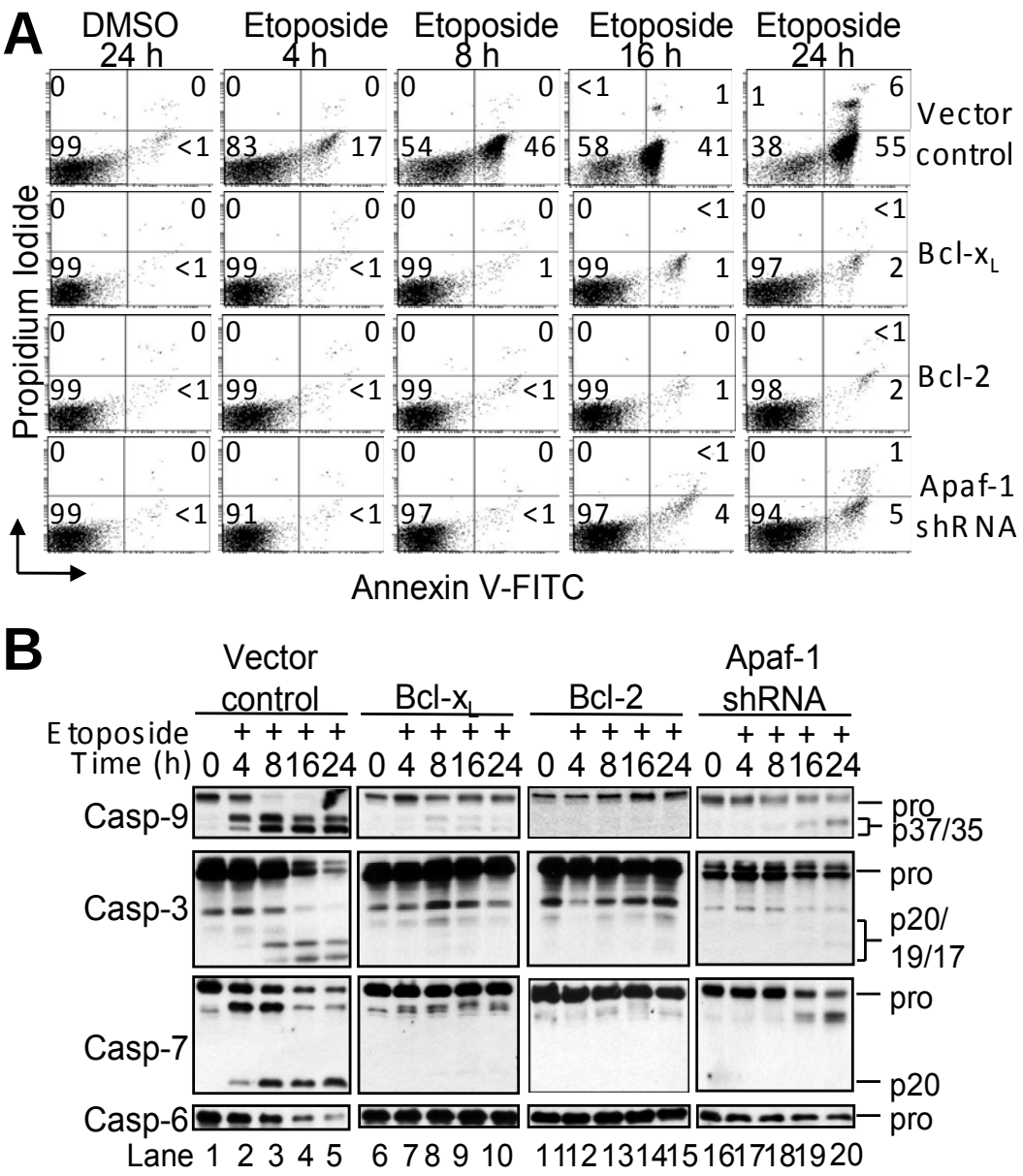
A
DMSO 24 h
Etoposide 4 h
Etoposide 8 h
Etoposide 16 h
Etoposide 24 h
Vector control
Bcl-xL
Bcl-2
Apaf-1 shRNA
Propidium Iodide
Annexin V-FITC
B
Vector control
Bcl-xL
Bcl-2
Apaf-1 shRNA
Etoposide
Time (h) 0 4 8 16 24
Casp-9
Casp-3
Casp-7
Casp-6
pro
p37/35
p20/19/17
p20
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Figure 21.

suggest that the Apaf-1-deficient cells are similarly resistant to etoposide up to 24 h as the Bcl-x_L- and Bcl-2-overexpressing cells.

Next, we examined whether caspase processing was also inhibited up to 24 h after etoposide treatment. As early as 4 h post-etoposide treatment in the vector control cells, processing of caspase-9, and -7, and the disappearance of the pro-form of caspase-6 were observed. Caspase-3 processing appeared after 8 h of etoposide treatment in the vector control cell line (Figure 21*B*, *lanes 2-5*). The Bcl-x_L- and Bcl-2-overexpressing cells lacked any caspase processing up to 24 h (Figure 21*B*, *lanes 6-15*). These data (Figure 21*B*) complement the data (Figure 21*A*), where only 3% of the cells were apoptotic. Even though the Apaf-1-deficient cells did not undergo death shown by annexin/propidium staining, they did exhibit minor caspase processing. In particular, the pro-form of caspase-9 disappeared and the inactive p37 fragment appeared (Denault et al., 2007) (Figure 21*B*, *lanes 16-20*), which is a cleavage event that can be mediated by caspase-3 after cleavage at the amino acid Asp^{330} (Srinivasula et al., 1998). However, because caspase-3 activation did not occur (Figure 21*B, lane 20*) to an appreciable extent in Apaf-1-deficient cells, it raises the possibility that other intracellular proteolytic activity is responsible for procaspase-9 cleavage. Additionally, there was disappearance of the pro-form of caspase-7 but no appearance of the active p20 fragment at 24 h with etoposide treatment (Figure 21*B, lane 20*). Lastly, a small amount of the pro-form of caspase-6

also disappeared (Figure 21*B, lane 20*). Even though none of the caspases were cleaved completely, indicating no fully activated caspases, our findings suggest that some amount of caspase activity was present in the Apaf-1-deficient cells insofar as caspases are known to be effective substrates for one another.

6.3.2 No Classical Mitochondrial Apoptotic Events in Gene-Manipulated Jurkat Cell lines After 24 h with Etoposide Treatment

The loss of ΔΨ is a common occurrence in cells undergoing intrinsic apoptosis. Thus, we next examined whether drug-resistant cells incubated with etoposide for 24 h had lost ΔΨ as determined by staining with $DilC_1(5)$. This dye accumulates in cells with normal ΔΨ but not in those cells that have lost ΔΨ. As illustrated in Figure 22*A* (*top row*), vector control cells experienced a robust loss of ΔΨ, whereas neither Bcl-2/Bcl-x_L-overexpressing or Apaf-1-deficient cells underwent a significant loss of ΔΨ. However, in the case of the Apaf-1-deficient cells, it is worth noting that, although mitochondria remained coupled on the whole, there was some heterogeneity in this response as illustrated by the peak flattening somewhat. In agreement with these results, incubation of vector control cells with etoposide (10 μM) caused a time-dependent release of cytochrome *c* and

Figure 22. Classical Mitochondrial Apoptotic Events are Inhibited in Apaf-1-Deficient and Bcl-2/Bcl-x_L-Overexpressing Jurkat Cells After 24 h of Etoposide Treatment.

A, vector control, Bcl-x_L- and Bcl-2-overexpressing, and Apaf-1-deficient Jurkat clones (5 x 10^5/ml) were cultured with DMSO or etoposide (10 μM) for up to 24 h and processed for mitochondrial membrane potential (ΔΨ) determination by flow cytometry. Reduced $DiIC_1(5)$ fluorescence is indicative of a loss of ΔΨ, and *numbers* refer to the percentage of cells that underwent a dissipation of ΔΨ. *B*, duplicate aliquots of cells were harvested and processed for subcellular fractionation. Supernatant (*s*) and pellet (*p*) fractions were analyzed by Western blotting. *shRNA, short hairpin RNA; Cyt c, cytochrome c.*

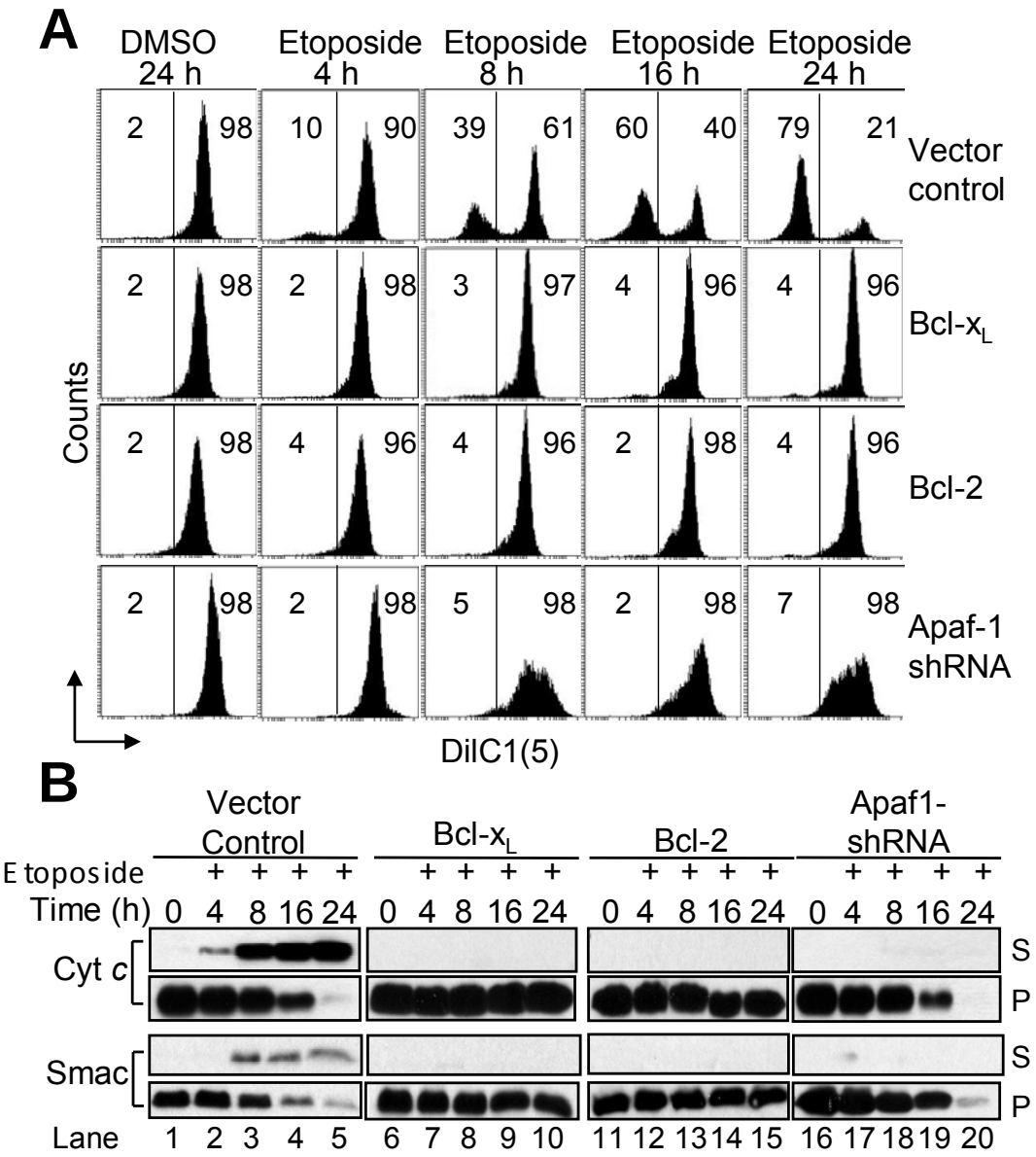
A
DMSO 24 h
Etoposide 4 h
Etoposide 8 h
Etoposide 16 h
Etoposide 24 h
2 98
10 90
39 61
60 40
79 21
Vector control
2 98
2 98
3 97
4 96
4 96
Bcl-xL
2 98
4 96
4 96
2 98
4 96
Bcl-2
2 98
2 98
5 98
2 98
7 98
Apaf-1 shRNA
Counts
DilC1(5)
B
Vector Control
Bcl-xL
Bcl-2
Apaf1-shRNA
Etoposide + + + + + + + + + + + + + + + +
Time (h) 0 4 8 16 24 0 4 8 16 24 0 4 8 16 24 0 4 8 16 24
Cyt c
Smac
S
P
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Figure 22.

Smac from the mitochondria (p) to the cytosol (s) that was nearly complete after 24 h (Figure 22*B, lanes 1-5*), and the Bcl-x_L-and Bcl-2-overexpressing cell lines (Figure 22*B, lanes 6-10 and 11-15, respectively*) never released cytochrome *c* or Smac in the cytosol. Remarkably, despite the apparent absence of etoposide-induced cytochrome *c* release in the Apaf-1-deficient cells, the mitochondrial (p) pool of cytochrome *c* in these cells began to decrease by 8 h and was entirely absent from the pellet fraction at 24 h (Figure 22*B, lanes 16-20*). Smac content also decreased from the mitochondrial fraction of the Apaf-1-deficient cells, although to a lesser extent. Combined, this suggested to us that these cells might be losing their mitochondrial content due to an induction of autophagy as described previously (Ferraro et al., 2008; Mizushima, 2007). In addition, these data largely contradict the view that the mitochondria-mediated apoptotic pathway is a strictly linear process (Chipuk et al., 2006; Goldstein et al., 2005; Goldstein et al., 2000; Waterhouse et al., 2001).

6.3.3 Total Cellular Cytochrome *c* and Smac Decreases Only in Apaf-1-Deficient Cells

Next, we wanted to rule out the possibility that the loss of cytochrome *c* and Smac in cells deficient in Apaf-1 had occurred during the process of subcellular fractionation. After incubation of cells for up to 24 h with

etoposide, whole-cell lysate was produced for Western blot analysis. The results indicated that three out of the four cell lines (vector control, Bcl-x_L-and Bcl-2-overexpressing cells) retained all of their cytochrome *c* and Smac out to 24 h with etoposide treatment (Figure 23, *lanes 1-5, 6-10, & 11-15, respectively*). Strikingly, Apaf-1-deficient cells had lost the majority of their cytochrome *c* at 16 h and a considerable amount of Smac at 24 h post-etoposide treatment (Figure 23, *lanes 16-20*). These data confirm and extend the subcellular fractionation data (Figure 22*B*) and indicate that these cells, indeed, lose their cellular cytochrome *c* and Smac.

6.3.4 Apaf-1-Deficient Cells Exhibit Biochemical Evidence of Autophagic Clearance of Damaged Mitochondria In Response to Etoposide

In the absence of caspase activation, even proliferating cells may retain the ability to survive MOMP and cytochrome *c* release, perhaps by triggering autophagic clearance of “leaky” mitochondria (Boya et al., 2005). However, whether autophagic removal of mitochondria promotes or prevents cell death is controversial. In general, if autophagy removes damaged or “leaky” mitochondria that could otherwise promote apoptosome-mediated activation of caspases, then autophagy could serve as an important pro-survival response to stress. A recent report showed that mitochondria that have undergone MOMP can be removed from cells by mitophagy so long as

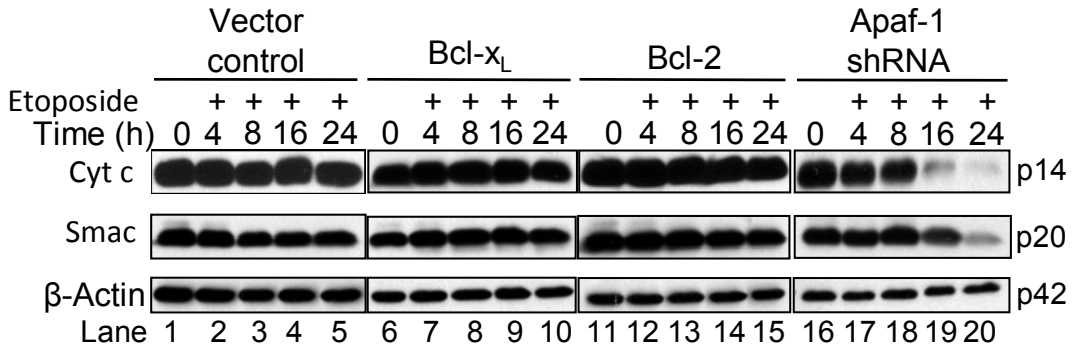

Figure 23. Total Cellular Cytochrome *c* and Smac Levels Decrease in Etoposide-Treated Apaf-1-Deficient Cells.

Vector control, Bcl-x_L- and Bcl-2-overexpressing and Apaf-1-deficient Jurkat clones (5 X 10^5/ml) were cultured with DMSO or etoposide (10 μM) for up to 24 h, harvested total cell lysate and performed Western blot analysis. β-actin was used as a loading control. *shRNA, short hairpin RNA; Cyt c, cytochrome c.*

caspases remain inactive and glyceraldehyde-3-phosphate dehydrogenase expression remains high (Colell et al., 2007). Thus, in our case, it was tempting to speculate that autophagic removal of damaged mitochondria might play a pro-survival role in cells lacking a functional apoptosome complex.

Autophagy will degrade damaged cellular contents such as organelles or proteins (Cuervo, 2004; Klionsky, 2005, 2007; Levine and Klionsky, 2004; Mizushima and Klionsky, 2007; Shintani and Klionsky, 2004). The damaged cellular contents are sequestered in a double-membrane vesicle called an autophagasome that is transported and fuses with a lysosome (Kroemer and Levine, 2008; Levine and Kroemer, 2008; Mizushima, 2007; Mizushima et al., 2008). LC3-I is a 19 kDa protein that is present constitutively in the cytosol, whereas LC3-II is a 17 kDa protein that is bound to autophagic vesicles. During the production of autophagic vesicles, LC3-I is converted to LC3-II (Kabeya et al., 2000). To determine if the disappearance of cytochrome *c* from the pellet fraction of Apaf-1-deficient cells (Figure 22*B*) might be due to autophagy, the disappearance of LC3-I was examined. After 16 h of etoposide treatment, the Apaf-1-deficient cells lost LC3-I (Figure 24*A, lanes 4 and 5, bottom row*). These data provided some biochemical evidence that autophagic vesicles were being produced in the Apaf-1-deficient cells after long exposure with etoposide.

Figure 24. Apaf-1-Deficient Cells Exhibit Some Biochemical Evidence of Autophagic Removal of Mitochondria in Response to Etoposide Treatment.

A, wild type, vector control, and Apaf-1-deficient Jurkat clones (5 x 10^5/ml) were cultured with DMSO or etoposide (10 μM) for up to 24 h, lysed and harvested for Western Blot analysis of LC3-I. *B,* Apaf-1-deficient Jurkat cells (5 x 10^5/ml) were cultured in the presence of etoposide (10 μM) for 24 h with or without 3-methyladenine (5 and 20 mM), lysed and harvested for Western blot analysis of LC3-I. *C*, duplicate aliquots of cells in *B* were lysed and harvested for Western blot analysis. *D*, vector control or Apaf-1-deficient cells were cultured in the presence of etoposide (10 μM) for 24 h with or without 3-methyladenine (5 and 20 mM), harvested and processed for subcellular fractionation. Supernatant (*s*) and pellet (*p*) fractions were analyzed by Western blotting. *shRNA, short hairpin; 3-MA, 3-methlyadenine; Cyt c, cytochrome c.*

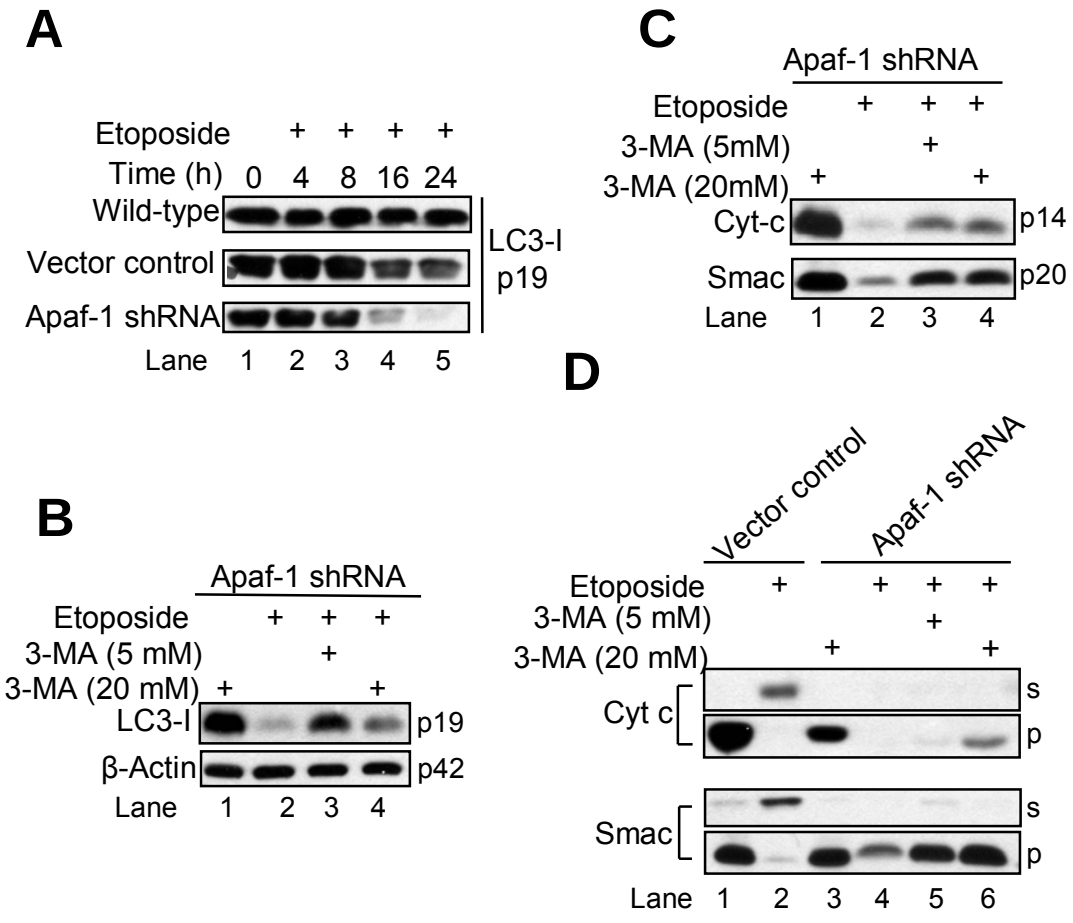
A
Etoposide + + + +
Time (h) 0 4 8 16 24
Wild-type
Vector control
Apaf-1 shRNA
LC3-I
p19
Lane 1 2 3 4 5
B
Apaf-1 shRNA
Etoposide + + +
3-MA (5 mM) +
3-MA (20 mM) + +
LC3-I
p19
β-Actin
p42
Lane 1 2 3 4
C
Apaf-1 shRNA
Etoposide + + +
3-MA (5mM) +
3-MA (20mM) + +
Cyt-c
p14
Smac
p20
Lane 1 2 3 4
D
Vector control
Apaf-1 shRNA
Etoposide + + + +
3-MA (5 mM) +
3-MA (20 mM) + +
Cyt c
s
p
Smac
s
p
Lane 1 2 3 4 5 6

Figure 24.

If damaged mitochondria in the Apaf-1-deficient cells had been cleared by autophagy, then inhibiting autophagy should help retain the total cellular cytochrome *c* and Smac. To extend this observation and to investigate the extent that we could inhibit the loss of LC3-I, we co-incubated cells for 24 h with etoposide and the autophagy inhibitor 3-methyladenine (3-MA) (Maiuri et al., 2007) (5 and 20 mM). At the end of 24 h, cells were harvested and subjected to Western blot analysis to look for LC3-I. When compared to treatment with etoposide (Figure 24*B, lane 2*) alone, co-treatment with 3-MA led to the retention of LC3-I (Figure 24*B, lane 3 and 4*).

Next, we were interested in determining whether co-incubation of cells with 3-MA would also lead to the retention of total intracellular cytochrome *c* and Smac. Indeed, the results indicated that co-treatment of Apaf-1-deficient cells with 3-MA and etoposide for 24 h led to some retention of total intracellular cytochrome *c* and Smac (Figure 24*C, lanes 3 and 4*). More precisely, subcellular fractionation of cells revealed that 3-MA (20 mM) led to an increased mitochondrial content of cytochrome *c*, while Smac was retained at the lower concentration (5 mM) (Figure *24D, lanes 5 and 6*). Overall, these data suggest that at least one reason total cellular cytochrome *c* and Smac disappear in Apaf-1-deficient cells is due to autophagy.

6.3.5 No Classical Autophagic Vesicles in the Apaf-1-Deficient Cells

Because we had observed some evidence of autophagy in etoposide-treated Apaf-1-deficient cells, which could be inhibited partially by 3-MA, we next employed the gold standard for studying autophagy (i.e. electron microscopy) and examined cells for the presence of classical autophagic vesicles. Autophagasomes can be identified by their double-membrane vesicles surrounding cytoplasmic organelles (Kroemer and Levine, 2008; Levine and Kroemer, 2008). After treatment with etoposide for up to 24 h, cells were harvested, processed, and examined with a J.E.O.L 100CXII transition electron microscope. Classical apoptotic morphology can be observed in the vector control cells after 24 h etoposide (Figure 25, *top row*). These events include chromatin condensation, maintenance of plasma membrane integrity, and a decrease in cytoplasmic contents. By comparison, etoposide-treated Bcl-2-overexpressing cells lacked all apoptotic morphology, and the mitochondria appeared to be normal and intact (Figure 25 *middle row*). Interestingly, Apaf-1-deficient cells exhibited neither classical apoptotic morphology nor robust autophagic vesicle formation. Strikingly, however, it is important to note that the mitochondria of drug-treated Apaf-1-deficient cells appeared to have been damaged as evidence by their distinctly engorged or bloated appearance (Figure 25, *bottom row*). Combined, these data indicate that even though there are some biochemical markers of autophagy, it appears that autophagy is not playing the major role in the disappearance of cytochrome *c* and Smac.

Figure 25. No Classical Autophagic Vesicles in the Apaf-1-Deficient Cells Incubated in the Presence of Etoposide for 24 h.

Vector control, Bcl-2-overexpressing, and Apaf-1-deficient Jurkat cells (5 x 10^5/ml) were cultured with DMSO or etoposide (10 μM) for up to 24 h and harvested for examination with J.E.O.L 100CXII TEM at 80 KV. Images were enlarged 7,200X. Scale bar represents 1 micron. *shRNA, short hairpin RNA.*

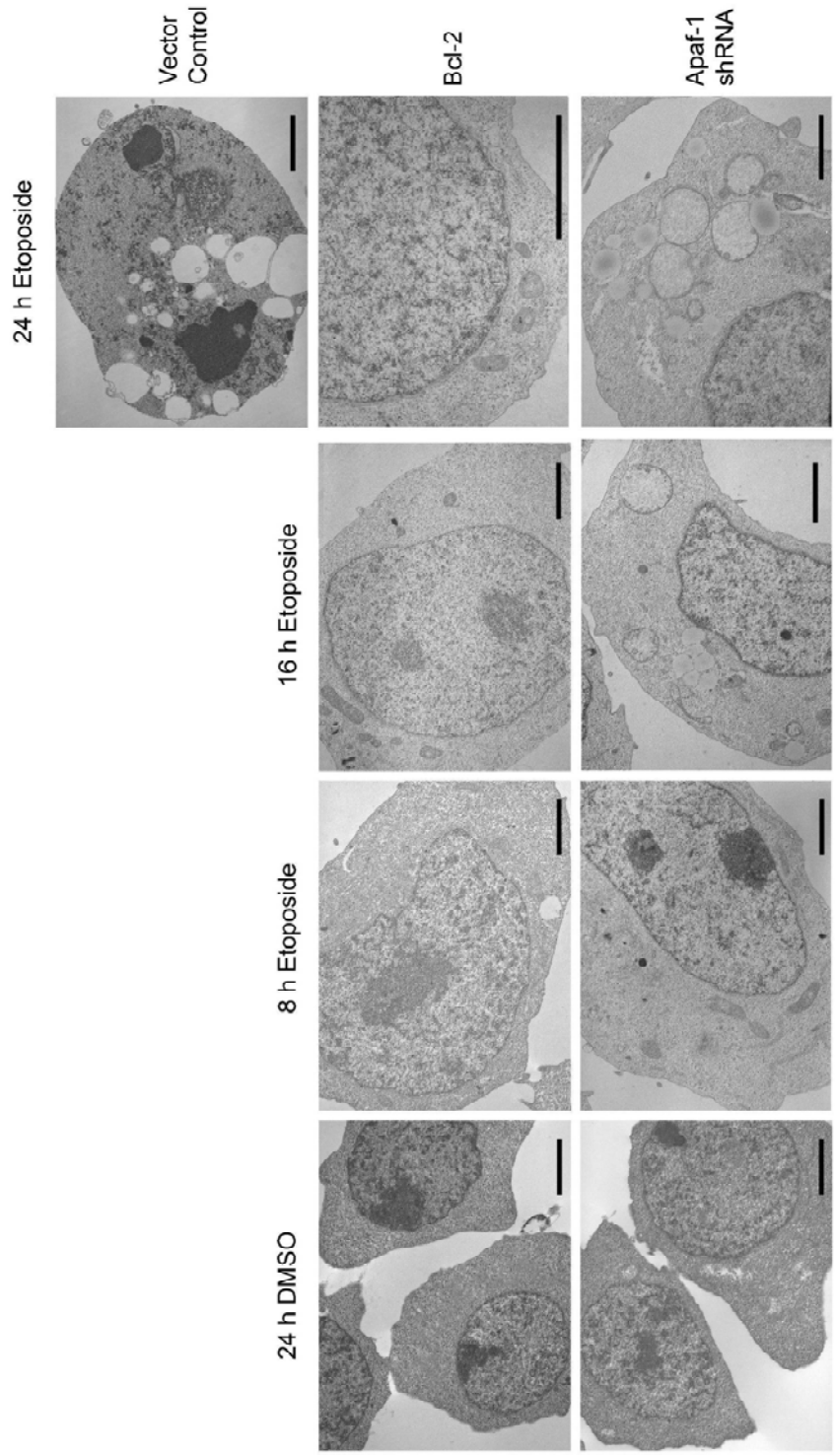
Vector Control
Bcl-2
Apaf-1 shRNA
24 h Etoposide
16 h Etoposide
8 h Etoposide
24 h DMSO

Figure 25.

6.3.6 Delayed Bak Activation in Apaf-1-Deficient Cells

Because autophagy could not explain the disappearance of all cytochrome *c* and Smac, we next investigated an alternative hypothesis. We knew that there was no appreciable accumulation of cytochrome *c* and Smac in the cytosol. Additionally, we knew that the mitochondria were damaged as indicated by the change in $\Delta\Psi$ and their swollen appearance by EM. Thus, we were interested to determine whether there was any evidence of MOMP induction in these cells. Although we had reported previously that Bak activation in etoposide-treated Apaf-1-deficient cells was inhibited when treated for 6 h (Shawgo et al., 2008), the question arose whether Bak activation would remain inhibited at longer time-points.

Thus, we incubated cells with etoposide for 0, 4, 8, 16, or 24 h and harvested for Bak activation. As reported previously, Jurkat (E6.1) cells do not express Bax (Shawgo et al., 2008). After treatment, cells were incubated with BMH, a protein cross-linker, and subjected to Western blot analysis. As mentioned earlier, Bak activation is associated with its homo-oligomerization. Both the Bcl-x_L-and Bcl-2-overexpressing cells never activated Bak, whereas vector control cells began activating Bak as early as 4 h after etoposide-treatment (Figure 26). Interestingly, Apaf-1-deficient cells were found to activate Bak beginning at 8 h post-treatment; however, the extent to which

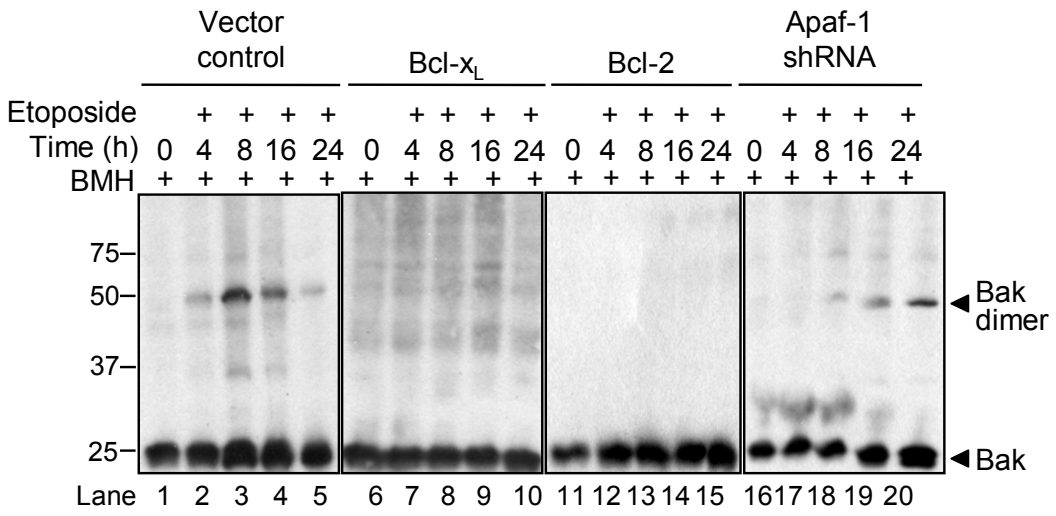

Figure 26. Delayed Bak Activation in Apaf-1-Deficient Cells After Treatment With Etoposide.

Vector control, Bcl-x_L- and Bcl-2-overexpressing, and Apaf-1-deficient Jurkat cells (5 x 10^5/ml) were cultured with DMSO or etoposide (10 μM) for up to 24 h and processed for determination of Bak oligermerization by Western blotting. *shRNA, short hairpin RNA; BMH, bismaleimidohexane.*

Bak was activated in cells lacking Apaf-1 was always less than that observed in vector control cells (Figure 26 *lane 20 versus 5*). Overall, these data indicate that etoposide-induced Bak activation is delayed, but not prevented, in the Apaf-1-deficient cells. Further, the fact that Bak was activated suggested that the mitochondrial outer membrane had been permeabilized to some extent and that the mitochondria are indeed damaged.

6.3.7 Inhibition of the 26S Proteasome Prevents the Loss of Cytochrome *c* and Smac in the Apaf-1-Deficient Cells

Since mitochondria in the Apaf-1-deficient cells appeared to have been permeabilized after longer exposures to etoposide, we hypothesized that pro-apoptotic factors were being released into the cytosol and rapidly degraded, perhaps by the 26S proteasome. To investigate this possibility, Apaf-1-deficient cells were co-treated with bortezomib (10, 20, 40 nM), an inhibitor of the 26S proteasome, and etoposide. Cells were then harvested for determination of total cellular cytochrome *c* and Smac levels. Compared to treatment with etoposide alone (Figure 27*A, lane 2*), co-treating with bortezomib led to a retention of cellular cytochrome *c* and Smac. Subsequently, we were curious to know in which subcellular compartment(s) bortezomib would lead to an increased cytochrome *c* and Smac content. The results indicated that cytochrome *c* and Smac were retained in both the

Figure 27. Inhibition of the 26S Proteasome Prevents the Loss of Cytochrome *c* and Smac in Etoposide-Treated Apaf-1-Deficient cells.

A, Apaf-1-deficient Jurkat clones (5 x 10^5/ml) were cultured with etoposide (10 μM) with and without bortezomib (10, 20, 40 nM) for 24 h, and harvested for Western blot analysis. *B*, vector control cells were treated with etoposide (10 μM) for 24 h and duplicate samples in *A* were harvested for subcellular fractionation. Supernatant (*s*) and pellet (*p*) fractions were analyzed by Western blotting. *C*, Apaf-1-deficient Jurkat clones (5 x 10^5/ml) were cultured with etoposide (10 μM) with and without MG132 (0.5, 1, 5 nM) for 24 h, and harvested for Western blot analysis. β-actin was used as a loading control. *shRNA, short hairpin RNA. Cyt c, cytochrome c.*

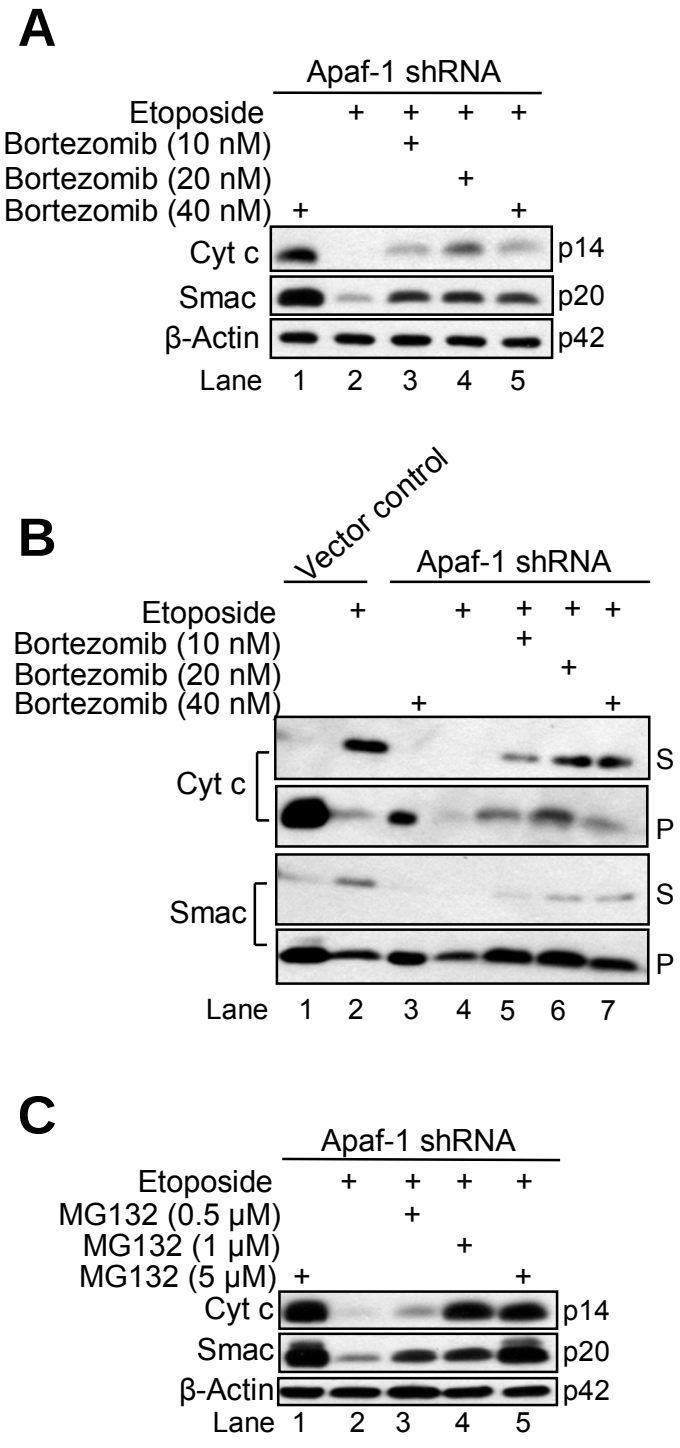
A
Apaf-1 shRNA
Etoposide + + + +
Bortezomib (10 nM) +
Bortezomib (20 nM) +
Bortezomib (40 nM) + +
Cyt c
Smac
β-Actin
p14
p20
p42
Lane 1 2 3 4 5
B
Vector control
Apaf-1 shRNA
Etoposide + + + + +
Bortezomib (10 nM) +
Bortezomib (20 nM) +
Bortezomib (40 nM) + +
Cyt c
Smac
S
P
S
P
Lane 1 2 3 4 5 6 7
C
Apaf-1 shRNA
Etoposide + + + +
MG132 (0.5 μM) +
MG132 (1 μM) +
MG132 (5 μM) + +
Cyt c
Smac
β-Actin
p14
p20
p42
Lane 1 2 3 4 5

Figure 27.

cytosol and mitochondrial fractions; however, Smac was retained to a greater extent than cytochrome *c* in the mitochondrial fraction (Figure 27*B*). Importantly, these findings were confirmed using another 26S proteasome inhibitor, MG132. Indeed, co-incubation of Apaf-1-deficient cells with MG132 (0.5, 1, 5 nM) and etoposide for 24 h led to the retention of intracellular cytochrome *c* and Smac in a concentration-dependent manner (Figure 27*C*). Combined, these data suggest that cytochrome *c* and Smac are released from mitochondria and degraded by the 26S proteasome in etoposide-treated Apaf-1-deficient cells.

6.4 Concluding Remarks

In summary, all the gene-manipulated cells were similarly resistant to etoposide up to 24 h evaluated by annexin/PI staining. However, only the Apaf-1-deficient cells exhibited some evidence of caspase activation after 24 h of etoposide treatment. Secondly, the Apaf-1-deficient cells appeared to have a modest change in $\Delta\Psi$ observed by staining with $DilC_1(5)$. The change in $\Delta\Psi$ led us to discover that total intracellular cytochrome *c* and Smac disappear from Apaf-1-deficient cells after treatment with etoposide for 16 - 24 h. When autophagy was explored as a possible mechanism for their disappearance, subtle biochemical evidence was observed but no gross appearance of classical autophagic vesicles could be detected. Otherwise,

the delayed Bak activation that was observed suggested that pro-apoptotic proteins could possibly be released into the cytosol. Indeed, the findings indicated that once cytochrome *c* and Smac were released into the cytosol in cells deficient in Apaf-1, they were rapidly degraded by the 26S proteasome. Combined, these data suggest that proteasomal degradation, and to a lesser extent autophagic removal of mitochondria, are responsible for the loss of intracellular cytochrome *c* and Smac in Apaf-1-deficient cells incubated with etoposide over extended time periods.

These findings are consistent with an emerging model of the intrinsic pathway in which after a cell's DNA becomes damaged, a small amount of Bak (or Bax) activation occurs, by a still poorly defined mechanism, to release a small amount of apoptogenic proteins into the cytosol. These pro-apoptotic factors will then help to activate caspases both directly (cytochrome *c*) and indirectly (Smac). To the extent this results in the activation of effector caspases, Bid can be cleaved to tBid to promote additional activation of Bak (or Bax) and the release of more apoptogenic proteins. This forms the basis of an essential feed forward loop among effector caspases, Bid, and the mitochondrial outer membrane. In the case of Apaf-1 deficiency, the initial release of cytochrome *c* would seem to serve no purpose since it cannot promote the activation of caspase-9 within the apoptosome complex. Likewise, there is no activated caspase-3, -7 or -9 that is being held up by an inhibitor of apoptosis protein for Smac to bind and thereby neutralize. In

instances where the release of cytochrome *c* and Smac do not result in the activation of caspases, our findings suggest that these pro-apoptotic factors present in the cytosol are mostly degraded by the 26S proteasome, and less so by a mechanism involving autophagic removal of damaged mitochondria. Overall, it is tempting to speculate that the degradation of these pro-apoptotic proteins might serve as a protective mechanism against inadvertent apoptosis induction.

CHAPTER 7: CONCLUSIONS AND FUTURE DIRECTIONS

7.1 How the New Conclusions Impact Controversies in the Field and Future Directions

It was once thought that mitochondria-mediated apoptosis involved only a few linear steps. In response to an apoptotic stimulus, the mitochondria would be permeablized to allow activation of caspases to cause cell death. However, as investigators have studied the molecular requirements and mechanisms of apoptosis, we now know that apoptotic signaling is not so straightforward. For example, considerable controversy persists over the precise mechanisms responsible for MOMP. Some of the unresolved questions that persist include: (i) Once one mitochondrion in a cell has its released pro-apoptotic proteins, do all the mitochondria in the cell release their pro-apoptotic factors? (ii) Are the different pro-apoptotic factors released simultaneously? (iii) What is considered to be the point of no return? (iv) Are there two waves of release of pro-apoptotic factors? The results of my dissertation research contribute toward the resolution of some of these questions.

7.1.1 Once One Mitochondrion in a Cell has Released Pro-Apoptotic Proteins Do All the Mitochondria in the Cell Release Their Pro-Apoptotic Proteins?

Some controversies in the field are how quickly do pro-apoptotic proteins get released into the cytosol and does one mitochondrion releasing its pro-apoptotic factors cause other mitochondria to release their pro-apoptotic factors. Much in the literature argues that if one of these pro-apoptotic factors is released, all of the pro-apoptotic proteins are released from all mitochondria. For instance, using GFP-labeled cytochrome *c*, it was shown that within 5 min all cytochrome *c* had been released in response to UV radiation, staurosporine, actinomycin D, or etoposide. This is where the "all or none" theory of MOMP was initially proposed (Goldstein et al., 2005; Goldstein et al., 2000). This theory was expanded to suggest that cytochrome *c* release can cause the release of cytochrome *c* from other mitochondria (Kagan et al., 2004). However, other studies have suggested that when one mitochondrion releases its pro-apoptotic factors, it does not affect the release by the neighboring mitochondria (Khodjakov et al., 2004; Lartigue et al., 2008). Zhou and Chang suggest that unlike cytochrome *c* that is all released at once, Smac is not released all at once (Zhou and Chang, 2008; Zhou et al., 2005). Lartigue *et al*. even suggested that cytochrome *c* release happens before Bax activation (Lartigue et al., 2008). We show that cytochrome *c* release is not instantaneous (Figure 13*B*, 15*B* and 21*B*). If

cytochrome *c* was released in an all-or-none manner, then inhibiting "downstream" events such as apoptosome formation (Figures 12 and 13) and/or the activation of effector caspases (Figures 14 and 15) would not have influenced the release of pro-apoptotic factors (Shawgo et al., 2008). Inhibition of "downstream" events that then inhibit "upstream events," such as MOMP, points to the existence of an essential feed forward loop. A feedback loop has also been suggested by a few groups (Boehning et al., 2003; Kroemer et al., 2007; Lartigue et al., 2008; Pacher and Hajnoczky, 2001). We show that effector caspase activation is required for the cleavage of Bid to tBid to feed forward and activate more Bak (Shelton et al., 2009). The release of pro-apoptotic factors not being an all-or-none event was further supported when we incubated our Apaf-1-deficient cells long-term with etoposide. Specifically, a slow time-dependent release of cytochrome *c* and Smac from mitochondria was observed as evidence by the disappearance of cytochrome *c* and Smac in the mitochondrial fraction (Figure 22*B*). In conclusion, our data suggest that the release of pro-apoptotic factors is not a rapid singular event. It is a process that occurs after "downstream events" (i.e., effector caspase activation) have occurred. These "downstream events" are essential for the complete release of pro-apoptotic proteins from the mitochondria to seal a cell's fate.

7.1.2 Are the Different Pro-Apoptotic Factors Released All Together?

Whether or not different pro-apoptotic factors are released at the same time is a second controversy in the field. That is, when cytochrome *c* is released do other pro-apoptotic factors such as Smac get released? The majority of the literature suggests that pro-apoptotic factors are released together. For example, it has been suggested that Smac and Omi are released at the same time as cytochrome *c* (Kashkar et al., 2002; Munoz-Pinedo et al., 2006; Twiddy et al., 2004). However, it has also been shown that cytochrome *c*-null cells do not release Smac after treatment with the DNA-damaging drug etoposide (Hansen et al., 2006).

Our data also suggest that when pro-apoptotic factors are released into the cytosol, their release into the cytosol is at least partially independent of each other. We first made the observation that Apaf-1-deficient cells are inhibited in their release of cytochrome *c* in response to etoposide. However, Smac release was not inhibited to the same extent that cytochrome *c* release was inhibited (Franklin and Robertson, 2007; Shawgo et al., 2008). This difference was extended when we incubated cells with etoposide for periods longer than 6 h (Figure 22*B*). We also saw a difference between the inhibition of cytochrome *c* and Smac release when we inhibited caspase activation by overexpressing XIAP and XIAP's BIR1/BIR2 domains (Figure 15*B*). This suggests that there may be a difference in their release and in the future this idea could be explored more.

7.1.3 What is Considered to Be the “Point of No Return”?

What is the “point of no return” during mitochondria-mediated apoptosis? Many researchers believe that MOMP is the “point of no return” within the intrinsic pathway, meaning that the cells are unable to recover once MOMP has occurred (Green and Kroemer, 2004; Kroemer and Reed, 2000). Apoptosome formation (Apaf-1, cytochrome *c*, dATP, pro-caspase-9) is widely thought to occur *strictly* downstream of MOMP. However, Franklin and Robertson (2007) recently reported that Apaf-1-deficient Jurkat T-lymphocytes fail to release pro-apoptotic factors into the cytosol in response to the DNA-damaging anti-cancer drug etoposide. It was also demonstrated that “downstream events” such as effector caspase activation (Figure 15; (Shawgo et al., 2008)) and Bid cleavage (Shelton et al., 2009) were required for full activation of MOMP and to allow the cell to undergo apoptosis. Additionally, when we extended the treatment of the Apaf-1-deficient cells out to 24 h, MOMP did occur; however, the cell did not undergo apoptosis (Figure 22*B*). These data suggest that MOMP is not the point of no return. However, it is very tempting to speculate that there is a threshold to how many pro-apoptotic proteins need to be released and how much effector caspase activation is required before the cell is irreversibly committed to apoptosis. Basic biochemical subcellular experiments will have to be done to determine exactly what that threshold is.

7.1.4 Are There Two Waves of Release of Pro-Apoptotic Factors?

If the release of pro-apoptotic factors is not instantaneous and MOMP is *not* the "point of no return" then there could be multiple waves of pro-apoptotic factors being released from the mitochondria into the cytosol. Those that do believe in multiple waves of pro-apoptotic protein release might examine the underlying mechanism(s). The redundancy of the function of Bak and Bax has been disputed for a long time. Originally, it was suggested that Bak and Bax have redundant roles because only double knockout mice are totally protected against various forms of mitochondria-mediated apoptosis (Wei et al., 2001; Youle and Karbowski, 2005). Single knockout mice show only partial protection from apoptosis. However, one obvious difference between Bax and Bak is their intracellular location. Bax is normally present in the cytosol, whereas Bak is loosely associated with the mitochondrial outer membrane. Indeed, it is now appreciated that Bax has to translocate to the mitochondria, insert into its outer membrane, and form a pore for the release of pro-apoptotic factors into the cytosol, while Bak just has to form a pore. Many consider the activation of Bax as occurring in one quick step (Antonsson et al., 2000; Antonsson et al., 2001; Gross et al., 1998; Saito et al., 2000; Wang et al., 1998). However, more recently these events have been shown to occur in multiple steps that can take some time (minutes-hours). To ensure that previous experimental procedures (use of certain detergents) have not interfered with the endogenous native form of Bax, one

study used a blue native page gel to show that there is another level of regulation of Bax activation. Bax first has to translocate to the outer mitochondrial membrane, oligomerize, and then subsequently become activated (Valentijn and Gilmore, 2004). This suggests that translocation or oligomerization does not mean that Bax is necessarily active. Unfortunately, they did not look at Bak in detail. Thus, there could be another unknown level of regulation to Bak activation to help explain what occurs during the essential feed forward amplification loop. The level of Bax/Bak redundancy was challenged by the work of Cheng *et al.* who demonstrated that Bak, but not Bax, co-immunoprecipitated with VDAC2 (Cheng et al., 2003; Upreti et al., 2008). Additionally, studies have demonstrated that Bak^{+}/Bax^{-} are more sensitive to apoptotic stimuli than Bak^{-}/Bax^{+} MEFs (Upreti et al., 2008). There is also controversy whether or not Bak and Bax hetero-oligomerize. There are a few groups that suggest that homo-oligomerized Bak and Bax can hetero-oligomerize to form larger complexes (Upreti et al., 2008; Zhou and Chang, 2008). If there are differences in the roles of Bax and Bak in MOMP, this could help explain how a biphasic release of pro-apoptotic factors takes place and could be investigated in the future. Perhaps, there is an initial release of pro-apoptotic factors that homo-oligomerize and then the major release occurs due to hetero-oligermerization of Bax and Bak. It is worth noting again that our Jurkat cells do not express Bax (Figure 13*C*). In the case of our data, the dramatic result of MOMP inhibition could be due to the

inability of our cells to make those hetero-oligermerized complexes. In conclusion, our data would suggest that there is a small release of pro-apoptotic factors into the cytosol that allows activation of effector caspases to feed forward to activate more Bak to release more pro-apoptotic factors. However, the exact amount of pro-apoptotic factors that are released initially *versus* how much is due to the feed forward amplification loop is not known and could be explored in the future. Determining the mechanism of the biphasic release of pro-apoptotic factors could also contribute to the understanding of how much of the pro-apoptotic players are needed to commit the cell to die.

7.2 Caspase-Mediated Bak Activation and Cytochrome *c* Release During Intrinsic Apoptotic Cell Death in Jurkat Cells

Overall our data support the hypothesis of a non-linear pathway for mitochondria-mediated apoptosis (Figure 28). We suggest that the commitment of a cell to undergo apoptosis depends on caspases and mitochondria forming an essential amplification circuit that functions as a central control point of genotoxic stress-induced apoptosis. After the apoptotic stimulus of DNA damage, mitochondria-mediated apoptosis is initiated. Initially, a small amount of a Bcl-2 multidomain pro-apoptotic protein (Bak) is activated by a BH3-only protein, most likely Bid or Bim. This allows

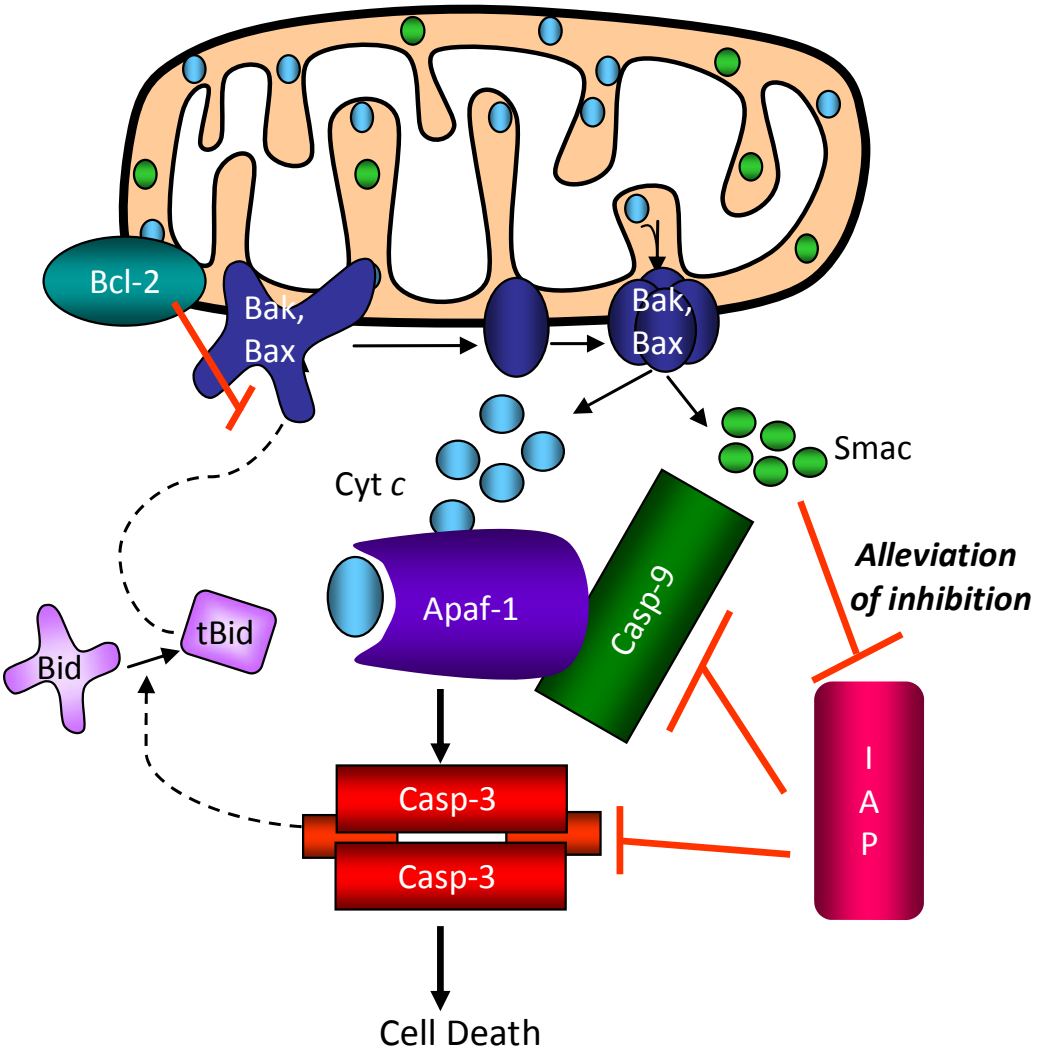

Figure 28. Feed Forward Perspective.

Caspases and mitochondria form an essential amplification circuit that functions as a central control point of genotoxic stress-induced apoptosis. *Cyt c, cytochrome c; tBid, truncated Bid; Casp, caspase; IAP, inhibitor of apoptosis proteins; Bid, BH3 interacting domain; Apaf-1, apoptotic protease activating factor 1; Bak, Bcl-2 antagonist/killer 1; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; Bcl-x_L, Bcl-2 like 1.*

the first pass through the mitochondria-mediated pathway to occur. Specifically, a small amount of pro-apoptotic factors (cytochrome *c* and Smac) are released into the cytosol. This allows for a few initiator caspase-9 molecules to become activated. These activated caspase-9 proteases, in turn, can cleave and activate caspase-3 to an extent that is sufficient to cleave Bid. The Bid that is cleaved allows for more Bak activation and thus causes a second more productive pass through the mitochondria-mediated pathway and so on. In other words, it is like the repeated passing of a roller coaster on a rail. If, the first time, the train of cars is almost empty, the lower weight will cause a lower maximum speed. If some more people jump in as the train passes through the station, the second time around with more weight the speed will increase. In a similar way, each time the cell releases more pro-apoptotic factors into the cytosol, the speed and extent to which the cell is degraded will increase.

7.3 Caspase-9 Activation by the Apoptosome Is Not Required for Fas-Mediated Apoptosis in Type II Jurkat Cells

It has been widely asserted that for a type II cell to undergo receptor-mediated apoptosis that it needs the mitochondria-mediated pathway to amplify the amount of caspase-3 activation that occurs initially. This is due to the low caspase-8 activation at the DISC in a type II cell. Caspase-8

activation in a type II cell is insufficient to cleave enough caspase-3 to cause cell death. However, what was less understood was what part of the mitochondria-mediated pathway was actually required to increase caspase-3 activation. We demonstrated by eliminating apoptosome-mediated activation of caspase-9 that what was needed from the mitochondria-mediated pathway was the release of Smac, not cytochrome *c*. Under normal conditions, as a way to protect the cell from accidental partial activation of caspases, IAPs reside in the cytosol waiting to sequester any inadvertantly activated caspases. There is a balance between how many activated caspases and how many IAPs are present. If a cell can activate enough caspases, then they can overcome the inhibition, but when they cannot, IAP will prevent cell death. In other instances, however, there is another level of complexity. Specifically, in type II cells, where initial caspase-8 activation is too low to induce cell death, Smac and/or Omi protein(s) need to be present to inhibit IAPs and promote cell death. In this case, after the stimulation of the receptor-mediated pathway, the low amount of caspase-3 activation that did occur was all inhibited by IAPs. Smac and Omi must be released into the cytosol to inhibit IAPs. This releases the activated caspase-3 to not only dismantle the cell, but also activate the feed forward amplification circuit that was described earlier.

7.4 Apaf-1 Deficiency Promotes the Elimination of Cytochrome *c* and Smac by Proteasomal Degradation, and to a Lesser Extent Autophagy, in Response to Genotoxic Stress

It would seem that the cell has developed another way to help protect itself from accidental apoptosis. A cell requires considerable energy to produce itself and presumably would want to be certain before committing itself to die, while reserving the ability to die if it cannot repair or recover from a given injury. This is evident when one considers the complexity of apoptotic regulation. Specifically, not only do apoptotic proteins regulate each other via protein-protein interactions (e.g. anti-apoptotic Bcl-2 family members inhibit pro-apoptotic Bcl-2 family members) but most apoptosis proteins are also internally regulated (i.e. post-translational modifications of Bid being cleaved to activate tBid). It is also clear that other cellular processes can be involved in regulation of apoptosis (i.e. increased transcription or translation). For example, up-regulation of Puma (p53-dependent pro-apoptotic Bcl-2 family member) has been reported during some forms of genotoxic stress-induced apoptosis.

My results also implicate another way in which a cell can protect itself from accidental apoptosis. A cell limits the length of time a pro-apoptotic factor released from the mitochondria can be retained in the cytosol. This time limit occurs when the cell is not actively undergoing apoptosis. This was

observed in the Apaf-1-deficient cells after prolonged incubation with the DNA-damaging drug etoposide. The initial amount of pro-apoptotic factors that were released into the cytosol in cells lacking Apaf-1, after being damaged with etoposide, have no purpose since caspase activation did not ensue. In this case, my data suggested that the released pro-apoptotic proteins are continuously degraded within the 26S proteasome (Figure 27). Lastly, another cellular process that can help protect the cell after an insult is autophagy. After prolonged incubation with etoposide total cellular cytochrome *c* and Smac disappeared in the Apaf-1-deficient cells. We see a partial retention of cytochrome *c* and Smac with autophagy inhibitors. These data suggest that autophagic removal of damaged mitochondria may play an important pro-survival role in eliminating damaged mitochondria after apoptotic stimuli.

7.5 Concluding Remarks

In conclusion, the mitochondrial pathway appears to *not* proceed in a strictly linear fashion after treatment with the DNA-damaging drug etoposide. What was once considered “downstream events”, such as caspase activation is now required for etoposide-induced mitochondrial apoptotic events. It appears that only the release of IAP antagonists are required from the mitochondria-mediated pathway after stimulation of a death receptor. The

apoptotic pathways are more complex than once thought. It is this “checks and balances” system that allows the body to be the most energetically proficient and ultimately determines if the cell will live or die. This new information about the process of MOMP presented in my dissertation provides important information that may help in drug development in the future.

CHAPTER 8: LITERATURE CITED

Acehan, D., Jiang, X., Morgan, D.G., Heuser, J.E., Wang, X., and Akey, C.W. (2002). Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. Mol Cell *9*, 423-432.

Adrain, C., Creagh, E.M., and Martin, S.J. (2001). Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by Bcl-2. EMBO J *20*, 6627-6636.

Antonsson, B., Montessuit, S., Lauper, S., Eskes, R., and Martinou, J.C. (2000). Bax oligomerization is required for channel-forming activity in liposomes and to trigger cytochrome c release from mitochondria. Biochem J *345 Pt 2*, 271-278.

Antonsson, B., Montessuit, S., Sanchez, B., and Martinou, J.C. (2001). Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. The Journal of biological chemistry *276*, 11615-11623.

Berger, A.B., Witte, M.D., Denault, J.B., Sadaghiani, A.M., Sexton, K.M., Salvesen, G.S., and Bogyo, M. (2006). Identification of early intermediates of caspase activation using selective inhibitors and activity-based probes. Mol Cell *23*, 509-521.

Boatright, K.M., Renatus, M., Scott, F.L., Sperandio, S., Shin, H., Pedersen, I.M., Ricci, J.E., Edris, W.A., Sutherlin, D.P., Green, D.R.*, et al.* (2003). A unified model for apical caspase activation. Mol Cell *11*, 529-541.

Boehning, D., Patterson, R.L., Sedaghat, L., Glebova, N.O., Kurosaki, T., and Snyder, S.H. (2003). Cytochrome c binds to inositol (1,4,5) trisphosphate receptors, amplifying calcium-dependent apoptosis. Nat Cell Biol *5*, 1051-1061.

Bonzon, C., Bouchier-Hayes, L., Pagliari, L.J., Green, D.R., and Newmeyer, D.D. (2006). Caspase-2-induced apoptosis requires bid cleavage: a physiological role for bid in heat shock-induced death. Mol Biol Cell *17*, 2150-2157.

Bossy-Wetzel, E., Newmeyer, D.D., and Green, D.R. (1998). Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. Embo J *17*, 37-49.

Boya, P., Gonzalez-Polo, R.A., Casares, N., Perfettini, J.L., Dessen, P., Larochette, N., Metivier, D., Meley, D., Souquere, S., Yoshimori, T.*, et al.* (2005). Inhibition of macroautophagy triggers apoptosis. Mol Cell Biol *25*, 1025-1040.

Brunelle, J.K., and Letai, A. (2009). Control of mitochondrial apoptosis by the Bcl-2 family. Journal of cell science *122*, 437-441.

Cain, K., Bratton, S.B., Langlais, C., Walker, G., Brown, D.G., Sun, X.M., and Cohen, G.M. (2000). Apaf-1 oligomerizes into biologically active approximately 700-kDa and inactive approximately 1.4-MDa apoptosome complexes. The Journal of biological chemistry *275*, 6067-6070.

Cartron, P.F., Gallenne, T., Bougras, G., Gautier, F., Manero, F., Vusio, P., Meflah, K., Vallette, F.M., and Juin, P. (2004). The first alpha helix of Bax plays a necessary role in its ligand-induced activation by the BH3-only proteins Bid and PUMA. Mol Cell *16*, 807-818.

Cerretti, D.P., Kozlosky, C.J., Mosley, B., Nelson, N., Van Ness, K., Greenstreet, T.A., March, C.J., Kronheim, S.R., Druck, T., Cannizzaro, L.A.*, et al.* (1992). Molecular cloning of the interleukin-1 beta converting enzyme. Science *256*, 97-100.

Certo, M., Del Gaizo Moore, V., Nishino, M., Wei, G., Korsmeyer, S., Armstrong, S.A., and Letai, A. (2006). Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. Cancer Cell *9*, 351-365.

Chai, J., Du, C., Wu, J.W., Kyin, S., Wang, X., and Shi, Y. (2000). Structural and biochemical basis of apoptotic activation by Smac/DIABLO. Nature *406*, 855-862.

Chai, J., Shiozaki, E., Srinivasula, S.M., Wu, Q., Datta, P., Alnemri, E.S., and Shi, Y. (2001). Structural basis of caspase-7 inhibition by XIAP. Cell *104*, 769-780.

Chao, Y., Shiozaki, E.N., Srinivasula, S.M., Rigotti, D.J., Fairman, R., and Shi, Y. (2005). Engineering a dimeric caspase-9: a re-evaluation of the induced proximity model for caspase activation. PLoS Biol *3*, e183.

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

Cheng, E.H., Sheiko, T.V., Fisher, J.K., Craigen, W.J., and Korsmeyer, S.J. (2003). VDAC2 inhibits BAK activation and mitochondrial apoptosis. Science *301*, 513-517.

Cheng, E.H., Wei, M.C., Weiler, S., Flavell, R.A., Mak, T.W., Lindsten, T., and Korsmeyer, S.J. (2001). BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol Cell *8*, 705-711.

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

Chipuk, J.E., Bouchier-Hayes, L., and Green, D.R. (2006). Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario. Cell death and differentiation *13*, 1396-1402.

Chipuk, J.E., and Green, D.R. (2006). Dissecting p53-dependent apoptosis. Cell death and differentiation *13*, 994-1002.

Chittenden, T., Flemington, C., Houghton, A.B., Ebb, R.G., Gallo, G.J., Elangovan, B., Chinnadurai, G., and Lutz, R.J. (1995). A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions. Embo J *14*, 5589-5596.

Cohen, G.M. (1997). Caspases: the executioners of apoptosis. Biochem J *326 (Pt 1)*, 1-16.

Colell, A., Ricci, J.E., Tait, S., Milasta, S., Maurer, U., Bouchier-Hayes, L., Fitzgerald, P., Guio-Carrion, A., Waterhouse, N.J., Li, C.W.*, et al.* (2007). GAPDH and autophagy preserve survival after apoptotic cytochrome c release in the absence of caspase activation. Cell *129*, 983-997.

Crook, N.E., Clem, R.J., and Miller, L.K. (1993). An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. J Virol *67*, 2168-2174.

Cuervo, A.M. (2004). Autophagy: in sickness and in health. Trends Cell Biol *14*, 70-77.

Danial, N.N., and Korsmeyer, S.J. (2004). Cell death: critical control points. Cell *116*, 205-219.

Denault, J.B., Eckelman, B.P., Shin, H., Pop, C., and Salvesen, G.S. (2007). Caspase 3 attenuates XIAP (X-linked inhibitor of apoptosis protein)-mediated inhibition of caspase 9. Biochem J *405*, 11-19.

Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B., and Martinou, J.C. (1999). Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. The Journal of cell biology *144*, 891-901.

Deveraux, Q.L., Takahashi, R., Salvesen, G.S., and Reed, J.C. (1997). X-linked IAP is a direct inhibitor of cell-death proteases. Nature *388*, 300-304.

Du, C., Fang, M., Li, Y., Li, L., and Wang, X. (2000). Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell *102*, 33-42.

Eckelman, B.P., and Salvesen, G.S. (2006). The human anti-apoptotic proteins cIAP1 and cIAP2 bind but do not inhibit caspases. The Journal of biological chemistry *281*, 3254-3260.

Fadeel, B., Orrenius, S., and Zhivotovsky, B. (1999). Apoptosis in human disease: a new skin for the old ceremony? Biochem Biophys Res Commun *266*, 699-717.

Ferraro, E., Pulicati, A., Cencioni, M.T., Cozzolino, M., Navoni, F., di Martino, S., Nardacci, R., Carri, M.T., and Cecconi, F. (2008). Apoptosome-deficient cells lose cytochrome c through proteasomal degradation but survive by autophagy-dependent glycolysis. Mol Biol Cell *19*, 3576-3588.

Fesik, S.W. (2005). Promoting apoptosis as a strategy for cancer drug discovery. Nat Rev Cancer *5*, 876-885.

Franklin, E.E., and Robertson, J.D. (2007). Requirement of Apaf-1 for mitochondrial events and the cleavage or activation of all procaspases during genotoxic stress-induced apoptosis. Biochem J *405*, 115-122.

Friesen, C., Herr, I., Krammer, P.H., and Debatin, K.M. (1996). Involvement of the CD95 (APO-1/FAS) receptor/ligand system in drug-induced apoptosis in leukemia cells. Nat Med *2*, 574-577.

Fuentes-Prior, P., and Salvesen, G.S. (2004). The protein structures that shape caspase activity, specificity, activation and inhibition. Biochem J *384*, 201-232.

Garrido, C., Galluzzi, L., Brunet, M., Puig, P.E., Didelot, C., and Kroemer, G. (2006). Mechanisms of cytochrome c release from mitochondria. Cell death and differentiation *13*, 1423-1433.

Gogvadze, V., Orrenius, S., and Zhivotovsky, B. (2006). Multiple pathways of cytochrome c release from mitochondria in apoptosis. Biochim Biophys Acta *1757*, 639-647.

Goldstein, J.C., Munoz-Pinedo, C., Ricci, J.E., Adams, S.R., Kelekar, A., Schuler, M., Tsien, R.Y., and Green, D.R. (2005). Cytochrome c is released in a single step during apoptosis. Cell death and differentiation *12*, 453-462.

Goldstein, J.C., Waterhouse, N.J., Juin, P., Evan, G.I., and Green, D.R. (2000). The coordinate release of cytochrome c during apoptosis is rapid, complete and kinetically invariant. Nat Cell Biol *2*, 156-162.

Green, D.R., and Evan, G.I. (2002). A matter of life and death. Cancer Cell *1*, 19-30.

Green, D.R., and Kroemer, G. (2004). The pathophysiology of mitochondrial cell death. Science *305*, 626-629.

Green, D.R., and Kroemer, G. (2005). Pharmacological manipulation of cell death: clinical applications in sight? J Clin Invest *115*, 2610-2617.

Gross, A., Jockel, J., Wei, M.C., and Korsmeyer, S.J. (1998). Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. Embo J *17*, 3878-3885.

Guo, Y., Srinivasula, S.M., Druilhe, A., Fernandes-Alnemri, T., and Alnemri, E.S. (2002). Caspase-2 induces apoptosis by releasing proapoptotic proteins from mitochondria. The Journal of biological chemistry *277*, 13430-13437.

Han, Z., Hendrickson, E.A., Bremner, T.A., and Wyche, J.H. (1997). A sequential two-step mechanism for the production of the mature p17:p12 form of caspase-3 in vitro. J Biol Chem *272*, 13432-13436.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Hansen, T.M., Smith, D.J., and Nagley, P. (2006). Smac/DIABLO is not released from mitochondria during apoptotic signalling in cells deficient in cytochrome c. Cell death and differentiation *13*, 1181-1190.

Hao, Z., Duncan, G.S., Chang, C.C., Elia, A., Fang, M., Wakeham, A., Okada, H., Calzascia, T., Jang, Y., You-Ten, A.*, et al.* (2005). Specific ablation of the apoptotic functions of cytochrome C reveals a differential requirement for cytochrome C and Apaf-1 in apoptosis. Cell *121*, 579-591.

Hegde, R., Srinivasula, S.M., Zhang, Z., Wassell, R., Mukattash, R., Cilenti, L., DuBois, G., Lazebnik, Y., Zervos, A.S., Fernandes-Alnemri, T.*, et al.* (2002). Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts inhibitor of apoptosis protein-caspase interaction. The Journal of biological chemistry *277*, 432-438.

Hinds, M.G., Norton, R.S., Vaux, D.L., and Day, C.L. (1999). Solution structure of a baculoviral inhibitor of apoptosis (IAP) repeat. Nat Struct Biol *6*, 648-651.

Huang, Y., Park, Y.C., Rich, R.L., Segal, D., Myszka, D.G., and Wu, H. (2001). Structural basis of caspase inhibition by XIAP: differential roles of the linker versus the BIR domain. Cell *104*, 781-790.

Hur, J., Bell, D.W., Dean, K.L., Coser, K.R., Hilario, P.C., Okimoto, R.A., Tobey, E.M., Smith, S.L., Isselbacher, K.J., and Shioda, T. (2006). Regulation of expression of BIK proapoptotic protein in human breast cancer cells: p53-dependent induction of BIK mRNA by fulvestrant and proteasomal degradation of BIK protein. Cancer Res *66*, 10153-10161.

Jeffers, J.R., Parganas, E., Lee, Y., Yang, C., Wang, J., Brennan, J., MacLean, K.H., Han, J., Chittenden, T., Ihle, J.N.*, et al.* (2003). Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. Cancer Cell *4*, 321-328.

Jin, Z., and El-Deiry, W.S. (2005). Overview of cell death signaling pathways. Cancer Biol Ther *4*, 139-163.

Johnstone, R.W., Ruefli, A.A., and Lowe, S.W. (2002). Apoptosis: a link between cancer genetics and chemotherapy. Cell *108*, 153-164.

Juo, P., Kuo, C.J., Yuan, J., and Blenis, J. (1998). Essential requirement for caspase-8/FLICE in the initiation of the Fas-induced apoptotic cascade. Curr Biol *8*, 1001-1008.

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y., and Yoshimori, T. (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. Embo J *19*, 5720-5728.

Kagan, V.E., Borisenko, G.G., Tyurina, Y.Y., Tyurin, V.A., Jiang, J., Potapovich, A.I., Kini, V., Amoscato, A.A., and Fujii, Y. (2004). Oxidative lipidomics of apoptosis: redox catalytic interactions of cytochrome c with cardiolipin and phosphatidylserine. Free Radic Biol Med *37*, 1963-1985.

Kandasamy, K., Srinivasula, S.M., Alnemri, E.S., Thompson, C.B., Korsmeyer, S.J., Bryant, J.L., and Srivastava, R.K. (2003). Involvement of proapoptotic molecules Bax and Bak in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced mitochondrial disruption and apoptosis: differential regulation of cytochrome c and Smac/DIABLO release. Cancer Res *63*, 1712-1721.

Kashkar, H., Kronke, M., and Jurgensmeier, J.M. (2002). Defective Bax activation in Hodgkin B-cell lines confers resistance to staurosporine-induced apoptosis. Cell death and differentiation *9*, 750-757.

Kasibhatla, S., Brunner, T., Genestier, L., Echeverri, F., Mahboubi, A., and Green, D.R. (1998). DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-kappa B and AP-1. Mol Cell *1*, 543-551.

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

Khodjakov, A., Rieder, C., Mannella, C.A., and Kinnally, K.W. (2004). Laser micro-irradiation of mitochondria: is there an amplified mitochondrial death signal in neural cells? Mitochondrion *3*, 217-227.

Kim, H., Rafiuddin-Shah, M., Tu, H.C., Jeffers, J.R., Zambetti, G.P., Hsieh, J.J., and Cheng, E.H. (2006a). Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. Nat Cell Biol *8*, 1348-1358.

Kim, H., Rafiuddin-Shah, M., Tu, H.C., Jeffers, J.R., Zambetti, G.P., Hsieh, J.J., and Cheng, E.H. (2006b). Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. Nat Cell Biol *8*, 1348-1358.

Kim, H.E., Du, F., Fang, M., and Wang, X. (2005). Formation of apoptosome is initiated by cytochrome c-induced dATP hydrolysis and subsequent nucleotide exchange on Apaf-1. Proc Natl Acad Sci U S A *102*, 17545-17550.

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

Klionsky, D.J. (2005). The molecular machinery of autophagy: unanswered questions. J Cell Sci *118*, 7-18.

Klionsky, D.J. (2007). Autophagy: from phenomenology to molecular understanding in less than a decade. Nat Rev Mol Cell Biol *8*, 931-937.

Kroemer, G., Galluzzi, L., and Brenner, C. (2007). Mitochondrial membrane permeabilization in cell death. Physiol Rev *87*, 99-163.

Kroemer, G., and Levine, B. (2008). Autophagic cell death: the story of a misnomer. Nat Rev Mol Cell Biol *9*, 1004-1010.

Kroemer, G., and Reed, J.C. (2000). Mitochondrial control of cell death. Nat Med *6*, 513-519.

Kuwana, T., Bouchier-Hayes, L., Chipuk, J.E., Bonzon, C., Sullivan, B.A., Green, D.R., and Newmeyer, D.D. (2005). BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. Mol Cell *17*, 525-535.

Kuwana, T., Mackey, M.R., Perkins, G., Ellisman, M.H., Latterich, M., Schneiter, R., Green, D.R., and Newmeyer, D.D. (2002). Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. Cell *111*, 331-342.

Lakhani, S.A., Masud, A., Kuida, K., Porter, G.A., Jr., Booth, C.J., Mehal, W.Z., Inayat, I., and Flavell, R.A. (2006). Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. Science *311*, 847-851.

Lartigue, L., Medina, C., Schembri, L., Chabert, P., Zanese, M., Tomasello, F., Dalibart, R., Thoraval, D., Crouzet, M., Ichas, F.*, et al.* (2008). An intracellular wave of cytochrome c propagates and precedes Bax redistribution during apoptosis. J Cell Sci *121*, 3515-3523.

Letai, A. (2009). Puma strikes Bax. The Journal of cell biology *185*, 189-191.

Letai, A., Bassik, M.C., Walensky, L.D., Sorcinelli, M.D., Weiler, S., and Korsmeyer, S.J. (2002). Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. Cancer Cell *2*, 183-192.

Letai, A.G. (2008). Diagnosing and exploiting cancer's addiction to blocks in apoptosis. Nat Rev Cancer *8*, 121-132.

Levine, B., and Klionsky, D.J. (2004). Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev Cell *6*, 463-477.

Levine, B., and Kroemer, G. (2008). Autophagy in the pathogenesis of disease. Cell *132*, 27-42.

Li, H., Bergeron, L., Cryns, V., Pasternack, M.S., Zhu, H., Shi, L., Greenberg, A., and Yuan, J. (1997). Activation of caspase-2 in apoptosis. The Journal of biological chemistry *272*, 21010-21017.

Li, H., Zhu, H., Xu, C.J., and Yuan, J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell *94*, 491-501.

Li, L., Thomas, R.M., Suzuki, H., De Brabander, J.K., Wang, X., and Harran, P.G. (2004). A small molecule Smac mimic potentiates TRAIL- and TNFalpha-mediated cell death. Science *305*, 1471-1474.

Li, L.Y., Luo, X., and Wang, X. (2001). Endonuclease G is an apoptotic DNase when released from mitochondria. Nature *412*, 95-99.

Li, S., Zhao, Y., He, X., Kim, T.H., Kuharsky, D.K., Rabinowich, H., Chen, J., Du, C., and Yin, X.M. (2002). Relief of extrinsic pathway inhibition by the Bid-dependent mitochondrial release of Smac in Fas-mediated hepatocyte apoptosis. J Biol Chem *277*, 26912-26920.

Lindsten, T., Ross, A.J., King, A., Zong, W.X., Rathmell, J.C., Shiels, H.A., Ulrich, E., Waymire, K.G., Mahar, P., Frauwirth, K.*, et al.* (2000). The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Mol Cell *6*, 1389-1399.

Liu, H., Chang, D.W., and Yang, X. (2005). Interdimer processing and linearity of procaspase-3 activation. A unifying mechanism for the activation of initiator and effector caspases. J Biol Chem *280*, 11578-11582.

Liu, X., Kim, C.N., Yang, J., Jemmerson, R., and Wang, X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell *86*, 147-157.

Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. (1998). Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell *94*, 481-490.

Maiuri, M.C., Le Toumelin, G., Criollo, A., Rain, J.C., Gautier, F., Juin, P., Tasdemir, E., Pierron, G., Troulinaki, K., Tavernarakis, N.*, et al.* (2007). Functional and physical interaction between Bcl-X(L) and a BH3-like domain in Beclin-1. Embo J *26*, 2527-2539.

Malladi, S., Challa-Malladi, M., Fearnhead, H.O., and Bratton, S.B. (2009). The Apaf-1*procaspase-9 apoptosome complex functions as a proteolytic-based molecular timer. Embo J.

Marani, M., Tenev, T., Hancock, D., Downward, J., and Lemoine, N.R. (2002). Identification of novel isoforms of the BH3 domain protein Bim which directly activate Bax to trigger apoptosis. Mol Cell Biol *22*, 3577-3589.

McStay, G.P., Salvesen, G.S., and Green, D.R. (2008). Overlapping cleavage motif selectivity of caspases: implications for analysis of apoptotic pathways. Cell death and differentiation *15*, 322-331.

Meier, P., Finch, A., and Evan, G. (2000). Apoptosis in development. Nature *407*, 796-801.

Mizushima, N. (2007). Autophagy: process and function. Genes Dev *21*, 2861-2873.

Mizushima, N., and Klionsky, D.J. (2007). Protein turnover via autophagy: implications for metabolism. Annu Rev Nutr *27*, 19-40.

Mizushima, N., Levine, B., Cuervo, A.M., and Klionsky, D.J. (2008). Autophagy fights disease through cellular self-digestion. Nature *451*, 1069-1075.

Munoz-Pinedo, C., Guio-Carrion, A., Goldstein, J.C., Fitzgerald, P., Newmeyer, D.D., and Green, D.R. (2006). Different mitochondrial intermembrane space proteins are released during apoptosis in a manner that is coordinately initiated but can vary in duration. Proc Natl Acad Sci U S A *103*, 11573-11578.

Muzio, M., Chinnaiyan, A.M., Kischkel, F.C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J.D., Zhang, M., Gentz, R.*, et al.* (1996). FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. Cell *85*, 817-827.

Norbury, C.J., and Zhivotovsky, B. (2004). DNA damage-induced apoptosis. Oncogene *23*, 2797-2808.

Ow, Y.P., Green, D.R., Hao, Z., and Mak, T.W. (2008). Cytochrome c: functions beyond respiration. Nat Rev Mol Cell Biol *9*, 532-542.

Pacher, P., and Hajnoczky, G. (2001). Propagation of the apoptotic signal by mitochondrial waves. Embo J *20*, 4107-4121.

Park, C.M., Sun, C., Olejniczak, E.T., Wilson, A.E., Meadows, R.P., Betz, S.F., Elmore, S.W., and Fesik, S.W. (2005). Non-peptidic small molecule inhibitors of XIAP. Bioorg Med Chem Lett *15*, 771-775.

Petak, I., Tillman, D.M., Harwood, F.G., Mihalik, R., and Houghton, J.A. (2000). Fas-dependent and -independent mechanisms of cell death following DNA damage in human colon carcinoma cells. Cancer Res *60*, 2643-2650.

Petersen, S.L., Wang, L., Yalcin-Chin, A., Li, L., Peyton, M., Minna, J., Harran, P., and Wang, X. (2007). Autocrine TNFalpha signaling renders human cancer cells susceptible to Smac-mimetic-induced apoptosis. Cancer Cell *12*, 445-456.

Pop, C., Fitzgerald, P., Green, D.R., and Salvesen, G.S. (2007). Role of proteolysis in caspase-8 activation and stabilization. Biochemistry *46*, 4398-4407.

Pop, C., Timmer, J., Sperandio, S., and Salvesen, G.S. (2006). The apoptosome activates caspase-9 by dimerization. Mol Cell *22*, 269-275.

Puthalakath, H., Huang, D.C., O'Reilly, L.A., King, S.M., and Strasser, A. (1999). The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. Mol Cell *3*, 287-296.

Ricci, J.E., Munoz-Pinedo, C., Fitzgerald, P., Bailly-Maitre, B., Perkins, G.A., Yadava, N., Scheffler, I.E., Ellisman, M.H., and Green, D.R. (2004). Disruption of mitochondrial function during apoptosis is mediated by caspase cleavage of the p75 subunit of complex I of the electron transport chain. Cell *117*, 773-786.

Riedl, S.J., Fuentes-Prior, P., Renatus, M., Kairies, N., Krapp, S., Huber, R., Salvesen, G.S., and Bode, W. (2001a). Structural basis for the activation of human procaspase-7. Proc Natl Acad Sci U S A *98*, 14790-14795.

Riedl, S.J., Renatus, M., Schwarzenbacher, R., Zhou, Q., Sun, C., Fesik, S.W., Liddington, R.C., and Salvesen, G.S. (2001b). Structural basis for the inhibition of caspase-3 by XIAP. Cell *104*, 791-800.

Riedl, S.J., and Shi, Y. (2004). Molecular mechanisms of caspase regulation during apoptosis. Nat Rev Mol Cell Biol *5*, 897-907.

Robertson, J.D., Fadeel, B., Zhivotovsky, B., and Orrenius, S. (2002). 'Centennial' Nobel Conference on apoptosis and human disease. Cell death and differentiation *9*, 468-475.

Rodriguez, J., and Lazebnik, Y. (1999). Caspase-9 and APAF-1 form an active holoenzyme. Genes Dev *13*, 3179-3184.

Saito, M., Korsmeyer, S.J., and Schlesinger, P.H. (2000). BAX-dependent transport of cytochrome c reconstituted in pure liposomes. Nat Cell Biol *2*, 553-555.

Samraj, A.K., Keil, E., Ueffing, N., Schulze-Osthoff, K., and Schmitz, I. (2006). Loss of caspase-9 provides genetic evidence for the type I/II concept of CD95-mediated apoptosis. J Biol Chem *281*, 29652-29659.

Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K.J., Debatin, K.M., Krammer, P.H., and Peter, M.E. (1998). Two CD95 (APO-1/Fas) signaling pathways. EMBO J *17*, 1675-1687.

Scaffidi, C., Schmitz, I., Zha, J., Korsmeyer, S.J., Krammer, P.H., and Peter, M.E. (1999). Differential modulation of apoptosis sensitivity in CD95 type I and type II cells. J Biol Chem *274*, 22532-22538.

Schmitz, I., Walczak, H., Krammer, P.H., and Peter, M.E. (1999). Differences between CD95 type I and II cells detected with the CD95 ligand. Cell Death Differ *6*, 821-822.

Scorrano, L., and Korsmeyer, S.J. (2003). Mechanisms of cytochrome c release by proapoptotic BCL-2 family members. Biochem Biophys Res Commun *304*, 437-444.

Scott, F.L., Denault, J.B., Riedl, S.J., Shin, H., Renatus, M., and Salvesen, G.S. (2005). XIAP inhibits caspase-3 and -7 using two binding sites: evolutionarily conserved mechanism of IAPs. Embo J *24*, 645-655.

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

Shawgo, M.E., Shelton, S.N., and Robertson, J.D. (2008). Caspase-mediated Bak activation and cytochrome c release during intrinsic apoptotic cell death in Jurkat cells. J Biol Chem *283*, 35532-35538.

Shelton, S.N., Shawgo, M.E., and Robertson, J.D. (2009). Cleavage of Bid by executioner caspases mediates feed forward amplification of mitochondrial outer membrane permeabilization during genotoxic stress-induced apoptosis in Jurkat cells. J Biol Chem *284*, 11247-11255.

Shintani, T., and Klionsky, D.J. (2004). Autophagy in health and disease: a double-edged sword. Science *306*, 990-995.

Shiozaki, E.N., Chai, J., Rigotti, D.J., Riedl, S.J., Li, P., Srinivasula, S.M., Alnemri, E.S., Fairman, R., and Shi, Y. (2003). Mechanism of XIAP-mediated inhibition of caspase-9. Mol Cell *11*, 519-527.

Shiozaki, E.N., Chai, J., and Shi, Y. (2002). Oligomerization and activation of caspase-9, induced by Apaf-1 CARD. Proc Natl Acad Sci U S A *99*, 4197-4202.

Shiozaki, E.N., and Shi, Y. (2004). Caspases, IAPs and Smac/DIABLO: mechanisms from structural biology. Trends Biochem Sci *29*, 486-494.

Srinivasula, S.M., Ahmad, M., Fernandes-Alnemri, T., and Alnemri, E.S. (1998). Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. Mol Cell *1*, 949-957.

Srinivasula, S.M., and Ashwell, J.D. (2008). IAPs: what's in a name? Mol Cell *30*, 123-135.

Srinivasula, S.M., Datta, P., Fan, X.J., Fernandes-Alnemri, T., Huang, Z., and Alnemri, E.S. (2000). Molecular determinants of the caspase-promoting activity of Smac/DIABLO and its role in the death receptor pathway. The Journal of biological chemistry *275*, 36152-36157.

Srinivasula, S.M., Hegde, R., Saleh, A., Datta, P., Shiozaki, E., Chai, J., Lee, R.A., Robbins, P.D., Fernandes-Alnemri, T., Shi, Y*., et al.* (2001). A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. Nature *410*, 112-116.

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

Strasser, A., O'Connor, L., and Dixit, V.M. (2000). Apoptosis signaling. Annu Rev Biochem *69*, 217-245.

Sun, C., Cai, M., Gunasekera, A.H., Meadows, R.P., Wang, H., Chen, J., Zhang, H., Wu, W., Xu, N., Ng, S.C*., et al.* (1999a). NMR structure and mutagenesis of the inhibitor-of-apoptosis protein XIAP. Nature *401*, 818-822.

Sun, H., Nikolovska-Coleska, Z., Yang, C.Y., Xu, L., Tomita, Y., Krajewski, K., Roller, P.P., and Wang, S. (2004). Structure-based design, synthesis, and evaluation of conformationally constrained mimetics of the second mitochondria-derived activator of caspase that target the X-linked inhibitor of apoptosis protein/caspase-9 interaction site. J Med Chem *47*, 4147-4150.

Sun, X.M., Bratton, S.B., Butterworth, M., MacFarlane, M., and Cohen, G.M. (2002). Bcl-2 and Bcl-xL inhibit CD95-mediated apoptosis by preventing mitochondrial release of Smac/DIABLO and subsequent inactivation of X-linked inhibitor-of-apoptosis protein. J Biol Chem *277*, 11345-11351.

Sun, X.M., MacFarlane, M., Zhuang, J., Wolf, B.B., Green, D.R., and Cohen, G.M. (1999b). Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. The Journal of biological chemistry *274*, 5053-5060.

Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Snow, B.E., Brothers, G.M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M.*, et al.* (1999). Molecular characterization of mitochondrial apoptosis-inducing factor. Nature *397*, 441-446.

Suzuki, M., Youle, R.J., and Tjandra, N. (2000). Structure of Bax: coregulation of dimer formation and intracellular localization. Cell *103*, 645-654.

Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K., and Takahashi, R. (2001). A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. Mol Cell *8*, 613-621.

Takahashi, R., Deveraux, Q., Tamm, I., Welsh, K., Assa-Munt, N., Salvesen, G.S., and Reed, J.C. (1998). A single BIR domain of XIAP sufficient for inhibiting caspases. The Journal of biological chemistry *273*, 7787-7790.

Thompson, C.B. (1995). Apoptosis in the pathogenesis and treatment of disease. Science *267*, 1456-1462.

Thornberry, N.A., Bull, H.G., Calaycay, J.R., Chapman, K.T., Howard, A.D., Kostura, M.J., Miller, D.K., Molineaux, S.M., Weidner, J.R., Aunins, J.*, et al.* (1992). A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. Nature *356*, 768-774.

Thornberry, N.A., Rano, T.A., Peterson, E.P., Rasper, D.M., Timkey, T., Garcia-Calvo, M., Houtzager, V.M., Nordstrom, P.A., Roy, S., Vaillancourt, J.P.*, et al.* (1997). A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. The Journal of biological chemistry *272*, 17907-17911.

Tsujimoto, Y., Finger, L.R., Yunis, J., Nowell, P.C., and Croce, C.M. (1984). Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. Science *226*, 1097-1099.

Tu, S., McStay, G.P., Boucher, L.M., Mak, T., Beere, H.M., and Green, D.R. (2006). In situ trapping of activated initiator caspases reveals a role for caspase-2 in heat shock-induced apoptosis. Nat Cell Biol *8*, 72-77.

Twiddy, D., Brown, D.G., Adrain, C., Jukes, R., Martin, S.J., Cohen, G.M., MacFarlane, M., and Cain, K. (2004). Pro-apoptotic proteins released from the mitochondria regulate the protein composition and caspase-processing activity of the native Apaf-1/caspase-9 apoptosome complex. The Journal of biological chemistry *279*, 19665-19682.

Twiddy, S.S., Holmes, E.C., and Rambaut, A. (2003). Inferring the rate and time-scale of dengue virus evolution. Mol Biol Evol *20*, 122-129.

Upreti, M., Chu, R., Galitovskaya, E., Smart, S.K., and Chambers, T.C. (2008). Key role for Bak activation and Bak-Bax interaction in the apoptotic response to vinblastine. Mol Cancer Ther *7*, 2224-2232.

Upton, J.P., Austgen, K., Nishino, M., Coakley, K.M., Hagen, A., Han, D., Papa, F.R., and Oakes, S.A. (2008). Caspase-2 cleavage of BID is a critical apoptotic signal downstream of endoplasmic reticulum stress. Mol Cell Biol *28*, 3943-3951.

Valentijn, A.J., and Gilmore, A.P. (2004). Translocation of full-length Bid to mitochondria during anoikis. The Journal of biological chemistry *279*, 32848-32857.

Vande Walle, L., Van Damme, P., Lamkanfi, M., Saelens, X., Vandekerckhove, J., Gevaert, K., and Vandenabeele, P. (2007). Proteome-wide Identification of HtrA2/Omi Substrates. J Proteome Res *6*, 1006-1015.

Varfolomeev, E., Blankenship, J.W., Wayson, S.M., Fedorova, A.V., Kayagaki, N., Garg, P., Zobel, K., Dynek, J.N., Elliott, L.O., Wallweber, H.J.*, et al.* (2007). IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. Cell *131*, 669-681.

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

Verdecia, M.A., Huang, H., Dutil, E., Kaiser, D.A., Hunter, T., and Noel, J.P. (2000). Structure of the human anti-apoptotic protein survivin reveals a dimeric arrangement. Nat Struct Biol *7*, 602-608.

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

Verhagen, A.M., Silke, J., Ekert, P.G., Pakusch, M., Kaufmann, H., Connolly, L.M., Day, C.L., Tikoo, A., Burke, R., Wrobel, C.*, et al.* (2002). HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins. The Journal of biological chemistry *277*, 445-454.

Villunger, A., Michalak, E.M., Coultas, L., Mullauer, F., Bock, G., Ausserlechner, M.J., Adams, J.M., and Strasser, A. (2003). p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. Science *302*, 1036-1038.

Vince, J.E., Wong, W.W., Khan, N., Feltham, R., Chau, D., Ahmed, A.U., Benetatos, C.A., Chunduru, S.K., Condon, S.M., McKinlay, M.*, et al.* (2007). IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. Cell *131*, 682-693.

Vincenz, C., and Dixit, V.M. (1997). Fas-associated death domain protein interleukin-1beta-converting enzyme 2 (FLICE2), an ICE/Ced-3 homologue, is proximally involved in CD95- and p55-mediated death signaling. The Journal of biological chemistry *272*, 6578-6583.

Wang, K., Gross, A., Waksman, G., and Korsmeyer, S.J. (1998). Mutagenesis of the BH3 domain of BAX identifies residues critical for dimerization and killing. Mol Cell Biol *18*, 6083-6089.

Waterhouse, N.J., Goldstein, J.C., von Ahsen, O., Schuler, M., Newmeyer, D.D., and Green, D.R. (2001). Cytochrome c maintains mitochondrial transmembrane potential and ATP generation after outer mitochondrial membrane permeabilization during the apoptotic process. The Journal of cell biology *153*, 319-328.

Wei, M.C., Lindsten, T., Mootha, V.K., Weiler, S., Gross, A., Ashiya, M., Thompson, C.B., and Korsmeyer, S.J. (2000). tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. Genes Dev *14*, 2060-2071.

Wei, M.C., Zong, W.X., Cheng, E.H., Lindsten, T., Panoutsakopoulou, V., Ross, A.J., Roth, K.A., MacGregor, G.R., Thompson, C.B., and Korsmeyer, S.J. (2001). Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science *292*, 727-730.

Wilkinson, J.C., Cepero, E., Boise, L.H., and Duckett, C.S. (2004). Upstream regulatory role for XIAP in receptor-mediated apoptosis. Mol Cell Biol *24*, 7003-7014.

Willis, S.N., Fletcher, J.I., Kaufmann, T., van Delft, M.F., Chen, L., Czabotar, P.E., Ierino, H., Lee, E.F., Fairlie, W.D., Bouillet, P.*, et al.* (2007). Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. Science *315*, 856-859.

Wu, G., Chai, J., Suber, T.L., Wu, J.W., Du, C., Wang, X., and Shi, Y. (2000). Structural basis of IAP recognition by Smac/DIABLO. Nature *408*, 1008-1012.

Wu, T.Y., Wagner, K.W., Bursulaya, B., Schultz, P.G., and Deveraux, Q.L. (2003). Development and characterization of nonpeptidic small molecule inhibitors of the XIAP/caspase-3 interaction. Chem Biol *10*, 759-767.

Yang, Q.H., Church-Hajduk, R., Ren, J., Newton, M.L., and Du, C. (2003). Omi/HtrA2 catalytic cleavage of inhibitor of apoptosis (IAP) irreversibly inactivates IAPs and facilitates caspase activity in apoptosis. Genes Dev *17*, 1487-1496.

Yi, C.H., and Yuan, J. (2009). The Jekyll and Hyde functions of caspases. Dev Cell *16*, 21-34.

Youle, R.J., and Karbowski, M. (2005). Mitochondrial fission in apoptosis. Nat Rev Mol Cell Biol *6*, 657-663.

Youle, R.J., and Strasser, A. (2008). The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol *9*, 47-59.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H.M., and Horvitz, H.R. (1993). The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. Cell *75*, 641-652.

Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S.J. (1996). Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). Cell *87*, 619-628.

Zha, J., Weiler, S., Oh, K.J., Wei, M.C., and Korsmeyer, S.J. (2000). Posttranslational N-myristoylation of BID as a molecular switch for targeting mitochondria and apoptosis. Science *290*, 1761-1765.

Zhou, L., and Chang, D.C. (2008). Dynamics and structure of the Bax-Bak complex responsible for releasing mitochondrial proteins during apoptosis. J Cell Sci *121*, 2186-2196.

Zhou, L.L., Zhou, L.Y., Luo, K.Q., and Chang, D.C. (2005). Smac/DIABLO and cytochrome c are released from mitochondria through a similar mechanism during UV-induced apoptosis. Apoptosis *10*, 289-299.

Zong, W.X., Lindsten, T., Ross, A.J., MacGregor, G.R., and Thompson, C.B. (2001). BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. Genes Dev *15*, 1481-1486.

Zou, H., Li, Y., Liu, X., and Wang, X. (1999). An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. The Journal of biological chemistry *274*, 11549-11556.