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Regulation of Apoptosis by XIAP Ubiquitin- Ligase Activity

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Virtually all animal cells have the known ability to self-destruct by undergoing apoptosis, a morphologically distinct form of programmed cell death. The proper regulation of apoptosis is critical for both development and tissue homeostasis, and inhibition of apoptosis contributes to the development and progression of cancer. Inhibitor of Apoptosis Proteins (IAPs) can bind to and inhibit caspases, the key executioners of apoptosis. Because IAPs are frequently over-expressed in human tumors, they have become major pharmacological targets for developing new cancer therapeutics. Many IAPs contain RING domains that function as E3 ubiquitin-ligases to regulate the abundance of IAPs themselves and their binding partners by engaging the ubiquitin system. The precise physiological function of individual mammalian IAPs and their role as E3-ubiquitin ligases *in situ* remain largely obscure.

Here, we investigated the function of XIAP ubiquitin-ligase activity by deleting the RING motif via gene targeting in the mouse. Mice expressing *XIAP ΔRING* were fertile, born in expected proportions, and

were not obviously prone to disease in a pathogen-free environment. Removing the RING finger motif stabilized XIAP protein in apoptotic thymocytes, demonstrating that XIAP ubiquitin-ligase activity is a major determinant of XIAP protein stability. However, consistent with earlier reports on *XIAP*-null mice, we found no detectable abnormalities in apoptosis of mutant thymocytes. On the other hand, *ΔRING* embryonic stem cells and fibroblasts had elevated caspase-3 enzyme activity and impaired ubiquitination of active caspase-3 during apoptosis. Furthermore, *XIAP ΔRING* embryonic fibroblasts were strongly sensitized to TNF-α-induced apoptosis. Similar results were obtained with *XIAP*-null mice. Finally, deletion of the RING also improved the survival of mice in the *Eμ-Myc* lymphoma model. The improved prognosis corresponded to increased apoptosis and decreased abundance of proliferating B-cells in the bone marrow, and a curtailed incidence of leukemia. This demonstrates a physiological requirement of XIAP E3 ubiquitin-ligase activity for the inhibition of apoptosis and for tumor suppression *in vivo*.

For my parents

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LIST OF ABBREVIATIONS

Apaf-1	apoptosis protease activating factor
ARTS	apoptosis related protein in TGF-β signaling pathway
BCL	B-cell lymphoma
BH	bcl homology
BIR	baculovirus iap repeat
CARD	caspase recruitment domain
CED	cell death abnormal
CHX	Cycloheximide
cIAP	cellular IAP
DD	death domain
DED	death effector domain
DIABLO	direct IAP binding protein with low pI
DIAP	*Drosophila* IAP
DISC	death inducing signaling complex
ES	embryonic stem
FLICA	fluorescent inhibitor of caspases
FLIP	flice-like inhibitor protein
GalN	D-galactosamine
IAP	inhibitor of apoptosis
IBM	IAP-binding motif
ICE	interleukin-1-beta converting enzyme-1
JNK	c-Jun N-terminal kinase
MALT	marginal zone mucosa-associated lymphoid tissue
MAP	mitogen activated protein
MEF	mouse embryonic fibroblast
MHC	major histocompatibility complex
ML-IAP	melanoma IAP
NF-κB	nuclear factor κB
NIK1	NF-κB interacting kinase
PARP	poly-ADP ribose polymerase
RFU	Relative fluorescence unit
RING	really interesting new gene
RIP1	receptor interacting kinase
RLU	relative luminescence unit
SMAC	second mitochondrial activator of caspases
TCR	T-cell receptor

TNF	tumor necrosis factor
TNF-R	TNF receptor
TRADD	TNF-R associated death domain
TRAF2	TNF-R associated factor 2
TRAIL	TNF-related apoptosis inducing ligand
TUNEL	terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling
XIAP	X-linked IAP

1 INTRODUCTION

1.1 Introductory comment

Cell death is a fundamental aspect of life in multicellular animals. Genetically encoded cell death pathways appear to function as early as the eight-cell blastocyst stage (Hardy et al., 1989), and continue during embryonic development to mold the shape and function of organs. Cell death functions throughout adult life as a cellular quality control mechanism by removing superfluous or harmful cells. Cell death can be considered a critical counterpoint to proliferation. Indeed, the two phenomena are linked in some situations: excessive proliferation can provoke cell death (Evan et al., 1992), while dying cells can signal their replacement by transmitting mitogenic signals (Ryoo et al., 2004).

Virtually all animal cells have the ability to self-destruct by undergoing apoptosis, a morphologically distinct form of programmed cell death. The conservation and diversification of apoptosis during the evolution of multicellular animals point to its involvement in many vital processes. An understanding of how cells die is important for controlling pathologically misregulated cell death pathways. For example, it is very likely that all tumor cells subvert cell death checkpoints in the process of becoming malignant (Hanahan and Weinberg, 2000). Diseases as diverse as

stroke, neurodegeneration, and HIV infection are characterized by excessive cell death with a strong apoptotic component as well (Thompson, 1995).

1.2 Historical perspective on programmed cell death

Nineteenth century biologists described cell deaths in developing embryos by noting cells with atypical appearances, mostly during metamorphosis (Clarke and Clarke, 1996). The physiological nature of cell death was noted as early as 1842, when the German naturalist Karl Vogt described the disappearance of the notochord during toad metamorphosis. A relationship between death and proliferation was later described in the rat retina (Tansley et al., 1937). Rita Levi-Montalcini and colleagues noted that extraneous neurons are culled by cell death during development (Hamburger and Levi-Montalcini, 1949), and that survival factors can determine which cells live and die (Cohen and Levi-Montalcini, 1957; Levi-Montalcini and Hamburger, 1951). Indeed, it was apparent by mid-century that death plays important roles in animal development (Glucksmann, 1951; Saunders, 1966). Richard Lockshin coined the phrase "programmed cell death" to describe an active death process during metamorphosis in the tobacco worm *Manduca* (Lockshin and Williams, 1965). This observation is very perceptive because it notes that death appears as a programmed fate. Studies on the regressing tadpole tail helped to generalize these findings and

emphasize the role of macromolecular synthesis in developmental cell death (Tata, 1966).

Apoptosis was the first form of programmed cell death to be defined by a distinct morphology common to diverse cell types during death (Kerr et al., 1972). Apoptosis was coined from a Greek word describing a "shedding off" of leaves to refer to a stereotyped pattern of changes that include membrane blebbing, nuclear condensation, cytoplasmic compaction and DNA fragmentation (Kerr et al., 1972; Wyllie et al., 1980). The original paper offers a wealth of prescient insights. For instance, the authors note that recurring apoptosis reflects a conserved physiological program in normal and diseased tissues. Apoptosis functions in the "focal elimination of cells during normal embryonic development", and "seems to be involved in cell turnover in many healthy adult tissues". Furthermore, they speculate that apoptosis may function in "therapeutically induced tumor regression" and "hyperplasia might sometimes result from decreased apoptosis rather than increased mitosis." Their ultrastructural analyses of apoptosis in different contexts also highlight phagocytosis, as all apoptotic cells appeared engulfed by other cells *in situ*. They also postulate that apoptosis is distinct from necrotic cell death, which occurs after insults provoke cell rupture and inflammation (Wyllie et al., 1980).

Early studies on the death of developing T-cells in the thymus implicated an intracellular nuclease in the orderly DNA fragmentation that occurs during apoptosis (Wyllie, 1980), but the biochemical underpinnings of apoptosis remained elusive long after the term was coined. Indeed, the importance of cell death remained somewhat understated until the discovery that *BCL-2*, a gene cloned at a chromosomal breakpoint in follicular lymphoma, can prolong the survival of cells deprived of cytokines and synergize with the proliferative oncogene *c-Myc* to immortalize B-cells (McDonnell et al., 1989; Vaux et al., 1988). Transgenic mouse models of cancer reveal that coexpressed *BCL-2* and *c-Myc* provoke high-grade lymphoma (McDonnell and Korsmeyer, 1991; Strasser et al., 1990). These novel discoveries showed that a factor involved in cell death could serve as an oncogene, and awakened interest in the field.

The molecular basis for apoptosis was uncovered in large part through genetic studies in the nematode worm *Caenorhabditis elegans*. *C. elegans* is well suited to the study of programmed cell death because each animal generates cells during development that die invariantly (Sulston and Horvitz, 1977). Genetic screens identified *ced-3* (cell death abnormal) and *ced-4* as genes required specifically for programmed cell death during development (Ellis and Horvitz, 1986). Another gene, *ced-9*, was identified with an

opposing pro-survival function: gain-of-function mutations prevent cell death, while loss of *ced-9* function kills cells that normally live (Hengartner et al., 1992). Some cell death genes have functions conserved throughout the evolution of multicellular animals; *ced-9* is homologous to human *BCL-2*, and *BCL-2* expression rescues loss of *ced-9* function (Hengartner and Horvitz, 1994; Vaux et al., 1992).

1.3 Caspases

The key insight into the mechanism of apoptotic cell death was the observation that *ced-3* encodes a cysteine protease similar to a mammalian protease, ICE (interleukin-1-beta converting enzyme-1) (Xue et al., 1996; Yuan et al., 1993). CED-3 and ICE (now caspase-1) are the founding members of the caspase family of proteases, which are so named because they harbor an active site containing a cysteine residue, and preferentially cleave their substrates after aspartate residues, although glutamate is also tolerated (Nicholson, 1999). There are least ten caspases in the human and mouse genomes, and many of them function in apoptosis (Thornberry and Lazebnik, 1998). Caspases are expressed as weakly active precursors that require allosteric activation or proteolysis by other caspases before they can cleave their substrates efficiently.

A typical caspase precursor contains an amino-terminal prodomain, a

large subunit, and a small subunit. Proteolysis between the domains releases the prodomain and allows the large and small subunits to dimerize; this unit proceeds to dimerize to form an active tetramer (Nicholson, 1999). All caspases described to date have a dimerization interface that appears to be critical for activity (Shi, 2002b). Caspases recognize a tetrapeptide sequence, P4-P3-P2-P1, and cleave after the carboxyl-terminal residue (P1). P1 is usually Asp (though sometimes Glu), while P3 is preferentially Glu for mammalian caspases. Different caspases have distinct but often overlapping preferences for the P4-P3-P2-P1 sequence.

The mammalian caspases involved in apoptosis can be grouped into two broad categories based on their domain structures. Caspases with large prodomains (e.g., caspases-8 and -9) generally function as initiator caspases, and depend on allosteric activation and/or association with other factors for their activity (Shi, 2002b). Caspases with shorter prodomains (e.g., caspases-3, -6. -7) appear to require cleavage, often by initiator caspases, to become active. The large prodomains in initiator caspases contain additional domains used for associating with activating complexes through homotypic interactions. Examples are the CARD (caspase recruitment domain) in caspase-9 and its allosteric activator, Apaf-1; and DED (death effector domain) in caspase-8 and its activator, FADD (Fas-associated death

domain).

Caspases are only weakly active in their proenzyme forms, and effector caspases appear to require intrachain cleavage to function. A framework for thinking about effector caspase activation comes from comparisons between the structures of inactive procaspase-7 and its active form (Chai et al., 2001b). The overall structures of inactive and active caspase-7 are similar and both are dimers, but the peptide loops that comprise the active site adopt a different conformation in the active caspase. Cleavage between the small and large subunits of caspase-7 frees one of the active site loops from a restrained covalent linkage, and allows it to adopt a conformation that is conducive to forming an active site. This mechanism applies to other initiator caspases, presumably (Shi, 2002b).

Activated caspases can cleave a broad range of proteins during apoptosis; however, it is largely unclear which substrates must be cleaved for apoptosis to proceed. The functional consequences of caspase cleavage are known for some substrates. Nuclear lamin, for example, is cleaved by caspases to facilitate degradation of the nucleus during apoptosis (Takahashi et al., 1996). The fragmentation of genomic DNA that can occur during apoptosis arises when ICAD, the inhibitor of the caspase-activated DNase CAD, is cleaved and inactivated (Liu et al., 1997; Sakahira et al., 1998).

Membrane blebbing and nuclear disintegration arise when caspases cleave and activate ROCK1, a regulator of actin dynamics (Coleman et al., 2001; Croft et al., 2005). Cleavage of β-catenin by caspases helps to promote detachment from cellular substrata, which may aid in clearance of apoptotic cells (Brancolini et al., 1997). Since more than 400 human caspase substrates have been identified to date (Luthi and Martin, 2007), it is likely that a wide range of proteolyzed substrates contribute to apoptosis.

Some caspases have functions that appear unrelated to apoptosis. Caspase-1, the founding member of the caspase family, processes a precursor of the interleukin-1 cytokine involved in immunity; *caspase-1* knockout mice are impaired in mounting a response to endotoxic shock (Li et al., 1995). *Caspase-8*-null mice have abnormalities in heart development that are not easily explained by defective apoptosis (Varfolomeev et al., 1998). A secondary role for caspases in lymphocyte development is revealed by T-cells lacking *caspase-8*: mutant mice are immunodeficient, and lymphocytes from young mice have proliferative defects (Salmena and Hakem, 2005). Furthermore, there are many documented cases where caspase activity does not lead to the death of a cell. An apoptosis-like process occurs during spermatid development in *Drosophila*, where caspase activity is locally restricted to eliminate bulk cytoplasm without causing

death (Arama et al., 2003). The differentiation pathway for macrophages (Sordet et al., 2002) and lens development in the eye (Ishizaki et al., 1998) require caspase activity as well.

1.4 Activation of caspases

There are two known routes to caspase activation in mammalian cells. The mitochondrial pathway (also known as the intrinsic pathway) is activated in response to diverse death stimuli that include genotoxic damage, oncogene activation, and developmental cues. This route to apoptosis is so-named because the activation steps appear to be controlled by factors localized in or near mitochondria. An alternate pathway is activated through death receptors and figures prominently in immunity. Some cell types require engagement of both pathways for efficient apoptosis.

1.4.1 *Activation through mitochondrial factors*

In addition to the *ced-3* caspase, *ced-4* is another gene required for programmed cell death of somatic cells in *C. elegans* (Ellis and Horvitz, 1986). Epistasis experiments place *ced-4* upstream of *ced-3* as its activator during apoptosis (Shaham and Horvitz, 1996a; Shaham and Horvitz, 1996b). Insight into CED-4 function came after its mammalian counterpart, Apaf-1 (apoptotic protease related factor-1), was identified biochemically as an

activator of caspase-9 which promotes caspase-3 cleavage and activation *in vitro* (Li et al., 1997; Zou et al., 1997). Activation of caspase-9 by Apaf-1 requires ATP/dATP plus an additional cofactor. To date, cytochrome c is the only known factor that can trigger the formation of an active caspase-9 holoenzyme complex, known as the apoptosome (Liu et al., 1996). The apoptosome forms after cytochrome c binds Apaf-1, and triggers a conformational change that permits recruitment of caspase-9 to Apaf-1 via homotypic interactions. This functional unit oligomerizes into a wheel-shaped structure with seven-fold symmetry (Acehan et al., 2002). The formation of an apoptosome is energy dependent and requires both an ADP-ATP nucleotide exchange and hydrolysis of ATP by the ATPase activity of Apaf-1 (Riedl et al., 2005). Apaf-1 is an allosteric activator of caspase-9 that stimulates its activity toward caspase-3 by several orders of magnitude (Rodriguez and Lazebnik, 1999). While dimerization is necessary for caspase-9 activity (Shiozaki et al., 2003), the intra-chain cleavage that occurs in the apoptosome is largely dispensable for its activity. Formation of an apoptosome appears to be a prerequisite for the recruitment and activation of caspase-3 (Bratton et al., 2001). Although CED-4 does not appear to require a cofactor like cytochrome c, it does oligomerize to promote CED-3 processing and activation (Seshagiri and Miller, 1997b;

Yang et al., 1998).

The best evidence for a linear cytochrome c-Apaf-1-caspase-9-caspase-3 pathway comes from studies using mouse embryonic fibroblasts (MEFs) from knockout animals. Cytochrome c is the only known activator of Apaf-1 following DNA damage in MEFs. After UVC irradiation, the apoptosome does not oligomerize in MEFs harboring a mutant *cytochrome c* allele that retains respiratory functions but has a diminished capacity to bind Apaf-1 (Hao et al., 2005). Caspase-3 cleavage is not seen and the cells are very resistant to apoptosis. When compared directly, this phenotype is slightly less severe than in cells lacking *Apaf-1*, which suggests that other activators of Apaf-1 may exist. Alternatively, it is possible that the mutant cytochrome c retains some residual capacity to activate Apaf-1. Caspase-9 cleavage is almost entirely impaired in *Apaf-1*-null MEFs (Ho et al., 2004), and caspase-3 cleavage is essentially absent in *caspase-9*-null MEFs (Kuida et al., 1998) and ES cells (Hakem et al., 1998).

Biochemical studies and knockouts of individual components (mostly in MEFs) order initiator and effector caspases in a cascade downstream of mitochondria, but this may be an overly simplistic view. Mice doubly deficient for *caspase-3* and *caspase-7* display postnatal lethality and defective heart development, while MEFs are broadly resistant to

mitochondrial and death receptor apoptosis (Lakhani et al., 2006). This finding argues that these caspases are required for apoptosis in MEFs and probably other cells. Remarkably, however, many steps that are presumed to act upstream of caspase activation are delayed in these double-knockout MEFs. For instance, during apoptosis in double-knockout MEFs, mitochondrial integrity is largely maintained; BAX translocation is diminished; and cytochrome c is released more slowly during apoptosis (Lakhani et al., 2006). These studies argue that mitochondria may function as amplifiers of an initial apoptotic signal in certain cell types.

There are probably other cell death pathways in thymocytes apart from the known apoptotic programs. Thymocytes deficient for *caspases-3* and *-7* are resistant (though not completely) to mitochondrial apoptotic stimuli, but die readily through receptor pathways. Thymocytes lacking the pro-apoptotic function of *cytochrome c* mount normal apoptotic responses to diverse stimuli, even though apoptosome formation does not occur; *Apaf-1*-null thymocytes are more broadly resistant (Hao et al., 2005). Although this cytochrome c mutant probably retains some residual capacity to activate Apaf-1, it is very likely that thymocytes have redundant death programs that require further elucidation. Caspase activation need not proceed directly from initiator to effector caspases; there is evidence that caspase-6 may

activate caspase-8, the caspase thought to be responsible for initiating death receptor apoptosis (see below)(Murphy et al., 2004).

1.4.2 *Activation through death receptors*

A second pathway leading to apoptosis is triggered through cell surface receptors that signal to activate caspases. Mouse and human cells express a variety of receptors in the TNF-α (tumor necrosis factor α) receptor superfamily, which includes Fas (APO-1/CD95). Death receptors span the plasma membrane, extending an extracellular cysteine-rich motif that binds cognate ligands and a cytoplasmic tail that signals to other factors. The expression of receptors is often regulated; for example, resting B-cells express low levels of the Fas receptor, but upregulate the receptor in the presence of cytokines or endotoxins (Watanabe et al., 1995). Death ligands such as Fas ligand (FasL) are trimers, and can be membrane bound on signaling cells or cleaved by extracellular proteases to become soluble factors. These ligands are usually expressed on the surface of activated T-cells and natural killer T-cells, where they have a cytotoxic function during immune responses (Suda et al., 1993).

Fas signaling is well characterized and also considerably simpler than the TNF-α pathway (discussed in Chapter 5). The Fas receptor was identified as the molecular target of an antibody (anti-APO-1) that induces

apoptosis in malignant lymphocytes (Trauth et al., 1989). The antibody functions as a FasL agonist that aggregates the receptor into homotrimers (Siegel et al., 2000), and triggers assembly of a DISC (death inducing signaling complex) on the cytosolic face of the receptor (Kischkel et al., 1995). The DISC forms when the receptor recruits the adaptor protein FADD (Fas-associated death domain) through homotypic DD interactions. FADD also contains death effector domains (DEDs) that recruit caspase-8 (also caspase-10 in human cells), also through homotypic interactions. Caspase-8 appears to require a DISC to dimerize and activate, because the isolated procaspase is an inactive monomer (Donepudi et al., 2003). Procaspase-8 is cleaved after binding the DISC, possibly by auto-proteolysis after changing conformation (Medema et al., 1997), but all of the subunits remain associated (Lavrik et al., 2003). The DISC appears to promote caspase-8 dimerization and cleavage, but cleavage may be dispensible for activity (Donepudi et al., 2003). Caspase-3 is cleaved and activated by caspase-8 in the DISC. Caspase-8 initiates Fas-dependent apoptosis in MEFs; *caspase-8*-null cells are not killed by receptor ligation (Varfolomeev et al., 1998). Caspase-8 activation is regulated by DISC assembly and a competitor, c-FLIP (cellular FLICE [caspase-8]-like inhibitor protein). c-FLIP exists as a short form (c-FLIP$_S$) with two DED domains, and a long

form (c-$FLIP_L$) that resembles caspase-8 with two DED domains and an non-functional active site. c-$FLIP_L$ may function as a dominant-negative inhibitor of caspase-8 activation in the DISC, and its roles are discussed in further detail in Chapter 5.

1.5 Regulation of cell death by anti-apoptotic proteins

Since the core apoptotic machinery appears to be universally expressed in most cells, it is not surprising that the steps leading to caspase activation are very tightly controlled. One such strategy involves the sequestration of pro-apoptotic factors in mitochondria. For example, cytochrome c, the only known activator of Apaf-1, is normally resident in the intermembrane space of mitochondria. The steps leading to translocation of cytochrome c from mitochondria to the cytosol are regulated by the BCL-2 family of proteins. Initially, the anti-apoptotic function of BCL-2 was apparent from its functional similarity to the CED-9 protein from *C. elegans*, which can prevent death. Early studies showed that BCL-2 localizes to mitochondria and prevents many manifestations of apoptosis without promoting cell division (Hockenbery et al., 1990).

The molecular cloning of *BCL-2* led to the identification of an extended family of homologues that include anti- and pro-apoptotic constituents. The principal anti-apoptotic family members are BCL-X_L

(Boise et al., 1993), MCL-1 (Kozopas et al., 1993), A1 (Choi et al., 1995) and BCL-W (Gibson et al., 1996). BAX was the first pro-apoptotic family member and identified as a binding partner of BCL-2 (Oltvai et al., 1993), while the related BAK was cloned by homology (Kiefer et al., 1995). Anti- and pro-apoptotic BCL-2 family members are classified by their domain organizations: factors that inhibit death seem to have four BH (BCL-2 homology) domains (BH1-4), while pro-death members have three (BH1-3). These factors can bind each other by virtue of a BH1-3 binding pocket on anti-apoptotic BCL-2 proteins that accommodates the BH3 domain of pro-death factors (Sattler et al., 1997).

BAX and BAK appear to require conformational changes to engage anti-apoptotic BCL-2 proteins and promote death (Sattler et al., 1997). BAX is present as a cytosolic or perimitochondrial monomer in healthy cells, but changes conformation, oligomerizes and inserts into mitochondrial membranes before apoptosis (Suzuki et al., 2000). BAK also shifts conformations and associates with mitochondria prior to apoptosis. The combined activation of BAX and BAK lead to release of pro-apoptotic factors like cytochrome c from mitochondria into the cytosol (Wei et al., 2001). How this happens is unknown, but recombinant BAX and BAK are capable of forming pores in isolated vesicles (Korsmeyer et al., 2000).

While mice deficient for either *BAX* or *BAK* are viable, the compound knockout results in neonatal lethality with a very high penetrance (Lindsten et al., 2000). This is probably because *BAX* and *BAK* double knockout cells are virtually resistant to apoptotic stimuli that involve mitochondrial factors (Wei et al., 2001).

Although it is still unclear how BAX and BAK are activated, their pro-death functions appear to depend on factors from a third class of BCL-2 family members that harbor only the third BH domain ("BH3-only"). This family has grown to include a large number of BH3-only proteins since the identification of the founding member, BID (Wang et al., 1996). The wide variety of regulatory steps that control individual BH3-only proteins stresses their roles in activating apoptosis in response to distinct stimuli. For example, the BH3-only genes *PUMA* (Nakano and Vousden, 2001) and *NOXA* (Oda et al., 2000) are upregulated by p53 following genotoxic stress; PUMA is the key transducer of apoptotic signaling to BAX and BAK after DNA damage in many cell types (Villunger et al., 2003). BIM is the key mediator of apoptosis triggered by cytokine withdrawal, and has a role in promoting the death of autoreactive lymphocytes during development (Bouillet et al., 1999). Additionally, the BH3-only protein BMF is poised to trigger apoptosis when cells detach from extracellular substrata by its

association with myosin bundles (Puthalakath et al., 2001). The BH3-only protein BID is required for death receptor apoptosis in cells like hepatocytes, where it amplifies the apoptotic signal through the mitochondrial pathway (Yin et al., 1999). BID is cleaved by caspase-8 during death receptor signaling; truncated BID inserts into mitochondrial membranes to promote BAX and BAK oligomerization and mitochondrial relocalization, which in turn promotes cytochrome c release (Desagher et al., 1999).

Individual BH3-only proteins bind distinct but overlapping subsets of pro- and anti-apoptotic BCL-2 proteins (Chen et al., 2005). This led to a hypothesis that certain BH3-only proteins like BIM and BID function as "activators" that directly activate BAX and BAK, while others like BAD and NOXA act as "sensitizers" by displacing anti-apoptotic BCL-2 factors from the activators (Letai et al., 2002). An alternative hypothesis stresses the point that BH3-only factors can activate apoptosis indirectly by inhibiting the anti-apoptotic BCL-2 proteins (Willis et al., 2005). The proposed mechanisms are not necessarily exclusive, and the interactions that lead to mitochondrial membrane permeabilization and cytochrome c release clearly require further research.

The central logic behind the apoptotic pathway appears throughout multicellular animals. *C. elegans* relies on a streamlined version of the

pathway described above. The *C. elegans* genome harbors an anti-apoptotic BCL-2 gene, *ced-9* (Metzstein et al., 1998), and at least two BH3-only genes, *egl-1* and *ced-13* (Schumacher et al., 2005). *ced-13* is transcriptionally upregulated in the germline by a p53 homologue, while *egl-1* is more broadly utilized in the soma; both act pro-apoptotically by preventing CED-9 from inhibiting CED-4 (Schumacher et al., 2005; Yan et al., 2005).

1.6 IAPs: negative regulators of caspases

In most cells, caspases are subject to at least two forms of negative control. The activation steps are regulated in the mitochondrial and receptor routes to apoptosis: BCL-2 proteins determine whether mitochondrial factors like cytochrome C translocate to the cytosol to activate caspases, while the death receptor pathway requires ligands to activate initiator caspases. Furthermore, caspases are subject to negative regulation by inhibitors. One important group of negative caspase regulators includes the Inhibitor of Apoptosis Proteins (IAPs) (Miller, 1999). The first IAPs were identified in a genetic complementation screen for genes in the baculovirus genome that could substitute for loss of *p35*, then the only known inhibitor of cell death in virally infected insect cells (Birnbaum et al., 1994; Crook et al., 1993). This effort led to the identification of *iap* in the genome of *Autographa*

californica nuclear polyhedrosis virus, as well as homologues like Op-*iap* from *Orgyia pseudotsugata* virus (Clem and Miller, 1994; Crook et al., 1993). Baculovirus IAPs are characterized by a unique zinc finger motif of about 80 amino acids, later known as the BIR (for baculovirus IAP repeat)(Crook et al., 1993). Insights into baculoviral IAP function came from the observation that Op-IAP inhibits the processing and activation of an insect effector caspase during viral infection (Seshagiri and Miller, 1997a). Subsequent work showed that BIR domains are principally involved in binding to and inhibiting caspases (Takahashi et al., 1998).

The discovery of related genes in insect and mammalian genomes suggested that IAPs might be part of a larger regulatory scheme (Salvesen and Duckett, 2002; Vaux and Silke, 2005). The cellular IAPs (cIAPs) 1 and 2 were identified biochemically in human cells as binding partners of the TRAF2 protein in TNF-α apoptosis (see introduction to Chapter 5)(Rothe et al., 1995), while the X-chromosome-linked IAP (XIAP) was identified by homology to other IAPs (Duckett et al., 1996). In mammals, the other BIR-containing proteins are ML-IAP (melanoma IAP) (Vucic et al., 2000), Bruce/apollon (Chen et al., 1999), Survivin (Ambrosini et al., 1997), and a testis-specific IAP (ts-IAP) present in great apes (Richter et al., 2001). All of these IAPs bear at least one BIR, while cIAP1, cIAP2, XIAP ts-IAP, and

ML-IAP harbor RING domains. cIAP1 and cIAP2 also have a CARD domain. The *Drosophila* genome harbors two IAPs, DIAP1 and DIAP2, with two BIR domains and a RING motif. The *C. elegans* genome has a Survivin-like gene, but Survivin probably does not function in cell death (Lens et al., 2006). The lack of any apparent IAPs in *C. elegans* might reflect the need for expanded control over caspases in more complex or longer-lived organisms.

1.6.1 *Inhibition of caspases by direct binding*

The discovery that XIAP can bind and inhibit caspases with BIR domains partly explains why IAP expression prevents death (Deveraux et al., 1997; Takahashi et al., 1998). XIAP is the most studied mammalian IAP, probably because it is the best inhibitor of caspases *in vitro* (Eckelman and Salvesen, 2006). XIAP can directly bind and inhibit caspases through BIR domains present in triplicate in the amino-terminal half of the protein. Deletion mapping and structural studies reveal how XIAP can inhibit different caspases. A peptide sequence that includes the second BIR domain binds to and inhibits active caspases-3 and -7 by a common mechanism (Chai et al., 2001a; Riedl et al., 2001; Sun et al., 1999). The initial structural studies revealed that a short peptide sequence on the immediate amino-terminal side of BIR2 is necessary for caspase inhibition. This section of the

linker between BIR1 and BIR2 forms a hydrophobic binding pocket that spans the caspase catalytic groove in the opposite orientation from caspase substrates. This interaction precludes access to the caspase active site. Subsequent studies have shown that the BIR2 makes additional contacts with caspases-3 and -7 that stabilize the interactions (Scott et al., 2005). Two closely related IAPs, cIAP1 and cIAP2, can bind caspases-3 and -7 but inhibit their enzymatic activity very poorly *in vitro* (Eckelman and Salvesen, 2006). The differences appear to lie in the linker region between BIR1 and BIR2, because replacing the cIAP1 or cIAP2 linker with the XIAP linker increases caspase inhibition.

XIAP inhibits caspase-9 by a different mechanism altogether (Shiozaki et al., 2003). XIAP associates with the apoptosome (Bratton et al., 2001), where the XIAP BIR3 makes a large continuous contact with a caspase-9 monomer. This interaction simultaneously maintains the caspase as an inactive monomer and precludes the active site from adopting a productive conformation. Sequestering caspase-9 as a monomer is especially inhibitory because caspases must dimerize to be active (Shi, 2002b). XIAP BIR3 forms a hydrophobic binding pocket that makes additional contacts with the amino-terminal tetrapeptide of the caspase-9 small subunit known as the IAP binding motif (IBM). Although cIAP1 and

cIAP2 also bind the caspase-9 IBM through a similar pocket in BIR3, they lack a suitable interface for holding caspase-9 in a monomeric state. This observation probably explains why these IAPs are poor inhibitors of caspase-9 (Eckelman and Salvesen, 2006).

1.6.2 *Inhibition of caspases through ubiquitination*

In addition to BIR domains that can bind and inhibit caspases, many IAPs also contain a RING (really interesting new gene) domain that bestows ubiquitin-ligase functions. RING domains are found in diverse proteins and impart substrate specificity to the ubiquitin system (Joazeiro and Weissman, 2000). The ubiquitin system is the principal mechanism for actively regulating the abundance of proteins in eukaryotic cells (Glickman and Ciechanover, 2002). Ubiquitin-ligases can target proteins for degradation by assembling ubiquitin polymers on specific substrates. Polyubiquitin chains are assembled through the sequential activity of three highly conserved classes of enzymes. In the first step, the E1 ubiquitin-activating enzyme forms a high energy intermediate between an active site cysteine side chain and a ubiquitin monomer. This step requires energy in the form of ATP. The activated ubiquitin is then transferred to an active site cysteine in the E2 ubiquitin-conjugating enzyme. The E3 ubiquitin-ligase simultaneously binds its cognate E2 and the substrate to be ubiquitinated,

and juxtaposes the two to promote ubiquitin transfer between the E2 and an internal lysine on the substrate. In the case of RING E3s, the ubiquitin is transferred without forming a RING-ubiquitin intermediate, as there are no catalytic residues. The RING may serve as an allosteric activator of the E2, or perhaps by forming a scaffold to structurally coordinate ubiquitin transfer (Glickman and Ciechanover, 2002). Generally, eukaryotic genomes harbor two E1 enzymes and several hundred E2s; mammalian genomes contain possibly as many as 1000 RING E3s. This observation highlights the central role that ubiquitination plays in regulating diverse cellular processes.

The way in which a RING ubiquitinates its substrate influences the outcome of ubiquitination (Hochstrasser, 2006). The majority of RINGs studied so far appear to form a polyubiquitin chain on their substrates through iterative addition of ubiquitins through Lys48 linkages (Pickart and Eddins, 2004). A typical polyubiquitin chain contains more than a dozen ubiquitin moieties, but extension of a Lys48 ubiquitin chain to four or more subunits appears to constitute a minimal recognition sequence for the proteasome, the elaborate protease complex that proteolyses ubiquitinated substrates (Thrower et al., 2000). Alternatively, a RING can mono-ubiquitinate a substrate, or form polyubiquitin linkages with different topologies that may not necessarily lead to degradation by the proteasome

(Vaux and Silke, 2005). An example of this is the Lys63-linked polyubiquitin chain assembled on RIP1 and TRAF2 to activate the canonical NF-κB pathway (discussed below in the introduction to Chapter 5). Additionally, ubiquitin was discovered as a covalent histone modification (Goldknopf and Busch, 1975) that does not promote degradation by the proteasome. Many aspects of the ubiquitin system are mysterious. For instance, it is not clear exactly how a RING E3 can activate its cognate E2 to promote ubiquitin transfer because the E2 does not appear to change conformation after binding an E3 (Hochstrasser, 2006).

IAP RINGs have been studied thoroughly in *Drosophila*, where DIAP1 functions as an E3-ubiquitin ligase for regulating cell death and survival. This includes targeting the caspase-9 ortholog DRONC for degradation by the proteasome in living cells, and promoting self-conjugation and DIAP1 degradation under apoptotic conditions (Ryoo et al., 2002; Ryoo et al., 2004; Wilson et al., 2002). Loss of DIAP1 RING function leads to late embryonic lethality accompanied by massive levels of apoptosis, which closely mimics a complete loss of DIAP1 function (Goyal et al., 2000; Lisi et al., 2000). Point mutations that inactivate RING ubiquitin-ligase function do not preclude DIAP1 from binding the initiator caspase DRONC, but they do prevent polyubiquitination of DRONC

(Wilson et al., 2002). In principal, loss of an anti-apoptotic function of the DIAP1 RING (e.g., polyubiquitinating DRONC) could be mitigated by the concurrent loss of a pro-apoptotic function (e.g., promoting self-conjugation and degradation). In the absence of DIAP1 RING function, however, DIAP1 is stabilized and its protein levels are increased, but the net outcome for most cells is still excessive cell death due to highly elevated DRONC levels (Ryoo et al., 2004).

Among mouse IAPs, XIAP, cIAP1, cIAP2 and ML-IAP all have RING domains, but their functions *in vivo* are largely unknown (Vaux and Silke, 2005). The RING domains of XIAP and cIAP1 regulate the abundance of these proteins during apoptosis in primary mouse thymocytes (Yang et al., 2000). XIAP and cIAP1 can both self-conjugate polyubiquitin chains *in vitro*. This is the first function assigned to IAP RINGs, and it is hypothesized to underlie the death of thymocytes because IAP downregulation occurs before many manifestations of apoptosis (Yang and Li, 2000). Consistent with this notion, a RING-deleted XIAP is stably expressed in a mouse T-cell hybridoma and imparts resistance to diverse death stimuli (Yang et al., 2000). There is evidence that the XIAP RING can regulate the abundance of active caspase-3 subunits during apoptosis (Suzuki et al., 2001), in a manner reminiscent of how DIAP1 RING

regulates DRONC (Wilson et al., 2002). These experiments are difficult to interpret because they involve ectopic expression of active-site mutants of caspase-3, and XIAP binds caspase-3 at its active site (Riedl et al., 2001). There are also *in vitro* and overexpression data implicating XIAP RING in regulating the abundance of the large subunit of active caspase-9 (Morizane et al., 2005) and active caspase-7 (Creagh et al., 2004). These experiments are also difficult to interpret because it is unclear how much of the observed changes are owing to overexpression artifacts.

1.7 IAP antagonists

Genetic studies in *Drosophila* identified a novel class of IAP antagonists that derepress caspases, at least in part by competitively displacing them from IAPs. The three founding members of the IAP antagonist family, *Reaper*, *Hid* (head involution defective) and *Grim*, were discovered as three tightly linked loci in a genomic region required for initiating all cell death during *Drosophila* development (White et al., 1994). Reaper, Hid and Grim function as integrators that relay diverse pro-apoptotic stimuli, ranging from developmental cues to genotoxic damage, to trigger cell death. All three proteins share a structurally conserved N-terminal IAP-binding-motif (IBM) that can bind BIR domains (Shi, 2002a). The IBM appears to be the basis for caspase derepression, since IAP antagonists can

liberate caspases from IAPs by binding BIR domains more tightly (Shi, 2002b; Srinivasula et al., 2001).

IAP antagonists appear in a central position to dictate the outcome of DIAP1 E3 activity. Expression of Reaper depletes DIAP1 through RING-dependent autoubiquitination, leading to apoptosis in the wing imaginal disc and other tissues (Ryoo et al., 2002). Furthermore, *Ubcd1*, which encodes a cognate E2 for DIAP1 RING, genetically interacts with *DIAP1* and is required for *Reaper*-dependent apoptosis (Ryoo et al., 2002). Structural studies offer some insights into how IAP antagonists might regulate DIAP1 RING ubiquitination of caspases (Chai et al., 2003). DRONC binds DIAP1 BIR2 using a peptide sequence that maps between the prodomain and the large subunit, but is distinct from the typical IBM interface. The DRONC and Reaper/Hid/Grim binding sites overlap, however, which allows IAP antagonists to compete for BIR2 using tighter binding affinities (Wu et al., 2001). In this manner, IAP antagonists liberate DRONC and prevent DIAP1 RING from ubiquitinating it. Although DIAP1 has intrinsic tendency to autoubiquitinate *in vitro*, it is still unclear how IAP antagonists stimulate this phenomenon during apoptosis. Perhaps DIAP1 has no other local substrate when bound to a small IAP antagonist like Reaper (65 amino acids).

Mammalian genomes harbor many genes that bear superficial

similarities to fruit fly IAP antagonists. Smac (second mitochondrial activator of apoptosis) was identified biochemically as a factor present in the detergent-soluble membrane fraction that promotes caspase-3 activation in cellular extracts (Du et al., 2000); the same protein was identified as a binding partner of XIAP, and named DIABLO (direct IAP binding protein with low pI) by a second group (Verhagen et al., 2000). Smac is a homodimer that localizes to the mitochondria in healthy cells and egresses to the cytosol during apoptosis, where it antagonizes IAPs and promotes caspase activation. The amino-terminal tetrapeptide motif (AVPI) of mature Smac shares sequence similarity to the amino-termini of fruit fly IAP antagonists (Reaper: AVAF; Hid: AVPR; Grim: AIAY). It is also very similar to the IBM of the caspase-9 small subunit (human: ATPF; mouse: AVPY), which suggests a mechanism for how Smac can competitively liberate caspase-9 from XIAP in the apoptosome (Srinivasula et al., 2001). Indeed, the crystal structure of a XIAP/Smac complex confirms that Smac binds the same hydrophobic IBM-binding grooves in BIR2 (Chai et al., 2000) and BIR3 (Liu et al., 2000; Wu et al., 2000) where caspases also bind (Scott et al., 2005; Shiozaki et al., 2003). While monomeric Smac suffices to liberate caspase-9 from BIR3, dimeric Smac appears to be required to derepress effector caspases probably because these caspases make additional

contacts with the BIR2 linker in addition to the BIR2 IBM pocket (Chai et al., 2000; Gao et al., 2007). IBM binding grooves appear to have a conserved function throughout evolution, as they are found in DIAP1 as well (Wu et al., 2001). The IBMs of Grim and Hid bind DIAP1 BIR2 (the counterpart to XIAP BIR3) similarly to how Smac binds XIAP (Chai et al., 2003). Another mammalian IAP binding protein, Omi, is a serine protease known to compete with XIAP for binding to caspases using similar principles (Vaux and Silke, 2003). ARTS (apoptosis related protein in TGF-β signaling pathway) is another IAP antagonist that can target XIAP (Gottfried et al., 2004; Larisch et al., 2000). Recently, a panel of other IAP antagonists have been identified as XIAP binding partners, and all appear to have IBM motifs (Verhagen et al., 2007). The functions of these proteins during apoptosis are largely unknown.

IAP antagonists function pro-apoptotically and their activity must be regulated to prevent spurious induction of cell death programs. Reaper, for example, is probably only present in cells that are doomed to die (Steller et al., 1994). Transcriptional activation of the *Reaper* locus appears to underlie many forms of apoptosis in *Drosophila*, ranging from developmental cell death (Jiang et al., 2000; Lohmann et al., 2002) to apoptosis following genotoxic damage (Brodsky et al., 2000; Nordstrom et al., 1996). Hid is

broadly expressed in most *Drosophila* cells, including viable cells, but is negatively regulated by survival signaling through the Ras pathway at the transcriptional (Kurada and White, 1998) and post-translational levels (Bergmann et al., 1998). Remarkably, all of the known mammalian IAP antagonists seem to localize to mitochondria and are released to the cytosol during apoptosis (Vaux and Silke, 2003). This finding emphasizes the logic behind sequestering pro-apoptotic factors in distinct subcellular compartments to discourage accidental cell death.

It is unclear if IAP antagonists play a central role in regulating IAP abundance in mammals as they do in *Drosophila*. Ectopic expression and *in vitro* experiments provide all of evidence that IAP antagonists can regulate IAP abundance. Ectopic expression of active (e.g., with a cleaved amino-terminal mitochondrial localization sequence) Smac efficiently depletes cIAP1 and cIAP2, but not XIAP, in HeLa cells. Expression of active Smac may actually antagonize XIAP E3 activity (Creagh et al., 2004; Silke et al., 2004). Similarly, small-molecule IAP antagonists deplete cIAP1 and cIAP2 efficiently but leave XIAP expression relatively unchanged (Varfolomeev et al., 2007; Vince et al., 2007). ARTS might function similarly as fruit fly IAP antagonists, because expression of ARTS can deplete XIAP from cells (Gottfried et al., 2004).

Drosophila IAP antagonists have apoptotic functions in mammalian cells. Expression of Grim in human 293T cells depletes XIAP efficiently but does not induce apoptosis (Silke et al., 2004); however, expressing Hid depletes XIAP (A. Kelkar, personal communication) and correlates with apoptotic cell death that can be inhibited by IAPs in Hela cells (Haining et al., 1999). Reaper also functions in mammalian cells by stimulating XIAP autoubiquitination (Holley et al., 2002). Some of the mammalian IAP antagonists probably do function like fruit fly antagonists because the functions of Reaper, Hid and Grim are conserved. It is clear that the biology of IAP antagonists requires more investigation.

1.8 Mouse models of IAP function

Flies lacking DIAP1 function die as embryos because of ubiquitous apoptosis (Goyal et al., 2000; Lisi et al., 2000). Surprisingly, however, individual genetic deletions of *XIAP* (Harlin et al., 2001; Olayioye et al., 2005), *cIAP1* (Conze et al., 2005), or *cIAP2* (Conte et al., 2006) by homologous recombination result in an essentially normal mouse on commonly used genetic backgrounds (C57BL/6 or 129/Ola). *XIAP*-null females show delayed lobuloalveolar development accompanied by reduced milk protein synthesis, but this effect does not seem related to apoptosis (Olayioye et al., 2005). The roles of individual IAPs in regulating apoptosis

during development may be masked by a combination of functional redundancy and compensatory upregulation. For example, there is evidence for IAP cross-regulation and possible redundancy in cells from the *cIAP1*-null mouse (Conze et al., 2005). cIAP2 levels increase post-translationally in *cIAP1*-null cells because cIAP1 negatively regulates the abundance of cIAP2 through ubiquitination and proteasomal degradation. XIAP expression is unaltered in cells from *cIAP1*-null mice, however, and its expression levels have yet to be addressed in *cIAP2*-null cells. *cIAP2*-null mice are resistant to endotoxic shock by bacterial lipopolysaccharide administration (Conte et al., 2006). This phenotype may arise because macrophages from *cIAP2*-null mice are sensitized to Fas apoptosis following endotoxic shock; *cIAP2* expression increases by about 30-fold in endotoxin treated *WT* cells, which may explain why these cells normally survive.

The role of functional redundancy could be addressed by generating compound mutant mice by interbreeding existing knockouts. The relatively unstudied members of the IAP family should be included in this effort. For example, ML-IAP harbors a RING ubiquitin-ligase domain and a BIR domain that binds caspases weakly but Smac strongly (Vucic et al., 2002). To our knowledge, *ML-IAP* has not been knocked out in mice. Another IAP, Bruce/Apollon, is unique not only for its size (528 kDa) but also

because it harbors an E2 ubiquitin-conjugating domain that can participate in polyubiquitinating Smac and caspase-9 when all are ectopically expressed (Hao et al., 2004). The functions of Bruce *in vivo* are hard to discern because the three knockout mice generated so far (two by gene targeting and one by gene trapping) yield conflicting results. All three *Bruce*-null mice are lethal *in utero*, though not at the same developmental stages (Hao et al., 2004; Lotz et al., 2004; Ren et al., 2005). One group (Ren et al., 2005) noted increased apoptosis *in situ* that associated with lethality in their gene-trapped knockout, while the others (Hao et al., 2004; Lotz et al., 2004) did not observe appreciable differences in death in gene-targeted embryos. Two groups (Hao et al., 2004; Ren et al., 2005) describe increased sensitivity to apoptotic stimuli in embryonic fibroblasts, while a third group reports no difference (Lotz et al., 2004). Bruce has other functions unrelated to apoptosis (Pohl and Jentsch, 2008), which is not surprising given the size of the protein. It is intriguing that the mutant with the most severe apoptosis phenotype (Ren et al., 2005) retains the BIR but not the E2 domain, while the other two mice lacked the entire gene. Studies on the fly homologue, dBruce, highlight the central importance of E2 domain in enhancing Reaper and Grim-dependent death (Vernooy et al., 2002). Perhaps the presence of the BIR is sufficient to subvert any compensatory mechanisms in place in

mouse cells. More work is clearly needed to address the role of Bruce and ML-IAP in relation to other mammalian IAPs.

XIAP-null thymocytes, splenocytes and embryonic fibroblasts die normally in response to many different apoptotic stimuli; however, post-mitotic cells cultured from *XIAP*-null mice show some apoptosis phenotypes that offer some deep insights into tissue-specific regulation by XIAP. Sympathetic neurons (Potts et al., 2003) and cardiomyocytes (Potts et al., 2005) from *XIAP*-null mice rapidly die by apoptosis after microinjection of cytochrome c, while cells from *WT* mice are completely insensitive. Dermal fibroblasts from *XIAP*-null and *WT* mice are equally sensitive to cytochrome c injection, however. XIAP inhibition is also necessary for apoptosis to occur following nerve growth factor withdrawal in cultured sympathetic neurons (Potts et al., 2003). Similar observations are seen in neuronally differentiated PC12 pheochromocytoma cells, which require inhibition of XIAP to die (Perrelet et al., 2002; Vyas et al., 2004). It is unknown if *XIAP*-null mice are prone to neurodegenerative diseases.

These observations show that XIAP is a non-redundant regulator of apoptosis in certain post-mitotic cells, some of which (e.g., neurons) can persist throughout life. Intriguingly, the expression levels of Apaf-1 are inversely related to how well XIAP can inhibit caspases and apoptosis after

cytochrome c release (Potts et al., 2005). Downregulation of Apaf-1 seems to be an effective strategy employed by post-mitotic cells to ensure that XIAP remains an effective inhibitor of caspases. Proliferative cells represent a greater danger to the organism as a whole; perhaps it is not surprising that there are alternate routes to apoptotic and non-apoptotic death that may be independent of IAPs.

The IAP antagonists *Smac* and *Omi* have been disrupted individually and together in the mouse (Martins et al., 2004; Okada et al., 2002). *Smac*-null mice are viable and fertile. Caspase-3 activation is impaired *in vitro* in *Smac*-null fibroblast lysates; however, apoptosis occurs normally in cultured primary and transformed fibroblasts, embryonic stem cells, hepatocytes and thymocytes (Okada et al., 2002). A role for Smac in apoptosis has emerged by comparing apoptosis in embryonic myoblasts and fibroblasts from *Smac*- and *Apaf*-deficient mice (Ho et al., 2007). XIAP is expressed at lower levels in myoblasts than fibroblasts. Release of Smac into the cytosol during mitochondria-dependent apoptosis is sufficient to neutralize XIAP in myoblasts, and caspase activation and apoptosis occur even the absence of *Apaf-1*. Simultaneous deletion of *Smac* and *Apaf-1* is necessary to block apoptosis in myoblasts. This type of regulation does not occur in fibroblasts because of the elevated level of XIAP; however, neutralization of XIAP by

small-molecule inhibitors allows apoptosis to occur in the absence of *Apaf-1*. These experiments reveal two independent pathways that can activate caspases and apoptosis in mammalian cells: one depends on Apaf-1, and the other is regulated by XIAP and IAP antagonists. Similar logic underlies caspase activation in *Drosophila* (Zhou et al., 1999).

The *Omi* knockout mouse suffers from a neurodegenerative disorder (Martins et al., 2004); however, this phenotype is owing to loss of *Omi* serine protease activity, rather than loss of IAP inhibitory function (Jones et al., 2003). Compound deletion of *Smac* and *Omi* does not exacerbate this phenotype. A physiological role of these proteins for regulating IAPs remains to be established, but this is possibly masked due to functional redundancy and the presence of additional IAP-antagonists. Indeed, there are far more potential IAP antagonists than IAPs in mammalian genomes (Vaux and Silke, 2003; Verhagen et al., 2007). Loss of *Sept4*, the locus encoding *ARTS*, results in male sterility accompanied by increases in XIAP expression in the testis (Kissel et al., 2005). ARTS can regulate XIAP expression by promoting auto-ubiquitination and degradation by the proteasome (Gottfried et al., 2004). Furthermore, loss of *ARTS* worsens prognosis on the *Eμ-Myc* lymphoma background corresponding to decreased apoptosis *in vivo* (M. García-Fernández and H. Steller, unpublished data).

1.9 Roles of IAPs outside of apoptosis

Many IAPs may be multifunctional proteins with roles distinct from regulating caspases and apoptosis. Overexpression of IAPs can activate diverse signaling pathways, including JNK, TGF-β and NF-κB (Lewis et al., 2004), but it is unclear if these are physiological observations. XIAP and cIAP1 are reportedly positive regulators of the NF-κB pathway downstream of the TNF-α cytokine, as cells with silenced expression of *XIAP* (Gaither et al., 2007) or *cIAP1* (Tang et al., 2003) cells show signs of reduced NF-κB activity. Primary *cIAP1*-null fibroblasts still activate NF-κB normally (Conze et al., 2005; Vince et al., 2007), so the observed effects may be cell type specific or possibly overexpression artifacts. XIAP may have a function in the bone morphogenetic protein (BMP) signaling pathway involved in developmental patterning. The carboxyl-terminus of XIAP can bind BMP receptors, and BIR1 can bind the adaptor protein TAB1 (Yamaguchi et al., 1999). TAB1 in turn recruits a member of the mitogen activated protein kinase (MAPK) kinase kinase TAK1, which in turn activates NF-κB signaling (Lu et al., 2007). The physiological significance of this interaction is unclear because the conclusions rely from overexpression experiments, and this has yet to be validated by genetic or other approaches.

1.10 IAPs in cancer and other diseases

Mammalian IAPs, and XIAP in particular, can inhibit apoptosis triggered through diverse stimuli when overexpressed (LaCasse et al., 1998). Elevated IAP expression is documented in human tumors (Tamm et al., 2000; Tamm et al., 2004), and correlates with high levels of active caspases (Yang et al., 2003). There are few published correlation studies, however, and the prognostic significance of XIAP overexpression is unclear (Fulda, 2007). The tightly linked *cIAP1* and *cIAP2* loci are frequently amplified in lung cancer (Dai et al., 2003), while *cIAP1* is amplified specifically in cervical cancer (Imoto et al., 2002) and correlates with resistance to radiotherapy. *cIAP1* is also amplified in human hepatocellular carcinoma and experimentally induced liver cancer in mice (Zender et al., 2006). Indeed, *cIAP1* overexpression has a pathogenic role in hepatocellular carcinoma (Zender et al., 2006). A chromosomal translocation common to extranodal marginal zone mucosa-associated lymphoid tissue (MALT) B-cell lymphomas juxtaposes the *cIAP2* BIR domains with a caspase-like gene, *MALT1* (Baens et al., 2000; Uren et al., 2000). The chimeric cIAP2/MALT protein displays enhanced NF-κB survival signaling (Zhou et al., 2005) and promotes tumorigenesis in a transgenic mouse model (A. Stoffel, unpublished data).

With the exception of *cIAP1* in liver cancer, the associations between IAP overexpression and tumorigenesis remain correlative. In spite of this, diverse strategies to inhibit IAPs have pro-apoptotic effects in many contexts. Downregulation of XIAP through antisense or interfering RNAs, or therapeutic peptides derived from IAP antagonists, sensitizes tumor cells to apoptosis and offers some promise for future therapy (Hunter et al., 2007). Smac-derived peptides (Fulda et al., 2002) or small-molecules (Petersen et al., 2007), or XIAP antisense treatments (Cummings et al., 2006), synergize with apoptotic stimuli to reduce tumor xenografts in mice. Small-molecule IAP antagonists have been developed by many groups, and they offer promising therapeutic advantages (Sun et al., 2006; Vucic and Fairbrother, 2007). Some of these antagonists have entered Phase I clinical trials for treatment in different malignancies.

IAPs may have roles in other disease contexts as well. Many neurodegenerative diseases have a strong apoptotic component, and blocking apoptosis could have therapeutic benefits (Mattson, 2000). XIAP gain-of-function models (e.g., overexpression) have been used to demonstrate involvement of IAPs in neurodegeneration. Overexpression of *XIAP* protects motor neurons in an experimental model of sciatic denervation (Perrelet et al., 2002). Transgenic expression of *XIAP* in neurons using the

neuron-specific enolase promoter (*nse-XIAP*) improves prognosis in a mouse model of Parkinson's disease (Crocker et al., 2003). Overexpression of *XIAP* also improves the survival of human pancreatic islets after transplantation in a mouse model of diabetes treatment (Emamaullee et al., 2005). Thus, IAP function may be modulated to either enhance apoptosis in diseases like cancer that display resistance to death, or reduce apoptosis in degenerative diseases.

1.11 Concluding remarks

Despite huge gains in our understanding of proteins involved in apoptosis, many aspects of cell death remain obscure. This is true both for particular aspects of cell death (e.g., regulation of IAPs), but also in broader contexts. For example, thymocytes lacking *Apaf-1* (Yoshida et al., 1998) or *caspase-9* (Hakem et al., 1998) are broadly resistance to apoptotic stimuli in culture, but thymus development is still largely normal. On a mixed genetic background (C57BL/6 / 129), mice lacking the pro-apoptotic functions of *cytochrome c* (Hao et al., 2005), *Apaf-1*, *caspase-9* and *caspase-3* (Kuida et al., 1996) die with many similar brain defects that include craniofacial abnormalities, exencephaly, reduced apoptosis and hyperplasia. While a common phenotype orders these genes in the same pathway, the penetrance is considerably lower on the C57BL/6 background and many of these

knockout mice can be viable and fertile (Leonard et al., 2002). The identity of the strain-specific modifier(s) is unknown.

These findings argue that there may be additional ways of activating apoptosis or other death programs apart from known pathways. Genetic arguments place the activation of any such pathway downstream of mitochondrial control points; *Apaf1*-null cells expressing BH3-only proteins can die a caspase-independent death (Cheng et al., 2001), while *BAX/BAK*-deficient cells are resistant (Wei et al., 2001). Some insights come from *C. elegans* and its streamlined version of the apoptotic cell death pathway, which contains counterparts of BH3-only (*egl-1, ced-13*), anti-apoptotic BCL-2 (*ced-9*), Apaf-1 (*ced-4*) and a caspase (*ced-3*). Even in this less complex system, there are other ways for *ced-4* to kill cells independently of *ced-3*, the only caspase that appears to function in cell death (Bloss et al., 2003; Shaham and Horvitz, 1996b). *Drosophila* may prove to be a valuable model system too, as novel cell death regulatory mechanisms have been identified in specialized cells like spermatids (Arama et al., 2003). Redundant death pathways may substitute for apoptosis during development as well. Apoptosis is the most studied cell death pathway, but it is certainly not the only form of programmed cell death (Abraham et al., 2007; Hetz et al., 2005; Overholtzer et al., 2007).

1.12 Prologue to the *XIAP ΔRING* project

The study described here seeks to extend our understanding of IAP RING functions in mammals. Mammalian XIAP shares several properties with *Drosophila* DIAP1, including the ability to bind to caspases, to Reaper-family proteins, and the ability to undergo auto-ubiquitination and proteasome-mediated degradation in response to apoptotic stimuli (Yang et al., 2000). Since these conclusions stem largely from overexpression and *in vitro* experiments, we decided to examine the role of XIAP E3-ligase activity for caspase regulation and apoptosis *in vivo*. For this purpose, we used gene targeting to generate a mouse *XIAP ΔRING* allele which uncouples the caspase-binding properties of XIAP from the ubiquitin system. Removing the RING stabilized XIAP in apoptotic thymocytes, demonstrating that XIAP E3 ubiquitin-ligase activity is a major determinant of XIAP protein stability *in vivo*. However, loss of RING function had no detectable consequences for thymocyte apoptosis, virtually reproducing the results from a previous report for *XIAP*-null mice (Harlin et al., 2001). On the other hand, *ΔRING* embryonic stem cells and fibroblasts had elevated caspase-3 enzyme activity and impaired ubiquitination of active caspase-3 during apoptosis. Furthermore, XIAP ΔRING embryonic fibroblasts were

strongly sensitized to TNF-α apoptosis. Therefore, XIAP RING function appears to play a physiological role in certain cell types, but not others. Finally, deletion of the XIAP RING also significantly increased apoptosis of proliferating B-cells and improved the survival of mice in a mouse lymphoma model. We conclude that the E3 ubiquitin-ligase activity of XIAP is important for the regulation of apoptosis in at least some cell types *in vivo*, and that this activity contributes to tumor suppression. These results suggest that the XIAP RING may be a promising drug target for developing a novel class of cancer therapeutics.

2 ENGINEERING THE *ΔRING* ALLELE BY GENE TARGETING

2.1 SUMMARY

We devised a strategy to inactivate the mouse XIAP RING ubiquitin-ligase motif by gene targeting. The RING was deleted by replacing the start of the domain with stop codons, while preserving the triplicate BIR domains that can bind and inhibit caspases. All stages of gene targeting were successful and a *ΔRING* colony was established. *ΔRING* mice were fertile and born at Mendelian frequencies for gender and genotype. Mutant mice were phenotypically normal and indistinguishable from wild-type mice when challenged *in vivo* by whole-body irradiation or experimental models of liver failure.

2.2 INTRODUCTION

Gene targeting based on homologous recombination is one of the most powerful tools in modern mouse genetics. Gene targeting has opened the mouse genome up to manipulations that range from chromosomal rearrangements to precise editing of individual codons (van der Weyden et al., 2002). We decided to use gene targeting to inactivate the XIAP RING motif for many reasons. First, we wanted to express the ΔRING protein

from the endogenous promoter, because XIAP is subject to uncommonly complex post-transcriptional and post-translational regulation in humans and mice (Lewis and Holcik, 2005; Vaux and Silke, 2005). Second, we reasoned this would minimize artifacts that arise when RING mutant constructs are overexpressed. For example, overexpression experiments in *Drosophila* would argue that RING mutations render DIAP1 a better inhibitor of apoptosis, while the same mutations in the endogenous gene actually cause lethality because of unrestrained apoptosis (L. Goyal and H. Steller, unpublished observations). Third, generating a mutant animal would allow us to address the consequences of RING inactivation in normal mouse development and disease models. Finally, it was relatively straightforward to create this allele because the RING is positioned at the far carboxyl terminus of the protein (Figure 2.1). Thus, we could truncate the XIAP protein at the start of the RING domain by introducing a stop codon in place of the codon (C449) that marks the first amino acid of the RING. This chapter describes the general strategy to generate a mouse *XIAP ΔRING* allele that lacks the RING but retains the triplicate BIR domains.

2.3 EXPERIMENTAL PROCEDURES

2.3.1 *Design and construction of the XIAP ΔRING targeting vector*

We devised a strategy to eliminate the XIAP RING (located at the far carboxyl terminus) using a homology-based "knock-in" replacement of the first two amino acids of the RING (C449 and K450) by stop codons. The pK-11 plasmid (a gift from Chingwen Yang) served as the backbone for assembling the ΔRING targeting vector. The targeting vector contained a removable positive selection cassette (*PGK-neomycin phosphotransferase*) in the sense orientation flanked by *FRT* sites that could be recognized and excised by the FLPe recombinase. A superfluous *lox*P site located downstream of the selection cassette was removed from pK-11 before cloning. A single 2.7 kB fragment containing both the final exon (encoding the RING) and the 2.0 kB 3' homology arm was amplified by PCR from an X-chromosome bacterial artificial chromosome (RPCI 23 207E14, C57BL/6 strain). The first two codons of the RING were mutated to tandem stop codons (opal and ochre) by site-directed mutagenesis and a diagnostic SacI restriction site was appended immediately downstream from the stop codons. This fragment was cloned into the SalI/ApaI sites located on the 3' site of the *FRT-Neo-FRT* cassette in pK-11. The 5' homology arm contained 6.0 kB of genomic DNA immediately upstream of the *ΔRING* mutation. A

NotI restriction site (for linearizing the vector) was appended to the 5' terminus during PCR, and the fragment was cloned into the SacII site on the 5' side of *FRT-Neo-FRT*. Finally, a *PGK-diphtheria toxin-A* negative selection cassette was amplified by PCR from the vector pDTA (a gift from Dónal O'Carroll) and ligated in the antisense origin into the KpnI site after the 3' homology arm. The Expand High-Fidelity DNA polymerase (Roche) was used for PCR amplification.

The vector was designed with the *FRT-Neo-FRT* cassette positioned in the final intron of the targeted locus, adjacent to the sixth exon encoding the RING. The final targeted locus (following excision of *FRT-Neo-FRT* by FLPe) would thus contain only the introduced stop codons (and diagnostic restriction site) in the final exon, and one residual intronic *FRT* site flanked by the SacII and SalI restriction sites introduced for cloning purposes. We presumed that this short, 55-nucleotide piece of DNA remaining in the intron would not affect the synthesis or splicing of the ΔRING mRNA. PCR genotyping primers that flank the insertion site of the residual DNA were chosen to distinguish *WT* from *ΔRING* alleles based on the larger size of the PCR product from the *ΔRING* locus (Forward primer sequence: 5'-TAA AGC CTT TAC CTT CTT CTC TAT TTC C-3'; reverse primer sequence: 5'-TGG GAC AGG TAG GAT TTA GTG CTT CG-3'; annealing

temperature of 55 C). The targeting vector was sequenced to confirm that the vector was assembled correctly and that no other mutations were introduced during PCR amplification of the BAC DNA template.

2.3.2 *Gene targeting, generation of chimeric mice, and establishment of a* ΔRING *colony*

100 μg of plasmid DNA were digested for 2 hours with NotI to linearize the vector, and then extracted by ethanol and resuspended in water. Gene targeting in mouse embryonic stem cells (E14 line, 129/Ola strain) was conducted by the Rockefeller University Gene Targeting Resource Center according to standard techniques (Nagy, 2003). Briefly, ES cells were electroporated with the linearized DNA, plated on a feeder layer of mitotically arrested neomycin-resistant fibroblasts, and then cultured in 200 μg/mL G418 to select for neomycin-resistant colonies. Individual neomycin-resistant, replica-plated colonies were selected and screened for homologous targeting of the *XIAP* locus by Southern blotting of SacI digested genomic DNA. For this purpose, we used a 312-bp probe created by PCR from outside the 3' homology arm (Forward primer sequence: 5'-TCG GAA GGT CAC AGA ATA ACC G; reverse primer sequence: 5'-CCC AGC GAA TCA CAT TGT AGT CAC; PCR annealing and Southern hybridization temperature of 55 C). Independently targeted clones were

expanded on feeder layers and karyotyped. Three ES clones with normal diploid genomes were injected into C57BL/6 blastocysts and implanted into pseudopregnant females by the Transgenic Services facility at Rockefeller University, using standard techniques (Nagy, 2003).

Strongly chimeric male mice (as gauged by the highest penetrance of the chinchilla coat color modifier gene from the 129/Ola background) were selected for backcrosses to transgenic C57BL/6 "FLPeR" females, expressing *FLPe* from the constitutive *ROSA26* locus (Farley et al., 2000), to remove the neomycin cassette *in vivo*. FLPeR mice were a gift from Dónal O'Carroll and genotyped by PCR (Forward primer sequence: 5'-CAC TGA TAT TGT AAG TAG TTT GC; reverse primer sequence: 5' CTA GTG CGA AGT AGT GAT CAG G) according to the published protocol (Farley et al., 2000).

The presence of any agouti pups in the litters indicated germline transmission of the mutant allele; this was confirmed by Southern blotting of tail biopsy DNA. Excision of the *Neo* cassette in progeny was assessed by Southern blotting of ApaLI digested genomic DNA from tail biopsies, using the same probe described above, and also by PCR using the genotyping primers. Animals positive for the mutant allele and *Neo* excision, and negative for the *FLPe* transgene, were selected to establish the colony.

Initial experiments were conducted using mice on a mixed 129/Ola / C57BL/6 genetic background. A congenic strain was established on the C57BL/6 background by serial backcrossing for over ten generations. Animals were housed in a specific pathogen free environment with unlimited access to food and water in the LARC vivarium at the Rockefeller University. The institutional animal care and use committee approved all experiments under animal protocols 04-058 and 07-059.

2.3.3 *Aging and irradiation experiments*

Aging cohorts of male mice on a mixed 129/Ola / C57BL/6 background (*WT*, n=14; *ΔRING*, n=19) were set aside and monitored periodically for the incidence of disease or death. Another cohort of mice derived after backcrossing to the C57BL/6 background for at least 5 generations (n=13 for both genotypes) was selected for an *in vivo* irradiation experiment. Mice were administered antibiotics (30 mg/L neomycin, 30 mg/L kanamycin, 10 mg/L gentamicin) in their drinking water for two days prior to a single 7-Gy dose of ionizing radiation from the LARC X-ray source (1.8 Gy/min). Irradiated mice were given continued access to the antibiotic drinking water and monitored for survival over time. Kaplan-Meier survival curves were constructed and analyzed with a log-rank statistical test in the Prism 4.0 software package (GraphPad Software, Inc.).

We also submitted pairs of littermate mice that ranged in age from 50 to 150 days for complete histopathological analysis by the Laboratory of Comparative Pathology at the Memorial Sloan-Kettering Cancer Institute. The pathologists were not informed of the genotypes of the mice or the nature of the project to ensure an unbiased examination.

2.3.4 *TNF-α and Fas-dependent liver apoptosis*

Agonstic anti-Fas antibody (10 μg Jo-2 antibody [BD Biosciences] in 200 μL sterile phosphate-buffered saline [PBS]), or 300 ng TNF-α (Peprotech) and 20 mg D-galactosamine (GalN; Sigma) in 200 μL PBS, was administered to age-matched mice by lateral tail vein injection. Mice were sacrificed by cervical dislocation, at a time (Fas, 4 hours; TNF-α/GalN: 6 hours) when mice were moribund but still alive. Livers were surgically removed and snap-frozen in liquid nitrogen. Livers were homogenized in lysis buffer (10 mM Tris [pH 7.5], 100 mM NaCl, 1mM EDTA, 0.01% Triton X-100) using a Polytron (Kinematica) and clarified by centrifugation at 14,000 RPM for 10 minutes at 4 C, before determining total protein concentration using the Bradford reagent (Bio-Rad). 150 μg of total protein from each sample were assayed for caspase-3 activity in bulk by measuring the linear rate of cleavage of a fluorescent Z-DEVD-R110 substrate using

the EnzChek Caspase-3 Assay Kit #2 (Invitrogen) and a SpectraMax M2 multiwell plate reader (Molecular Devices).

2.3.5 *Statistical treatments*

All bar graphs values are displayed as means with standard error unless noted otherwise.

2.4 RESULTS

2.4.1 *Generation of* ΔRING *mice by gene targeting and blastocyst injection*

We used homology-based gene targeting in mouse embryonic stem (ES) cells to create a *XIAP ΔRING* mutant allele by replacing the first two amino acids (C449 and K450) of the RING domain with stop codons (Figure 2.1). This mutation was designed to truncate the RING and the thirteen amino acid sequence between the end of the RING and the carboxyl terminus, while leaving intact the triplicate BIR domains in the amino-terminal half of XIAP. After targeting the XIAP locus in the E14 embryonic stem (ES) cell line with a conventional replacement strategy, 6 of 96 neomycin-resistant ES colonies showed restriction fragment length polymorphisms consistent with precise homologous targeting; an example Southern blot autoradiograph is shown in Figure 2.2A. Correct insertion of the stop codons was confirmed by directly sequencing genomic DNA from

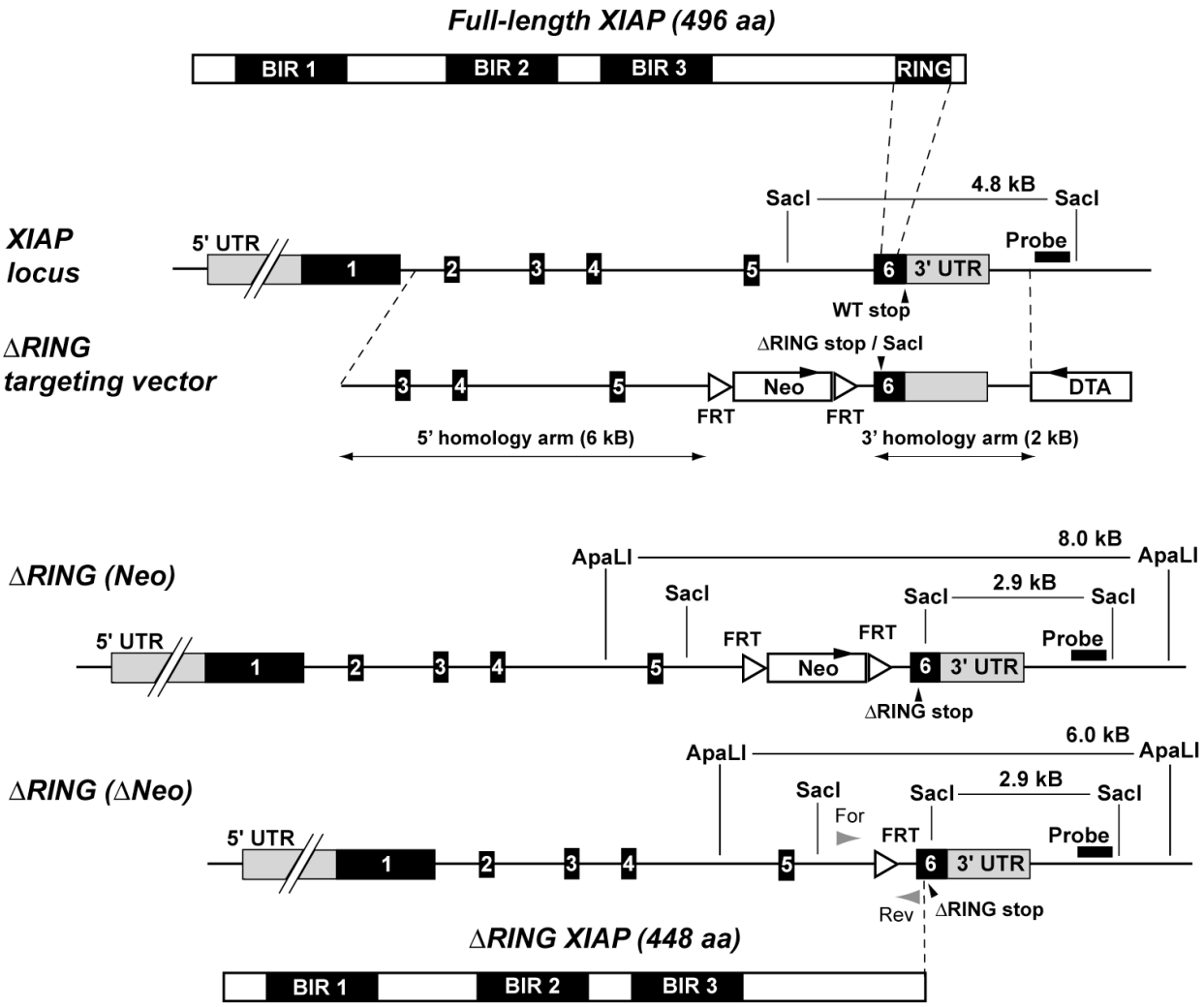


Figure 2.1 Gene targeting strategy. WT XIAP protein structure is shown at top. Second line: genomic organization of the *XIAP* locus, indicating *WT* stop codon and the location of the Southern probe. Third line: targeting vector, indicating *ΔRING* stop and *FRT* sites (open triangles); DTA denotes negative selection cassette. Third line: Targeted *ΔRING* (*Neo*) locus. Fourth line: Final *ΔRING* (*ΔNeo*) locus after FLPe excision of *Neo*; grey arrowheads indicate location of genotyping primers that flank residual *FRT* site. ΔRING XIAP protein structure is shown at bottom.

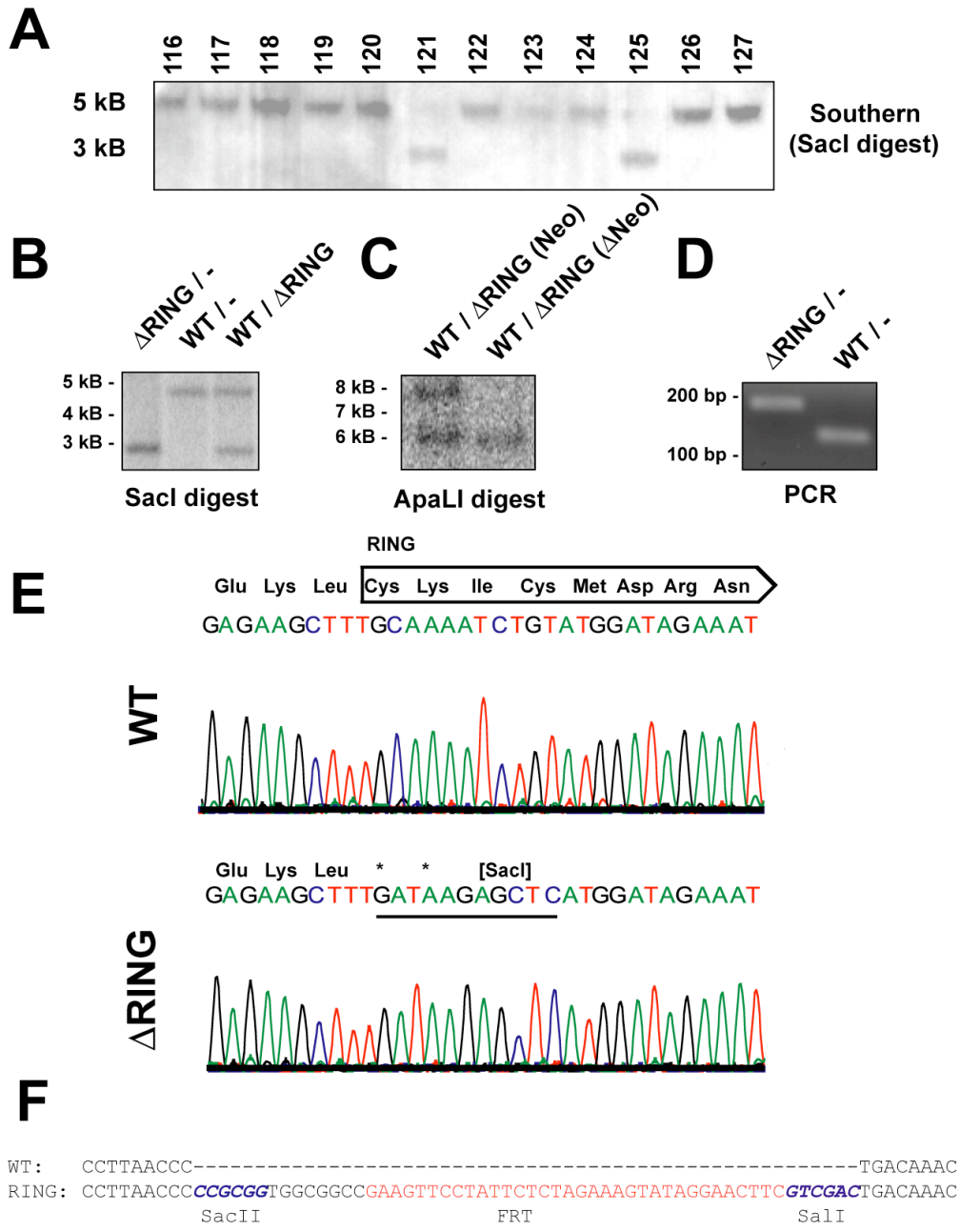


Figure 2.2 Gene targeting results. (A) Southern blot of SacI-digested DNA from Neo-resistant ES colonies using the probe in Figure 2.1; numbers indicate individual colonies **(B)** Southern blot of SacI-digested tail biopsy DNA. **(C)** Southern blot of ApaLI-digested DNA from mice crossed to FLPeR deletor strain. **(D)** PCR genotyping of mice. **(E)** DNA sequencing chromatograph from Exon 6 in *WT* and *ΔRING* alleles in ES cells; asterisks indicate stop codons. **(F)** DNA sequence of residual intronic DNA in *ΔRING* allele.

targeted clones (Figure 2.2E). We decided to remove the *Neo* cassette *in vivo* to minimize the time ES cells spent in culture; therefore, the ES cells used for blastocyst injection had the genomic organization shown in the *ΔRING (Neo)* line in the targeting schematic (Figure 2.1).

Two of three independently targeted clones with normal diploid karyotypes (#49, #129) yielded mice with strongly chimeric coat colors after blastocyst injection and implantation into foster mothers from the C57BL/6 inbred strain; a third clone (#165) produced no chimeras. Chimeric males from both founder lines (line 49: 3 of 7 chimeras; line 129: 5 of 11 chimeras) transmitted the mutant allele through the germline when backcrossed to C57BL/6 "FLPeR" females expressing the FLPe recombinase. This was evident by the presence of pups with the dominant agouti coat color and verified by Southern blotting of SacI digested DNA tail biopsies (Figure 2.2B). Excision of the *Neo* cassette was confirmed in these animals by Southern blotting of ApaLI digested DNA (Figure 2.2C). This was corroborated by PCR genotyping across the residual *FRT* site in a *Neo*-deleted locus (Figure 2.2D) and direct DNA sequencing (Figure 2.2F). Collectively, the genotyping results confirmed that we had derived mice with the final, correctly targeted *XIAP ΔRING* (*ΔNeo*) locus. Mice derived from targeted clone #129 were chosen arbitrarily to found the colony.

2.4.2 *Analysis of an aging cohort of mice*

ΔRING mice were fertile and born at expected Mendelian ratios for gender and genotype (Table 1). We submitted age-matched wild-type and mutant mice (mixed 129/Ola / C57BL/6 genetic background) for

Sex	Number in litters	Predicted number	Genotype	Number with genotype	Predicted
Female	42	40	*WT/WT*	19	20
			WT/ΔRING	23	20
Male	38	40	*WT/-*	18	20
			ΔRING/-	20	20

Table 1 Distributions of gender and genotype in ΔRING litters. Tally of the number of mice of different gender and genotype in 11 litters (n=80 mice). Parental crosses: *WT*/- male x *WT/ΔRING* female.

comprehensive, unbiased histopathological examinations by an outside laboratory. *ΔRING* mice were indistinguishable from *WT* littermates at different ages when examined in a blind manner. Moreover, *ΔRING* mice have shown no overt susceptibility to disease or premature death in our pathogen free mouse colony after more than 24 months of age (Figure 2.3). These observations are consistent with those of *XIAP*-null mice, which develop mostly normally (Harlin et al., 2001; Olayioye et al., 2005). These results argue that the RING domain was dispensable for fertility and normal

development on this mixed genetic background. We derived a congenic mutant strain by repeatedly backcrossing (n>10 generations) to the C57BL/6 inbred strain; these mice are also fertile and phenotypically normal.

2.4.3 *Whole-body irradiation*

Treatment of mice with a single whole-body dose of ionizing radiation leads to mortality over the course of roughly two weeks, probably in response to p53-dependent apoptosis of all hematopoietic precursors in the bone marrow, in addition to other radiosensitive renewable tissues (Westphal et al., 1998). We irradiated a cohort of WT and mutant mice to determine if loss of the RING domain influenced survival in a paradigm of DNA damage induced apoptosis. The survival curves for mice of both genotypes were comparable following a single 7-Gy dose of X-irradiation (Figure 2.3). The median survival time for *WT* mice was 12 days and 11 days for *ΔRING* mice. Since survival kinetics were unaltered in mutant mice, the loss of XIAP E3 ubiquitin-ligase activity did not affect death that occurs following DNA damage caused by X-irradiation *in vivo*.

2.4.4 In vivo *hepatocyte apoptosis*

Antibodies against the Fas death receptor function as agonists that trigger Fas-dependent apoptosis in hepatocytes when administered intravenously

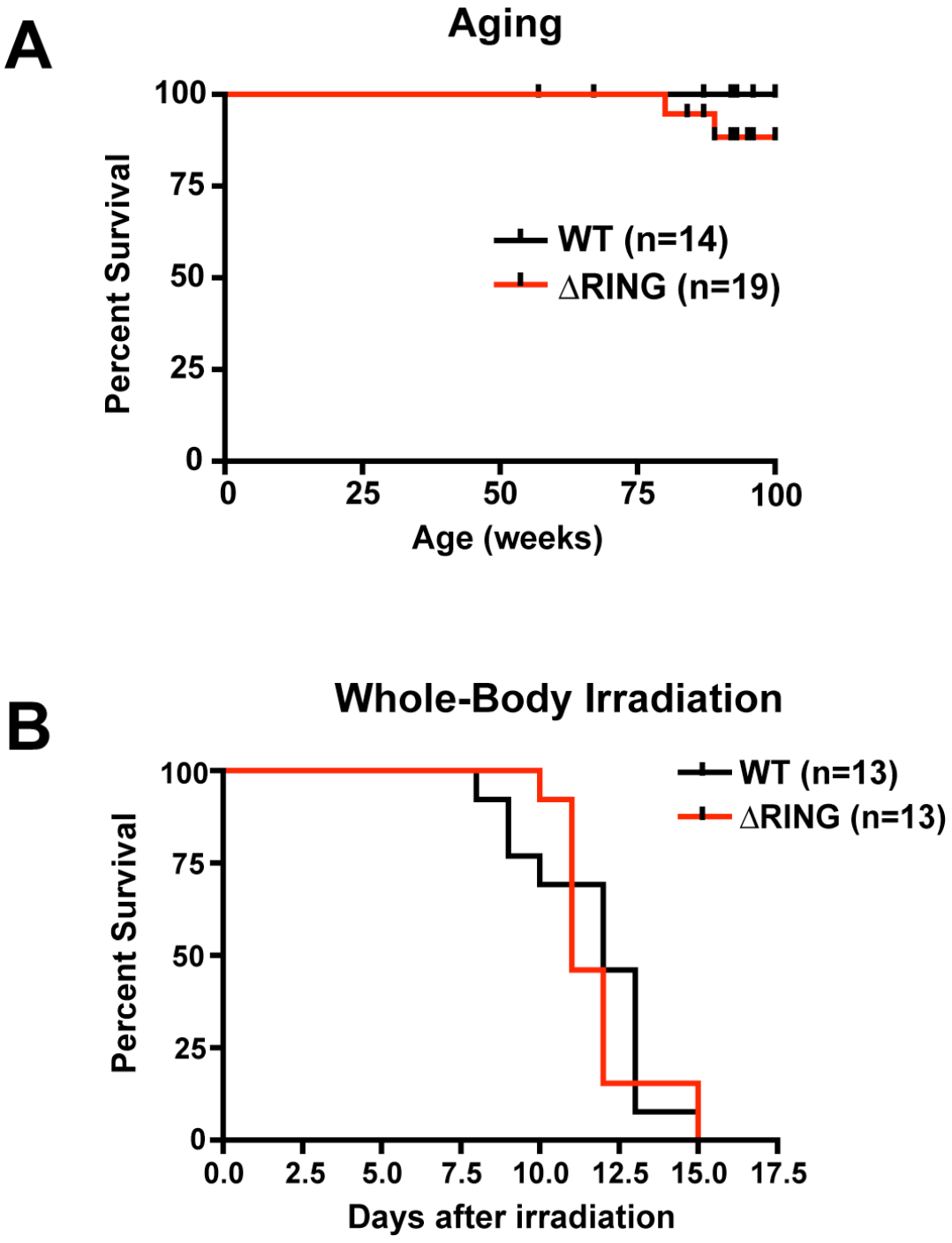


Figure 2.3 Aging and Whole-Body Irradiation Survival Curves. (A) Kaplan-Meier survival curve of an aging cohort of mice; p=0.25. **(B)** Kaplan-Meier survival curve of mice following 7-Gy of whole-body irradiation; p=0.44. Log-rank test was used for significance.

(Ogasawara et al., 1993). Likewise, administration of the TNF-α cytokine in combination with an inhibitor of transcription (e.g., D-galactosamine, "GalN") provokes acute hepatitis accompanied by severe apoptosis, ultimately leading to the death of the animal within hours (Leist et al., 1994). Both are models for death receptor apoptosis *in vivo*. Although the downstream effector pathways are distinct for Fas- and TNF-α-dependent apoptosis, activation of either pathway leads to caspase-3 activation. We administered either treatment to *WT* and *ΔRING* mice to see if the RING domain influenced these *in vivo* paradigms of hepatocyte apoptosis. Animals were sacrificed 4 hours after Fas or 6 hours after TNF-α/GalN treatment and assayed for caspase-3-like activity in the liver, as an endpoint readout for hepatocyte apoptosis. *WT* and *ΔRING* mice showed comparable caspase-3 activity in response to these treatments. *WT* mice became moribund roughly at the same time as *ΔRING* mice. Although we did not perform any TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling) to corroborate the caspase assay, these experiments show that deletion of the RING domain did not affect caspase activity *in vivo* in response to different death receptor signaling paradigms.

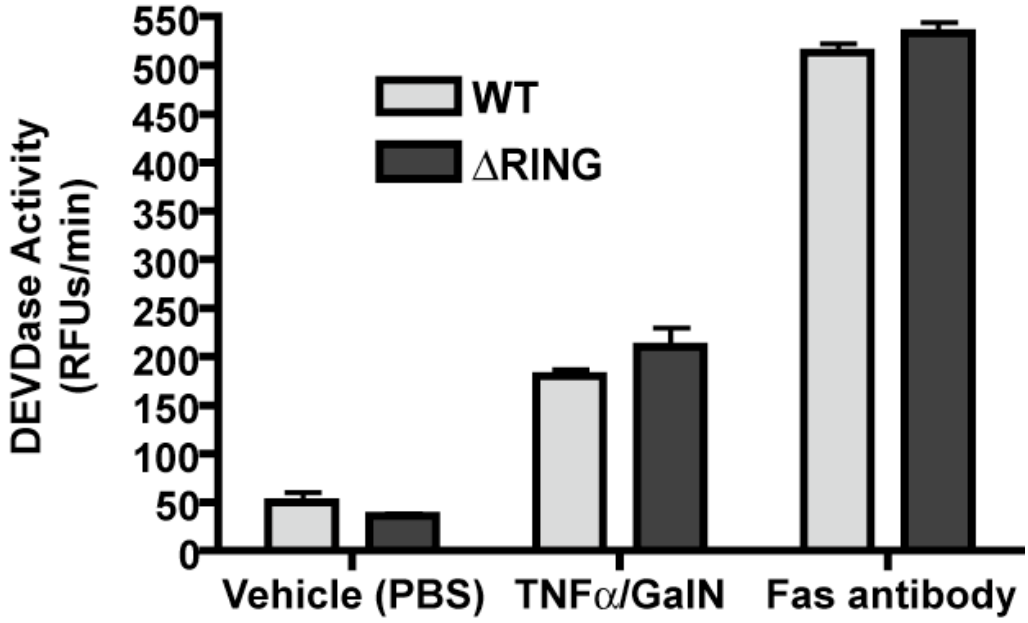


Figure 2.4 Liver apoptosis. Caspase-3-like activity measured in liver lysates after tail vein injections.

2.5 DISCUSSION

The results shown here imply that XIAP ubiquitin-ligase function was dispensable for development and survival on mixed and congenic backgrounds. The *XIAP ΔRING* mouse is similar in these respects to the two *XIAP*-null mice that have been engineered, neither of which displays any severe developmental defects (Harlin et al., 2001; Olayioye et al., 2005). To date, the only phenotype described at the level of the whole animal is delayed lobuloalveolar development in the mammary gland, but *XIAP*-null dams are still able to nurse litters (Olayioye et al., 2005). The lack of an apoptosis phenotype in the liver following Fas or TNF-α/GalN administration contrasts with the results presented in Chapter 5, where a sensitivity to TNF-α-dependent apoptosis is described in embryonic fibroblasts.

3 APOPTOSIS IN ΔRING THYMOCYTES AND OTHER PRIMARY HEMATOPOETIC CELLS

3.1 SUMMARY

The evidence that RING domains promote IAP turnover was first uncovered by studying mouse thymocytes undergoing apoptosis. We used *ΔRING* thymocytes to test the hypothesis that RING-mediated turnover can facilitate cell death. The RING was the principal determinant of XIAP stability during thymocyte apoptosis. This is consistent with the notion that the XIAP RING functions as an E3 ubiquitin-ligase to promote XIAP turnover. Enhancing XIAP stability by removing the RING did not affect caspase activation or activity, and *ΔRING* thymocytes underwent normal apoptosis. T- and B-cell development in the thymus and bone marrow, respectively, was unaltered in *ΔRING* mice. Collectively, these data imply that the RING was important for XIAP stability, but increasing the abundance of XIAP by deleting the RING did not impede apoptosis or alter hematopoietic development.

3.2 INTRODUCTION

3.2.1 *General comment on apoptosis in immunity*

Cell death pathways regulate the development and homeostasis of the immune system. Virtually every step in B- and T-cell development is

governed by checkpoints that can activate apoptosis when developmental programs go awry, thereby eliminating non-functional or auto-reactive lymphocytes. Lymphocyte survival depends on the proper integration of cell-autonomous and external signals. Lymphocyte apoptosis involves both mitochondrial- and receptor-dependent death pathways (Rathmell and Thompson, 2002). These pathways function in development and maturation of lymphocytes, and to attenuate the immune response after an infection (Opferman, 2007). Proper regulation of apoptosis in the lymphoid system is important for preventing autoimmunity and cancer as well (Krammer, 2000).

3.2.2 *Apoptosis in developing lymphocytes*

T-cell development is regulated by control checkpoints that can trigger cell death to maintain the quality of the T-cell pool. T-cell progenitors originate in the bone marrow but hone to the thymus for development and maturation (Jotereau et al., 1987) (Carlyle and Zuniga-Pflucker, 1998). Stringent quality control results in the death of approximately 97% of developing T-cells in the thymus without any evident inflammation (Shortman et al., 1990). Indeed, a high index of apoptosis is seen *in situ*, while apoptotic thymocytes are cleared very rapidly (Surh and Sprent, 1994). Apoptosis is evident at the earliest stages of thymocyte development as T-cell precursors compete for the survival factor interleukin-

7 (Baird et al., 1999): cells deprived of interleukin-7 undergo apoptosis that depends on the BH3-only gene *BIM* (Bouillet et al., 1999) and is blocked by *BCL-2* overexpression (von Freeden-Jeffry et al., 1997).

One of the hallmark events of T-cell development is the somatic rearrangement of T-cell receptor (TCR) genes to create specificities for diverse antigens. Thymocytes that fail to arrange a functional TCR undergo "death by neglect", although the molecular mechanism remains unclear (Ashwell et al., 2000). A prevailing hypothesis holds that signaling through the TCR, possibly transduced by Ras through the ERK (extracellular signal-related kinase) effector pathway (Alberola-Ila et al., 1996), rescues thymocytes from apoptosis that would otherwise occur by default in response to ambient glucocorticoids in the thymic microenvironment (Zacharchuk et al., 1990). Thymocytes expressing a functional TCR escape death by neglect, upregulate the CD4 and CD8 surface antigens, and become subject to positive and negative selection.

The TCR recognizes antigens presented by major histocompatibility complexes (MHC) on dendritic cells and macrophages, and this interaction is necessary for T-cell function. A TCR that recognizes a MHC with the proper avidity initiates Ras survival signalling in the process known as positive selection (Yang and Ashwell, 1999). Cells that fail positive

selection die in the thymus (Huesmann et al., 1991) (Merkenschlager et al., 1997). Negative selection eliminates T-cells that bind a cognate MHC too tightly and helps ensure a pool of self-tolerant T-cells (Kappler et al., 1987). Thymocytes that undergo positive selection and survive negative selection proceed to downregulate either CD4 or CD8 to become cytotoxic or helper T-cells, respectively, and migrate to the periphery.

The mitochondrial pathway of apoptosis has a prominent role in thymocyte apoptosis. Negative selection depends on the BH3-only protein BIM, as *BIM*-null mice fail to eliminate many self-reactive thymocytes (Bouillet et al., 2002) and develop autoimmunity (Bouillet et al., 1999). BAX and BAK together control most of the death that occurs in T-cell development, and mice genetically deficient for both genes accumulate T-lymphocytes across many developmental stages (Lindsten et al., 2000). *BCL-2* overexpression inhibits the death of double-positive thymocytes that do not undergo positive selection (Strasser et al., 1994), but not death by neglect that occurs in the absence of a functional TCR (Maraskovsky et al., 1997). *BCL-2* overexpressing thymocytes remain self-tolerant, so other mechanisms are likely in place to ensure deletion of autoreactive thymocytes (Sentman et al., 1991).

Analogous apoptotic checkpoints function during B-cell development, which occurs primarily in the bone marrow. B-cells also depend on cytokine signaling for survival and to avoid death by neglect (Baird et al., 1999). Developing B-cells that receive sufficient interleukin-7 upregulate the antiapoptotic BCL-2 family member, *MCL1*, and this is believed to impart resistance to apoptosis at this developmental stage (Opferman et al., 2003). Apoptosis functions in eliminating B-cells that do not generate a functional, self-tolerant B-cell receptor during development in the bone marrow (Melchers et al., 2000). As with T-cells, the death of autoreactive B-cells depends largely on *BIM* (Bouillet et al., 1999), and almost entirely on *BAX* and *BAK* (Takeuchi et al., 2005). *BCL-2* overexpression prevents many of these deaths (Hartley et al., 1993).

3.2.3 *Apoptosis in peripheral T- and B-cells*

The numbers of peripheral lymphocytes are tightly controlled, in large part by apoptosis that balances out proliferation in cells involved in antigen-dependent immune responses. Death receptor signaling appears to have a more prominent role in maintaining homeostasis in the periphery than it does during lymphocyte development (Rathmell and Thompson, 2002). For example, Fas death receptor signaling instructs the death of clonal populations of activated T-cells in a waning immune response (Krammer,

2000). Mice and humans with deficiencies in the Fas receptor pathway develop autoimmunity and accumulate peripheral self-reactive lymphocytes. Mice that lack both *Fas* and *BIM* show the most striking accumulation of peripheral T-cells (Hughes et al., 2008), which points to the combined effects of these pathways in regulating immune homeostasis. Antibody-producing plasma B-cells also die during a waning immune response. The molecular underpinnings of death in these cells probably involve both *Fas* and *BIM* as well (Hutcheson et al., 2008; Rothstein et al., 1995).

3.2.4 *Roles of IAPs in thymocyte apoptosis*

The first functions described for the XIAP and cIAP1 RING domains were the capacity to serve as E3 ubiquitin ligases that promote autoubiquitination and IAP degradation during mouse thymocyte apoptosis (Yang et al., 2000). Additionally, a ΔRING XIAP mutant protein is resistant to ubiquitination, stably expressed, and imparts resistance to apoptosis when expressed in a mouse T-cell hybridoma (Yang et al., 2000). The observation that efficient thymocyte apoptosis requires the proteasome (Grimm et al., 1996) led to a hypothesis that IAP RINGs may act proapoptotically in these cells by engaging the ubiquitin system to relieve a break on cell death pathways by degrading IAPs (Yang and Li, 2000). Studies on DIAP1 in *Drosophila* provide a precedent for a pro-apoptotic

RING function during apoptosis. Expression of the DIAP1 antagonist Reaper depletes DIAP1 through RING-dependent autoubiquitination, leading to apoptosis in the wing imaginal disc (Ryoo et al., 2002). Furthermore, Ubcd1, the cognate E2 ubiquitin-conjugating enzyme for DIAP1, is required for Reaper-dependent apoptosis (Ryoo et al., 2002).

Thymocytes from mice deficient in *XIAP*, *cIAP1* or *cIAP2* are not strongly sensitized to apoptotic stimuli *in vivo* or under culture conditions, although there are subtle reductions in thymus size in *cIAP1*-null mice (Conte et al., 2006; Conze et al., 2005; Harlin et al., 2001). In contrast, the total thymocyte count increases and T-cells accumulate in the spleen when human *XIAP* is expressed transgenically in mice by the thymocyte-specific *lck* promoter (Conte et al., 2001). Thymocytes from the *lck-XIAP* transgenic mouse are also resistant to diverse apoptotic stimuli *in vivo* and in culture, including an experimental mimic of negative selection and glucocorticoid treatment. The CD4:CD8 (single-positive) T-cell ratio is also inverted in the thymus; similar observations are seen in *lck-Bcl2* mice (Sentman et al., 1991). The authors attribute the resistance to apoptosis to the increased stability of human *XIAP* overexpressed from the mouse *lck* promoter (Conte et al., 2001). Therefore, data from transgenic mice suggest that the

persistence of stabilized XIAP during thymocyte apoptosis may inhibit death.

In this chapter, we describe experiments designed to test the hypothesis that the XIAP RING can act pro-apoptotically in thymocytes by serving as a ubiquitin-ligase to direct XIAP downregulation. We reasoned that genetic deletion of the RING would prevent autoubiquitination and ubiquitination in trans by other IAPs (Silke et al., 2005), and that stabilized XIAP may impair lymphocyte cell death by continuing to inhibit caspases.

3.3 EXPERIMENTAL PROCEDURES

3.3.1 *Isolation and culture of primary lymphocytes*

Adolescent (4-6 week old) littermate mice were selected for thymocyte experiments because the thymus undergoes involution and decreases in size after adolescence. Animals were sacrificed using CO_2 gas and thymic lobes were dissected under sterile conditions in 2% (v/v) fetal bovine serum (FBS; Hyclone) in PBS (pH 7.4). Single cell thymocyte suspensions were prepared by mincing thymi in FBS/PBS through a 40 μm nylon mesh cell strainer. Cells were collected by centrifugation (3 minutes, 1000 RPM, 4 C), then treated with erythrocyte lysis buffer (9 volumes of 150 mM NH_4Cl, 1 volume of 130 mM Tris-Cl [pH 7.65]) for 3 minutes at room temperature. Thymocytes were collected again by centrifugation and

resuspended in thymocyte medium (RPMI medium supplemented with 10% FBS, 100 U/mL penicillin [Gibco], 100 μg streptomycin [Gibco], and 2 mM L-Glutamine[Gibco]) at a density of 5 x 10^6 cells/mL, generally in T25 flasks or 6-well plates.

B-cells were purified from spleens by first creating single-cell splenocyte preparations and lysing erythrocytes as described above. Cells were resuspended at a density of 1 x 10^7 cells per 40 μL FBS/PBS and incubated for 10 minutes at 4 C with a biotin-conjugated antibody cocktail that labelled the predominant non-B-cell populations in the spleen (Miltentyi). Non-B-cells were labelled secondarily with anti-biotin magnetic beads and depleted by flowing samples through a magnetic column. Isolated splenic B-cell populations in the flow-through were used for downstream assays; purity was verified by fluorescent immunostaining and flow cytometry as described below. B-cells were cultured in B-cell medium (45% Iscove's MEM, 45% DMEM, 10% FBS, 100 U/ml penicillin, 100 g/ml streptomycin, 4 mM l-glutamine, and 25 μM β-mercaptoethanol [Sigma]) at 37 C and 5% CO_2.

3.3.2 *Apoptosis assays*

Thymocytes were treated in culture with 10 μM etoposide (Sigma) or 1 μM dexamethasone (Sigma) to induce apoptosis, or left untreated in thymocyte medium as a control for spontaneous death under cell culture conditions. Thymocytes were also treated with 100 ng/mL TNF-α with and without 10 μM cycloheximide (Sigma). B-cells were treated with 10 μM etoposide or left untreated in B-cell medium. For some experiments, the peptide aldehyde proteasome inhibitor MG-132 (Calbiochem) or the pan-caspase inhibitor zVAD-fmk (Alexis) was added at a final concentration of 20 μM.

3.3.3 *FLICA labeling of the caspase-3 active site*

To analyze of caspase-3 activation, thymocytes were treated with dexamethasone or left untreated in 96-well plates for 5 or 7 hours, then incubated with the FAM-DEVD-FMK FLICA (caspase-3, principally) or FAM-LEHD-FMK (caspase-9, but crossreacts with other caspases) reagent (Immunochemistry Technologies; diluted from 150X stock) for an additional hour. Unbound FLICA reagent was removed by pelleting cells through centrifugation in the 96-well plate, decanting the medium, and washing twice with the 1X wash buffer supplied in the FLICA kit. Cells were

resuspended in 100 μL wash buffer supplemented with 40 ng/mL propidium iodide (PI). The number of apoptotic cells was determined with a FACScalibur flow cytometer (BD Biosciences) by gating PI-negative, carboxyfluorescein-positive cells among at least 4000 total cells.

3.3.4 *Annexin-V/propidium iodide and TUNEL apoptosis assays*

Thymocytes were treated with dexamethasone or left untreated in thymocyte medium in 96-well plate for 8 hours, then collected by centrifuging the entire plate and decanting culture medium. For TUNEL, cells were washed twice in 0.5% bovine serum albumin (BSA; Sigma) in PBS (pH 7.4) and fixed in 2% paraformaldehyde for 10 minutes at 37 C. Cells were washed twice in BSA/PBS and permeabilized in 70% ethanol for 30 minutes at -20 C. Cells were washed twice in BSA/PBS before performing the TUNEL reaction for 60 minutes at 37 C according to the manufacturer's directions (MBL International). Cells were washed again before flow cytometry. The percentage of apoptotic cells was determined by counting the number of fluorescein-positive cells among 4000 cells per sample. For annexin-V/propidium iodide labelling, cells were collected and labelled in flow cytometry tubes (100 μL) with 4 μL propidium iodide (50 μg/mL stock) and 2 μL annexin-V-FITC (BD Biosciences) for 10 minutes at room temperature before flow cytometry.

3.3.5 *Caspase-3-like (DEVDase) enzyme activity assay*

$3x10^7$ thymocytes were treated in T25 flasks with etoposide or dexamethasone, or left untreated, for 8 hours before collecting cells by centrifugation. Cell pellets were first frozen at -80 C and then lysed in DEVDase assay lysis buffer (described in Chapter 2) for 20 minutes on ice. Lysates were clarified by centrifuging tubes for 5 minutes at 5000 RPM at room temperature, and the protein concentrations were determined using the Bradford reagent (Bio-Rad). Bulk caspase-3-like activity was determined from 50 μg total protein as described in Chapter 2.

3.3.6 *Immunoblotting*

Cells were homogenized in lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1mM EDTA, 1 mM EGTA, 1% Triton X-100) supplemented with protease inhibitors (1 mM PMSF, 2 μg/mL leupeptin, 100 nM benzamidine, 1 μM pepstatin A). Lysates were incubated on ice for 30 minutes and clarified by centrifugation (14,000 RPM, 10 minutes, 4 C) before normalizing protein concentrations using the Bradford assay. Protein samples were resolved on SDS-PAGE Ready Gels (Bio-Rad) and electroblotted on Immobilon-P membranes (Millipore). Membranes were blocked for one hour at room temperature in blocking buffer (5% [w/v]

nonfat milk in 0.5% [v/v] Tween 20/PBS) and incubated overnight at 4 C with antibodies dissolved in blocking buffer. Membranes were washed with 0.5% Tween 20/PBS wash solution, incubated for 30 minutes at room temperature with appropriate secondary antibodies (conjugated to horseradish peroxidase and diluted in blocking buffer), before washing again. Signals were detected with the West Femto chemiluminescent kit (Pierce Biotechnology) on Bio-Max film (Kodak).

3.3.7 *Analyzing the cellular compositions of the thymus, spleen and bone marrow*

The distribution of major thymocyte subpopulations was determined in single-cell suspensions by flow cytometry, using fluorescently labelled antibodies against surface markers CD4 and CD8. Total cell counts in the thymus were calculated using a hemocytometer. The spleen was analyzed in an analogous manner by creating single-cell splenocyte suspensions and lysing erythrocytes, then staining with antibodies directly conjugated to fluorophores. The composition of the bone marrow was determined by flushing the marrow from femurs and tibias, treating with erythrocyte lysis buffer and staining with antibodies. Cells were labelled with antibodies (BD Biosciences) for 20 minutes on ice; dilutions were CD19-FITC (1:100); CD90-PE (1:100); B220-APC (1:50); IgD-FITC (1:100); IgM-PE (1:200);

CD43-FITC (1:100); CD8-FITC (1:100); CD4-PE (1:200); cKIT-FITC (1:100); Sca1-PE (1:200).

3.4 RESULTS

3.4.1 *RING ubiquitin-ligase activity was the primary determinant of XIAP stability during thymocyte apoptosis*

If RING-mediated downregulation of IAPs is necessary for efficient apoptosis, we reasoned that genetic deletion of the XIAP RING would allow us to determine if IAP RINGs can act pro-apoptotically. Indeed, ΔRING XIAP was more stable than full-length XIAP in cultured thymocytes dying in response to the glucocorticoid dexamethasone, a physiological trigger of cell death, or the topoisomerase inhibitor etoposide, which elicits DNA breaks (Figure 3.1A). The basal expression of ΔRING XIAP was greater, and it was cleaved over time during apoptosis. Deletion of the RING did not alter the basal expression or downregulation of cIAP1 during apoptosis (Figure 3.1A). XIAP and cIAP1 can bind each other in a RING dependent manner (Silke et al., 2005), and our genetic evidence showed that the XIAP RING did not influence the abundance of cIAP1 in thymocytes. This result

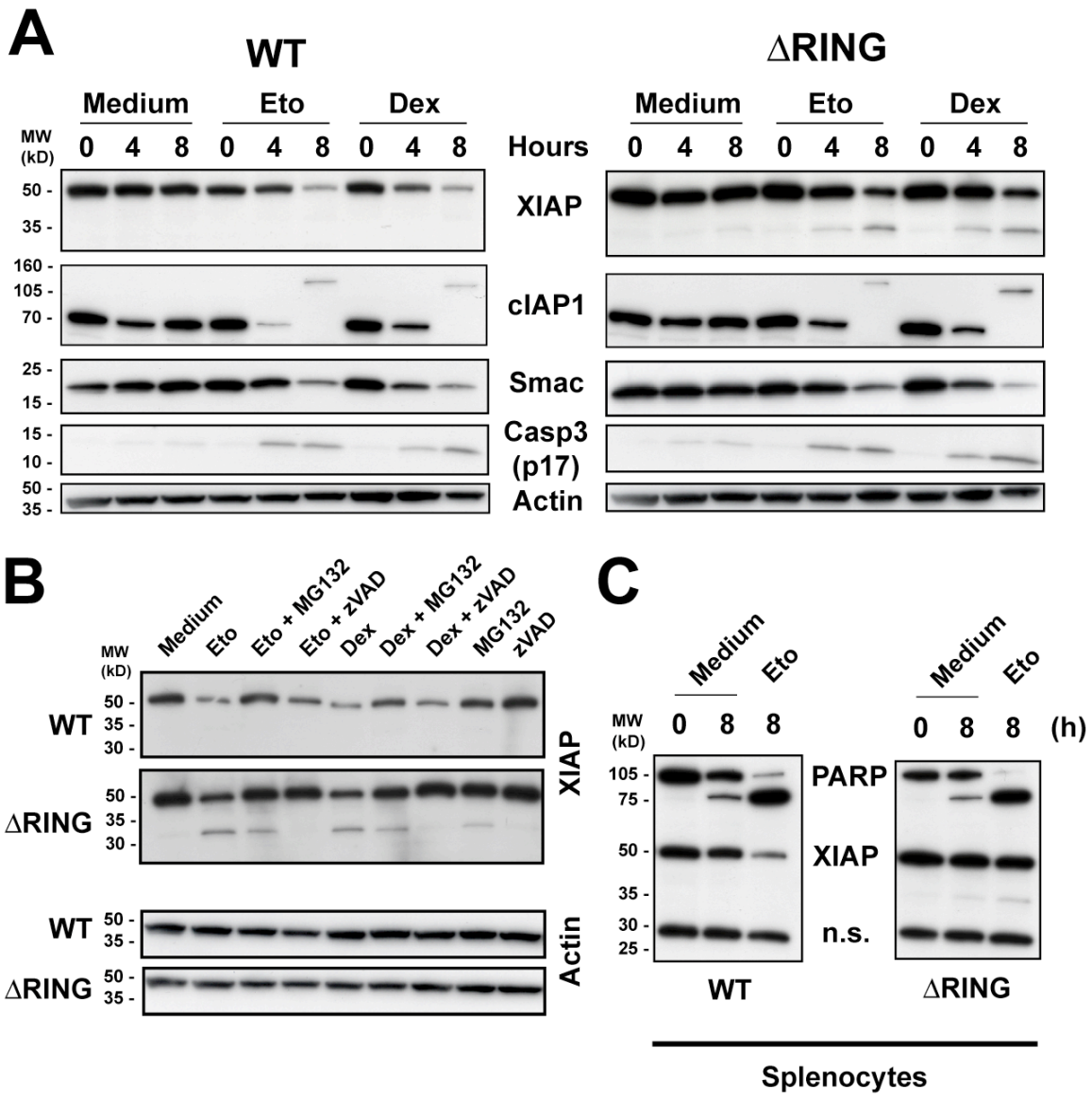


Figure 3.1 The RING was the primary determinant of XIAP stability in thymocytes and splenocytes. (**A)** Immunoblotting of *WT* and *ΔRING* thymocytes treated with etoposide, dexamethasone or left untreated for indicated times. (B) Immunoblotting of thymocytes treated for 8 hours with etoposide or dexamethasone, with or without the proteasome inhibitor MG-132 or the pan-caspase inhibitor zVAD-fmk. (C) Splenocytes were treated with etoposide for 8 hours or left untreated before immunoblotting. n.s. indicates a non-specific band detected by the XIAP antibody in B-cells, used as a loading control.

is consistent with the finding that cIAP1 expression is unchanged in *XIAP*-null thymocytes (Conze et al., 2005). It is unclear if XIAP ubiquitinates its binding partner Smac/DIABLO (Creagh et al., 2004; MacFarlane et al., 2002). Smac/DIABLO expression was unchanged in non-apoptotic *ΔRING* thymocytes and it was still downregulated during apoptosis (Figure 3.1A). Although XIAP can ubiquitinate active caspase-3 in certain situations and this can lead to decreased subunit abundance (Suzuki et al., 2001), we found caspase-3 was activated with comparable kinetics and to similar extents (Figure 3.1A).

Consistent with prior reports (Yang et al., 2000), a peptide aldehyde inhibitor of the proteasome, MG-132, blocked the decrease of full-length XIAP during apoptosis while the pan-caspase inhibitor zVAD-fmk had negligible effects (Figure 3.1B). In striking contrast, ΔRING XIAP expression was far more sensitive to caspase inhibition, as zVAD-fmk fully prevented the decrease in protein levels and abolished the cleavage product, while MG-132 had a much lesser effect by comparison (Figure 3.1B). This result supports the hypothesis that the RING engages the ubiquitin system to promote XIAP turnover in apoptotic thymocytes. cIAP1 and Smac/DIABLO protein levels were not affected by the RING deletion, and they remained labile during apoptosis (Figure 3.1A). This leaves open the

possibility that other IAP E3-ligases may be responsible for targeting these proteins for degradation.

We also isolated splenocytes (a heterogeneous population of approximately 50% B-cells and 30% T-cells; see Figure 3.6) and cultured them with etoposide to induce apoptosis. We also observed enhanced stability of ΔRING XIAP during apoptosis in splenocytes (Figure 3.1C). PARP (poly ADP-ribose polymerase) cleavage was used as a readout for caspase-3 activity; the native 100 kDa form of PARP was cleaved similarly in *WT* and *ΔRING* cells as splenocytes died spontaneously in the culture medium and in response to etoposide (Figure 3.1C).

3.4.2 *Genetic deletion of the XIAP RING did not influence thymocyte apoptosis*

We next asked if stabilizing XIAP had any impact on thymocyte cell death, since ΔRING XIAP efficiently blocks glucocorticoid-induced apoptosis when expressed ectopically in a T-cell hybridoma (Yang et al., 2000). We counted the numbers of cells harboring active caspases using fluorescent inhibitor of caspases (FLICA) reagents that covalently label caspase active site cysteines with fluorophores. We used a FAM-DEVD-FMK reagent to label effector caspases (principally caspase-3, but also -6 and -7 to lesser extents) or a FAM-LEHD-FMK reagent to label caspase-9 (though it cross-

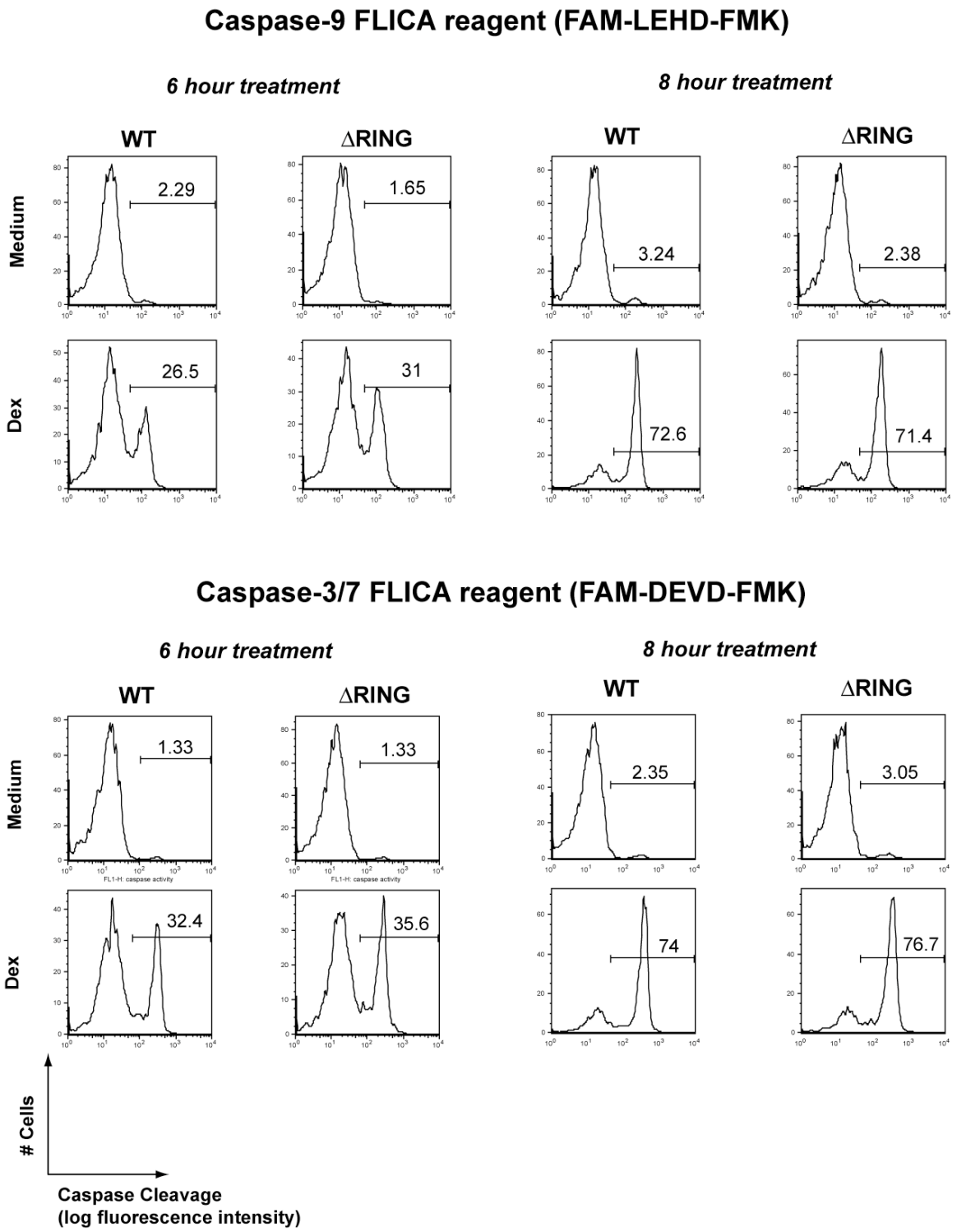


Figure 3.2 FLICA labeling of thymocyte active caspases. Histogram plots of fluorescence intensity after caspase active sites were labeled with FLICA affinity tags in thymocytes treated as indicated**.**

reacts with other caspases). We did not see any differences in caspase induction using these reagents when thymocytes were induced to die with dexamethasone or left untreated for different times (Figure 3.2). Moreover, caspase-3 enzyme activity was comparable in both untreated and apoptotic *WT* and *ΔRING* (Figure 3.3A). These results show that deleting the XIAP RING did not affect caspase activation or activity in thymocytes.

We characterized apoptosis using annexin-V/propidium iodide ("PI") labelling as a readout for membrane integrity, and TUNEL as a readout for cleavage of genomic DNA largely dependent on and downstream of caspase-3 (Woo et al., 1998). Neither annexin-V/PI (Figure 3.3B) nor TUNEL (Figure 3.3C) revealed any differences in etoposide- or dexamethasone-induced apoptosis in *ΔRING* thymocytes. This finding is in line with our results showing unaltered caspase activation and activity in mutant cells. We also cultured thymocytes for 24 hours to characterize the amount of spontaneous death *ex vivo*. AnnexinV/PI staining showed no difference in apoptosis in this situation, and FLICA staining revealed that effector caspases were activated similarly (Figure 3.3D). Additionally, treating cells with TNF-α with or without cycloheximide (CHX) did not reveal any differences in apoptosis. CHX alone prevented cell death in these

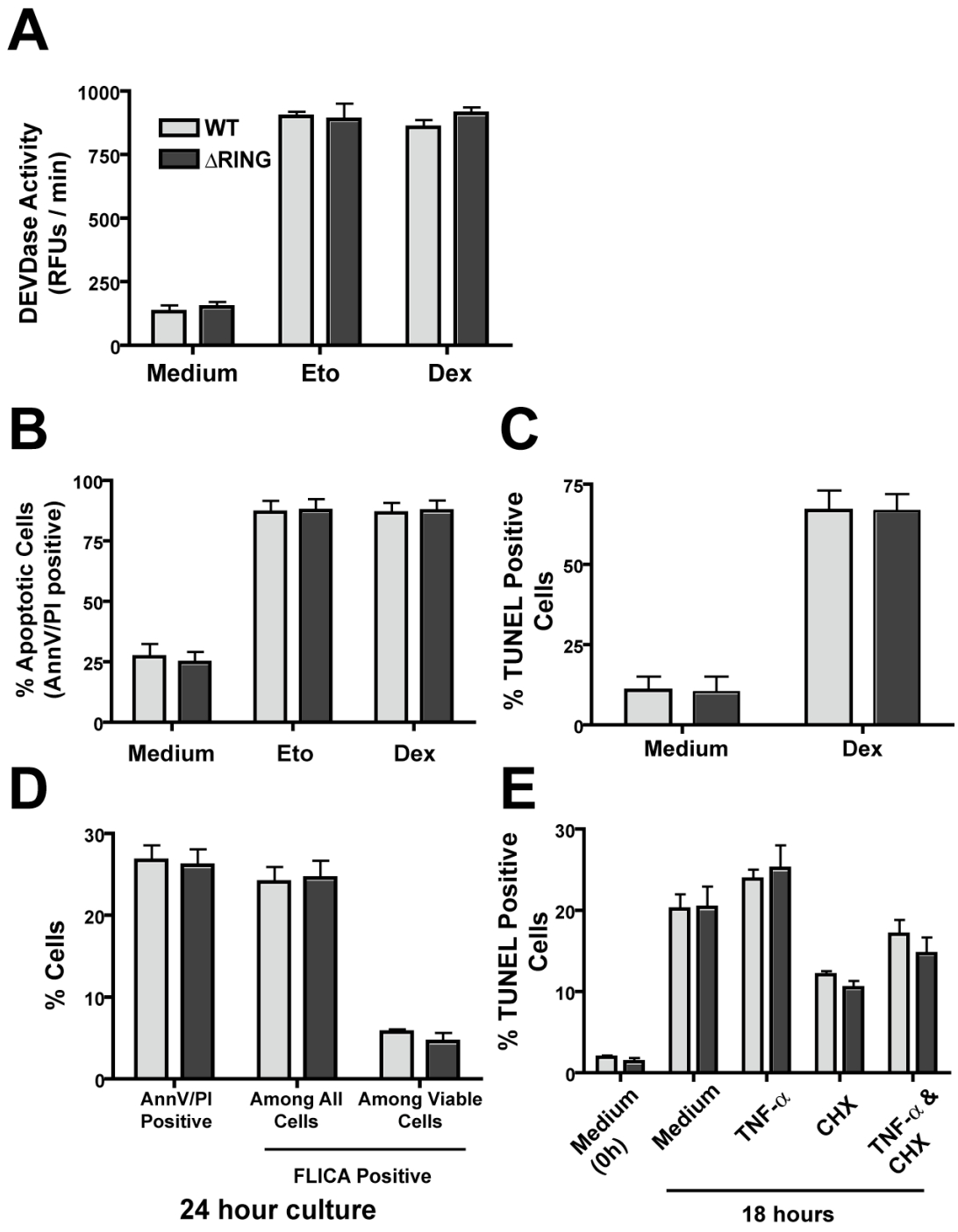


Figure 3.3 Lack of apoptosis phenotype in *ΔRING* thymocytes. (A) Bulk caspase-3-like activity after 8 h treatment. **(B)** Annexin-V/propidium iodide labeling of untreated or treated thymocytes after 8h. **(C)** TUNEL of apoptotic thymocytes after 8h. **(D)** Annexin-V/propidium iodide labeling and FLICA staining of thymocytes left untreated for 24h. **(E)** TUNEL in thymocytes treated with TNF-α/CHX.

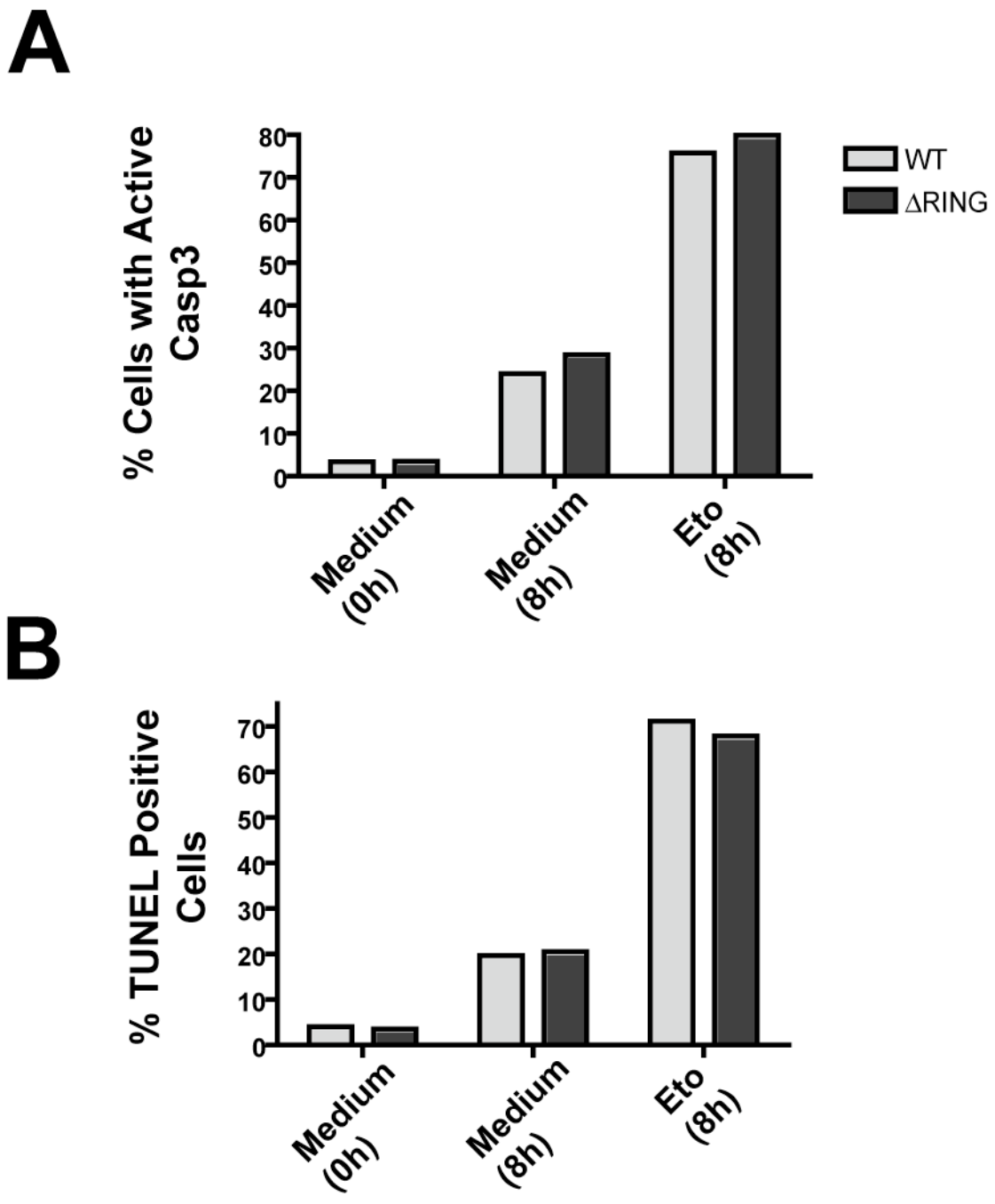


Figure 3.4 Apoptosis in B-cells. Purified B-cells were treated as indicated then assayed for caspase-3 activation by indirect immunofluorescence **(A)** or TUNEL **(B)**.

cells, which suggests that a pro-apoptotic factor may need to be synthesized for effective thymocyte apoptosis to ensue (Figure 3.3E). We also purified B-cells from the spleens of *WT* and *ΔRING* mice to study apoptosis in this cell type. Isolated B-cells of both genotypes activated caspase-3 (Figure 3.4A) and underwent apoptosis (Figure 3.4B) similarly when treated with etoposide or left untreated (Figure 3.4).

Collectively, the data here imply that the RING was important for XIAP protein stability in thymocytes, but that its removal affected neither the induction of caspases nor apoptosis in response to several stimuli. In this cell type, removing XIAP RING function did not reveal any specific pro-apoptotic role for ubiquitin-mediated XIAP turnover during cell death.

3.4.3 *Lymphoid compartments were distributed normally in ΔRING mice*

Defective apoptosis can cause imbalances in the cell numbers and cell distributions of sites where lymphocytes mature and accumulate, because cell death regulates development in hematopoietic cells (Opferman, 2007). We determined the cellular compositions of the thymus, spleen and bone marrow in mutant mice to see if genetic deletion of the RING altered lymphocyte development *in situ*. The developing T-cell compartment in the thymus was normally distributed in *ΔRING* mice, as assessed by surface

staining of CD4 and CD8 antigens (Figure 3.5); cells bearing either or both of these surface makers constitute nearly all of the thymocyte populations. Double-positive thymocytes formed the bulk of cells, and single-positive cells were normally abundant. Moreover, total thymocyte counts were unperturbed in mutant mice (Figure 3.5). The normal composition of the thymus may reflect the lack of any clear apoptosis defects in cultured thymocytes. Our results accord with a previously published description of *XIAP*-null mice that does not note any defects in thymocyte development (Harlin et al., 2001).

The B-cell lineage differentiates in the bone marrow after embryonic development. We investigated the distribution of major cell types in the bone marrow using combinations of fluorophore-conjugated antibodies against major surface markers (Figure 3.5). Hematopoietic stem cells (LSK-, Sca1+,cKit+), pro-B cells (B220+, CD43+) and pre-B cells (B220+, CD43-) were present in the bone marrow of *ΔRING* mice in similar proportions as *WT* mice. The numbers of mature and immature B-cells were also comparable. Spleens from mutant mice showed normal distributions of the major lymphocyte types (Figure 3.6). The cell types we examined included: B-cells (B220+, CD90-) and T-cells (B220-, CD90+); immature (B220 low,

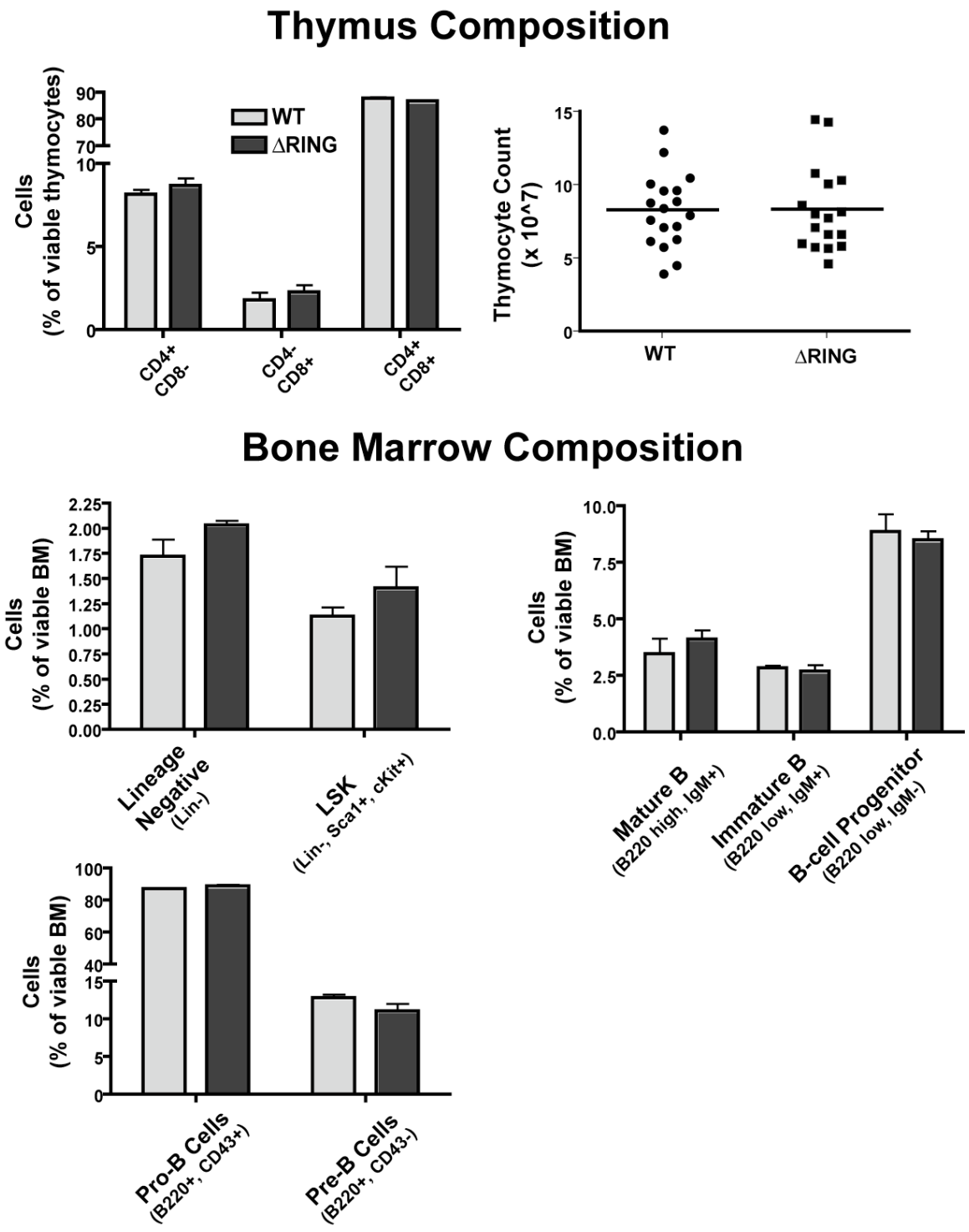


Figure 3.5 Normal thymus and bone marrow in *ΔRING* mice. Distributions of cell types as inferred by direct fluorescent labeling of cell surface proteins.

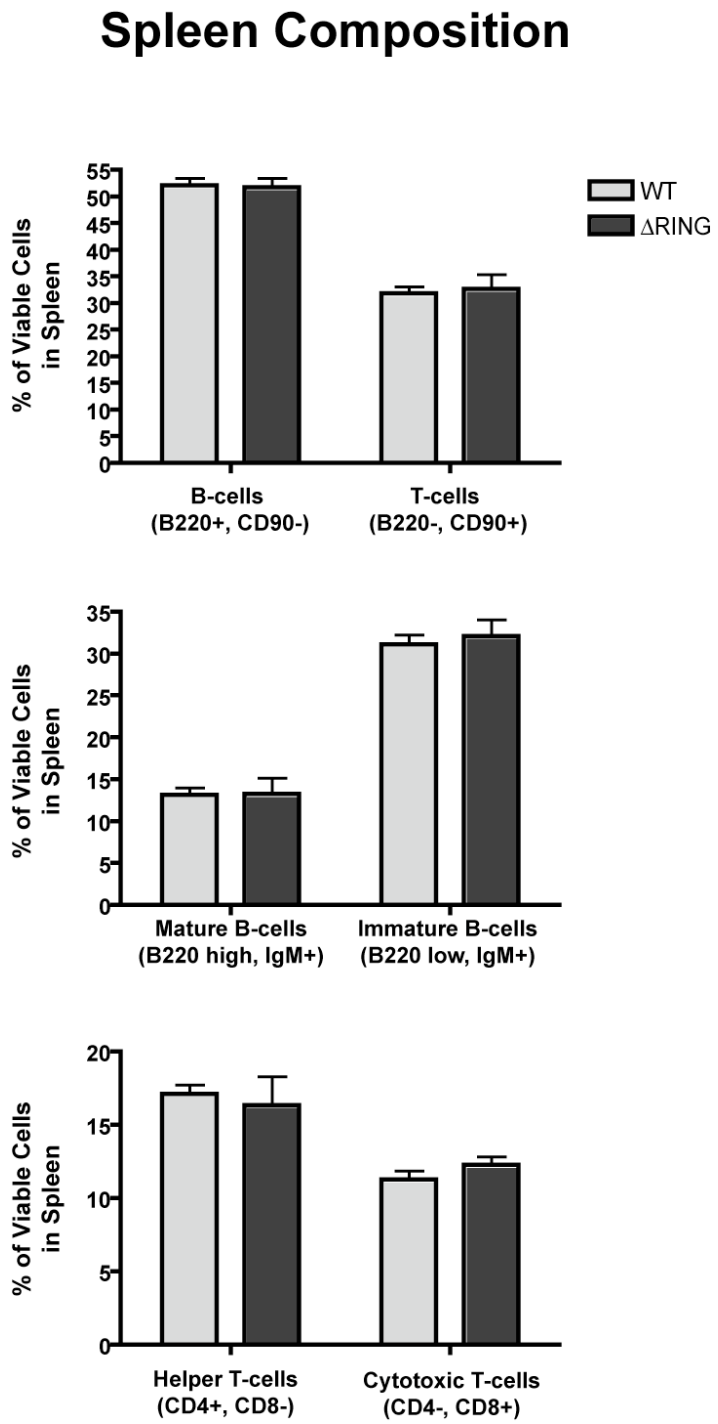


Figure 3.6 Normal spleens in *ΔRING* mice. Cellular composition of the spleen as inferred from direct immunofluorescent labeling of surface antigens

IgM+) and mature (B220 high, IgM+) B-cells; and helper-T (CD4+, CD8-) and cytotoxic-T (CD4-, CD8+) cells (Figure 3.5B). Taken together, these results indicate that deletion of the RING domain did not alter major lymphoid compartments in mice.

3.5 DISCUSSION

We used thymocytes genetically deficient for XIAP RING ubiquitin-ligase function to test the hypothesis that RING domains promote IAP degradation through autoubiquitination (Yang et al., 2000). A prevailing notion holds that RING-mediated degradation of IAPs might underlie apoptosis in thymocytes, since the proteasome is necessary for death in this cell type (Grimm et al., 1996; Yang and Li, 2000). We found that RING ubiquitin-ligase activity was indeed the primary determinant of XIAP; however, the persistence of XIAP in dying thymocytes did not affect caspase activation or enzymatic activity, and *ΔRING* cells died normally. These results argue that the RING did not exert any appreciable pro-apoptotic functions in dying thymocytes. Our results are more in line with data from *XIAP*-null thymocytes, which die normally, than with thymocytes overexpressing human *XIAP*, which are resistant to death (Conte et al., 2001). This highlights a discrepancy between different experimental approaches used to study IAP function; heterologous expression systems

may interfere with homeostatic mechanisms that control IAP levels, which may introduce artifacts that confound the interpretation of results.

The proteasome is required for apoptosis in thymocytes, but the identity of the labile (and presumably anti-apoptotic) factor(s) responsible for this phenomenon remains elusive. Our results argue that XIAP is not that factor. Another candidate is MCL-1, a short-lived anti-apoptotic BCL-2 family member that is degraded by the proteasome during apoptosis following genotoxic stress (Nijhawan et al., 2003). Although MCL-1 deficiency in lymphocytes results in increased apoptosis and profound reductions in cell numbers, thymocyte development arrests at the double-negative stage; thus, it is not yet possible to examine the role of MCL-1 degradation in the double-positive thymocyte population that is most sensitive to apoptosis and makes up the bulk of the thymus (Opferman et al., 2003).

Removing the RING domain rendered XIAP more susceptible to caspase cleavage, and this accounted for almost all of the processing of ΔRING XIAP that occurred during apoptosis. XIAP can homotrimerize through its RING; trimerization allows Smac to derepress caspases more efficiently *in vitro* (Gao et al., 2007); however, Smac was downregulated in apoptotic *ΔRING* cells and caspase activity was unaltered in mutant

thymocytes. XIAP also binds cIAP1 through homotypic RING-RING interactions (Silke et al., 2005) and XIAP is detected in high molecular weight complexes *in vivo* (Bratton et al., 2001). It is not known if XIAP forms higher order complexes in thymocytes, but the existing data argue that a ΔRING mutant would be unable to oligomerize (Silke et al., 2002). In principal, this could render XIAP more susceptible to proteolysis if steric hindrance through oligomerization or ubiquitination normally excludes caspases from IAP complexes. This is a plausible explanation because WT XIAP was not cleaved by caspases during thymocyte apoptosis. We also note that XIAP downregulation may depend on transubiquitination (e.g., by the cIAP1 RING) instead of autoubiquitination, since the ΔRING mutation also eliminated the binding surface for cIAP1 (Silke et al., 2005). Our mutant could not rule out this hypothesis, but it could be tested easily in *cIAP1*-null thymocytes (Conte et al., 2006).

4 ELEVATED ACTIVITY AND REDUCED UBIQUITINATION OF CASPASE-3 IN *ΔRING* EMBRYONIC CELLS

4.1 SUMMARY

We used embryonic fibroblasts and stem cells to study apoptosis triggered through mitochondria. We found that absence of XIAP RING function led to elevated effector caspase activity during apoptosis. Deletion of the RING did not affect the activation steps that led to caspase-3 cleavage, however, suggesting that the RING acts on activated effector caspases. Consistent with this notion, we found evidence that XIAP RING can serve as a ubiquitin-ligase for caspase-3, because ubiquitination of caspase-3 subunits was impaired in apoptotic fibroblasts in the absence of a functional XIAP RING. This phenomenon may underlie the increased caspase activity. In spite of elevated caspase activity and reduced ubiquitination, cells tolerated higher caspase activity without any increased apoptosis. Taken together, our findings show that XIAP can regulate caspases through ubiquitination, in addition to direct binding and inhibition.

4.2 INTRODUCTION

This chapter describes experiments designed to investigate the function of XIAP ubiquitin-ligase activity in embryonic cells, with an

emphasis on stimuli that use factors associated with mitochondria as the major means for activating apoptosis. The mitochondrial pathway to apoptosis is well characterized in mouse embryonic fibroblasts (MEFs) and embryonic stem (ES) cells, largely because of gene targeting efforts to inactivate major components of the pathway. Genotoxic agents are stereotypical activators of the mitochondrial pathway. Examples include etoposide, which inhibits topoisomerase II to promote DNA strand breaks, and UVC irradiation, which causes pyrimidine dimers and photoadducts. Following genotoxic damage, p53 transduces apoptotic signaling by transcriptionally activating BH3-only genes *PUMA* (Nakano and Vousden, 2001) and *NOXA* (Oda et al., 2000). While MEFs deficient for either gene are refractory to apoptosis caused by genotoxic agents, *PUMA* deficiency offers the greatest protection against the widest range of apoptotic stimuli (Villunger et al., 2003). *PUMA* is upstream of *BAX* and *BAK*, because cells lacking *BAX* and *BAK* together are insensitive to DNA-damage induced apoptosis (Wei et al., 2001). XIAP can inhibit apoptosis caused by genotoxic agents by antagonizing caspase-3 when it is overexpressed (LaCasse et al., 1998). We used mouse ES cells and MEFs to test how XIAP RING deletion affects the response to stimuli that depend largely on factors associated with mitochondria to activate apoptosis.

4.3 EXPERIMENTAL PROCEDURES

4.3.1 *Embryonic stem cell culture*

The *Neo* cassette was removed from the hemizygous *ΔRING* (*Neo*) embryonic stem (ES) cell line #125 by expressing the FLPe recombinase (pCAGGS-FLPe, a gift from Chingwen Yang) transiently using the mouse ES cell Nucleofection kit (program A-23; Amaxa). Nucleofected ES cells were seeded on mitotically arrested embryonic fibroblasts and colonies were allowed to grow for four days. *ΔRING* (*ΔNeo*) clones were identified by screening replica-plated colonies by Southern blotting of ApaLI digested genomic DNA using the same probe described in Chapter 2. Excision of the *Neo* cassette was verified by direct sequencing, as described in Chapter I. A *ΔRING* (*ΔNeo*) clone and the isogenic wild-type E14 parental line used for gene targeting were cultured on gelatinized dishes without feeder layers in ES cell medium (DMEM with 15% ES-qualified FBS [Chemicon], 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-Glutamine, 100 mM non-essential amino acids, 25 μM β-mercaptoethanol) with 10 ng/mL leukemia inhibitory factor (Chemicon). Mitotically arrested fibroblasts were essentially absent from the culture after serially passaging ES cells three times in 1:10 splits. There was no need to re-target the *XIAP* locus because

the E14 ES cell line used for gene targeting had an XY karyotype and hence was hemizygous with respect to *XIAP*.

4.3.2 *Isolation and culture of mouse embryonic fibroblasts*

Primary MEFs were isolated from embryos 12.5 days after timed matings; the presence of a copulation plug denoted day 0.5. Livers and extraembryonic tissues were dissected away from the embryos, and heads were reserved for genotyping. Embryos were passed twice through 18G needles and once through 21G needles before plating on 10-cm dishes. Primary MEFs were used through the fifth passage. Polyclonal pools of transformed fibroblasts were obtained by infecting primary MEFs with a bicistronic pBabe RasV12/E1A vector (gift from Joe Rodriguez) packaged into an ecotropic Phoenix retrovirus. Primary and transformed MEFs were cultured in MEF medium (DMEM with 15% FBS [Hyclone], 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-Glutamine, 100 mM non-essential amino acids, 25 μM β-mercaptoethanol).

4.3.3 *Induction of apoptosis in embryonic cells*

ES cells were treated in culture with 100 μM etoposide or 1 μM staurosporine for 10 hours, or left untreated in fresh medium. Primary MEFs were treated with 120 mJ/cm^2 UVC (Spectrolinker XL-1500;

Spectronics Corporation) or mock-irradiated by placing cells in the irradiator without UVC light. Transformed MEFs were treated with 100 μM etoposide or 60 mJ/cm^2 UVC for 5 hours, or left untreated in fresh medium.

4.3.4 *Apoptosis assays (indirect immunofluorescence)*

Trypsinized single cell ES and MEF suspensions were collected with any detached dead cells, washed twice in 0.5% BSA/PBS (pH 7.5), and fixed in 2% paraformaldehyde in PBS for 10 minutes at 37 C. Cells were washed twice and permeabilized in 90% methanol on ice for 30 minutes, before washing twice more. Cells were blocked in 0.5% BSA/PBS for 10 minutes at room temperature before incubating with cleaved caspase antibodies (caspase-3: Cell Signaling 9661, 1:200 dilution; caspase-8: Cell Signaling 18C8, 1:50 dilution) for one hour at room temperature. Antibodies were detected using FITC-conjugated secondary antibodies and analyzed by flow cytometry. TUNEL and DEVDase assays were performed as described in Chapter 3.

4.3.5 *Analysis of caspase-3 ubiquitination state*

Two 90% confluent 10-cm plates of MEFs were irradiated with 120 mJ/cm^2 UVC or mock-irradiated, then harvested after 7 hours; 20 μM MG-132 was added to cells 30 minutes before collection. Cell pellets were lysed

in hot 1% (w/v) SDS to dissociate protein/protein interactions, then diluted ten-fold in 1% Triton-X100 lysis buffer described in Chapter 3. Lysates were clarified by centrifugation and normalized for total protein content before rotating overnight at 4 C with a caspase-3 antibody (p32/p17, Cell Signaling 8G10 rabbit monoclonal, 1:100). Immunocomplexes were collected with 25 μL of pre-washed protein A-magnetic beads (New England Biolabs) by rotating for 2 hours at 4 C. Beads were collected using a magnetic tube rock, washed three times in cold PBS and eluted in SDS-PAGE sample buffer (New England Biolabs) for 5 minutes at 95 C before immunoblotting.

4.4 RESULTS

4.4.1 *Expression of* XIAP ΔRING *in ES cells*

We investigated the expression of the XIAP ΔRING protein in hemizygous ES cells. We obtained a *XIAP ΔRING (ΔNeo)* ES cell line by transiently expressing the FLPe recombinase to delete the *Neo* cassette. After screening 96 individual colonies, we found most of the colonies showed clonal excisions of *Neo* (Figure 4.1A). Immunoblotting confirmed that the *XIAP ΔRING (ΔNeo)* allele encoded a truncated protein of the expected size in ES cells (Figure

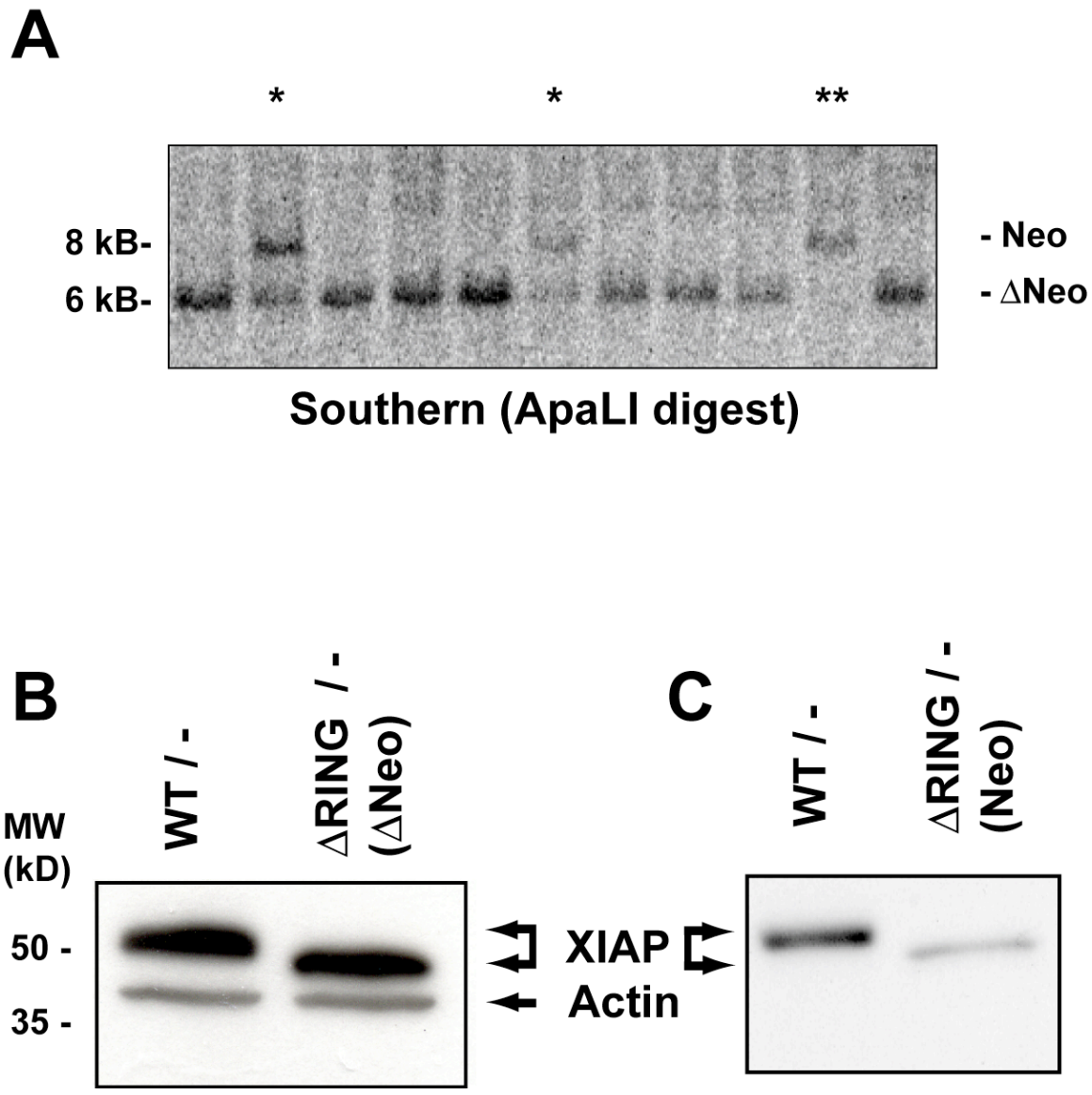


Figure 4.1 Excision of *Neo* cassette from *ΔRING* ES cells. (A) Southern blot of ApaLI-digested DNA from individual *ΔRING* ES colonies after FLPe expression. *, mixed *Neo/ΔNeo* colony; **, unexcised *Neo* colony. **(B)** Immunoblot of *ΔRING (ΔNeo)* ES clone and isogenic *WT* line. **(C)** Expression of ΔRING XIAP prior to deleting *Neo*.

4.1B). The steady state expression level was comparable to that of the full-length protein in isogenic *WT* cells. Thus, removing the RING did not influence basal expression of XIAP in ES cells, while ΔRING XIAP is expressed at higher basal levels in thymocytes (Figure 3.1). The expression of ΔRING XIAP was lower with *Neo* still present in the final intron (Figure 4.1C). This finding stresses the importance of using a removable positive selection cassette in gene targeting.

4.4.2 *Elevated caspase-3 activity in cultured* ΔRING *embryonic cells during apoptosis*

We investigated how ES cells and primary MEFs responded to different apoptotic stimuli that involve mitochondrial factors. The mitochondrial pathway of apoptosis functions primarily through caspase-9 and effector caspases-3 and -7, all of which can be inhibited by XIAP *in vitro*. Caspase-3 enzyme activity was significantly elevated in *ΔRING* ES cells treated with staurosporine or etoposide (Figure 4.2A, left) and in MEFs irradiated with UVC (Figure 4.2A, right). This result was obtained from bulk cellular lysates, so it does not offer any insights into caspase activity or activation in individual cells. For this reason, we used an antibody against a neoepitope exposed on the large subunit of cleaved, active caspase-3 to

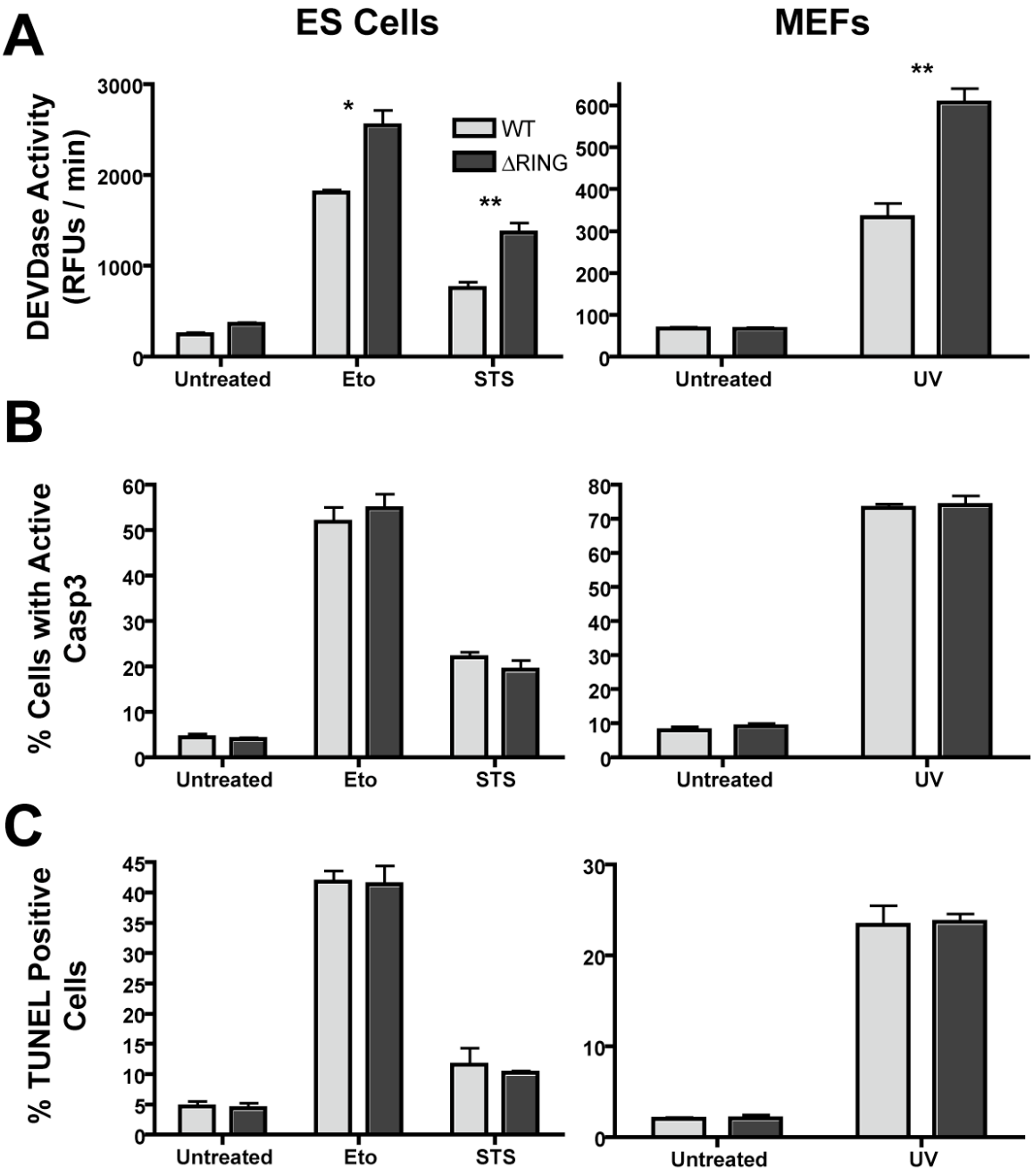


Figure 4.2 Elevated caspase activity but normal apoptosis in *ΔRING* embryonic cells. (A) Caspase-3-like enzyme activity in bulk populations of *WT* and *ΔRING* ES cells treated for 10 hours with etoposide or staurosporine (left) and MEFs irradiated with UVC after 8 hours (right). **(B)** Percentages of cells with active caspase-3 detected by indirect immunofluorescence. **(C)** Apoptosis measured by the TUNEL assay. (*) $P < 0.05$; (**) $P < 0.01$, paired two-tailed Student's t-test.

count the number of apoptotic cells by indirect immunofluorescence and determined whether the increased caspase activity was due to a higher rate of death for the *ΔRING* cells. We found equal numbers of *WT* and *ΔRING* cells harboring active caspase-3 at times when caspase activity was greater in *ΔRING* cells (Figure 4.2B). This result suggests that the steps that lead to caspase-3 cleavage and activation were not accelerated in embryonic *ΔRING* cells exposed to different stimuli that activated the mitochondrial pathway of apoptosis. Likewise, cells of both genotypes died similarly, as assessed by TUNEL (Figure 4.2B).

In spite of elevated caspase-3 enzyme activity in *ΔRING* cells, the native and cleaved forms of different caspases were expressed similarly in untreated and apoptotic ES cells (Figure 4.3A) and primary MEFs (Figure 4.3B). XIAP, cIAP1 and Smac/DIABLO became labile during thymocyte apoptosis, but the expression levels of these proteins did not change appreciably during ES cell apoptosis (Figure 4.3A).

4.4.3 *Impaired caspase-3 subunit ubiquitination in irradiated* ΔRING *fibroblasts*

We asked if genetic deletion of the RING affected caspase-3 ubiquitination in apoptotic cells, because XIAP can polyubiquitinate caspase-3 when it is overexpressed (Suzuki et al., 2001). Additionally, the

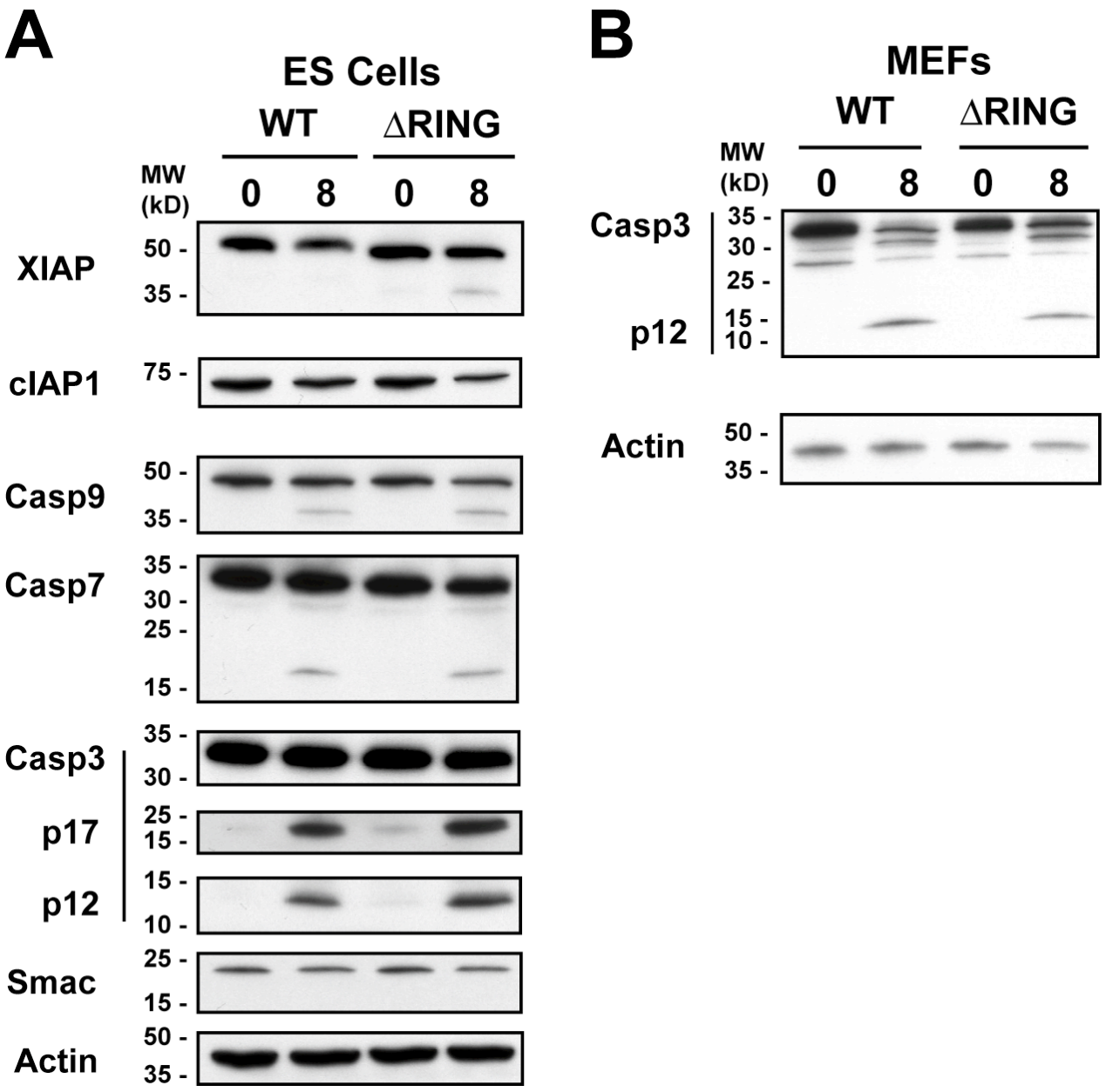


Figure 4.3 MEF and ES cell immunoblots. (A) ES cells were treated for the indicated time (hours) with staurosporine and immunoblotted as indicated. **(B)** MEFs were irradiated with UVC and immunoblotted as indicated.

DIAP1 RING has an essential function in ubiquitinating the caspase-9 ortholog DRONC in *Drosophila* (Wilson et al., 2002). We used an antibody that recognizes both full-length caspase-3 and the cleaved large subunit to immunoprecipitate the caspase from mock- and UVC-irradiated cells, then we immunoblotted with either an antibody against ubiquitin (Figure 4.4A, left), or the large subunit of caspase-3 (Figure 4.4A, right). Both antibodies detected a smear that typically indicates polyubiquitination when caspase-3 was immunoprecipitated from irradiated *WT* MEFs. This smear was largely absent in irradiated *ΔRING* MEFs and in mock-irradiated *WT* and *ΔRING* cells. These observations suggest that caspase-3 ubiquitination depends on both apoptosis and the presence of an intact RING.

We also measured the chymotrypsin-like activity of the proteasome in MEFs. Proteasome functions were comparable in *WT* and *ΔRING* MEFs both with and without UVC treatment (Figure 4.4B). The decrease in activity is consistent with caspase-mediated inactivation of the proteasome during apoptosis (Sun et al., 2004). The observed activity reflected genuine proteasome activity because lactacystin, a highly specific small-molecular inhibitor of the proteasome, abolished the signal (Figure 4.4B).

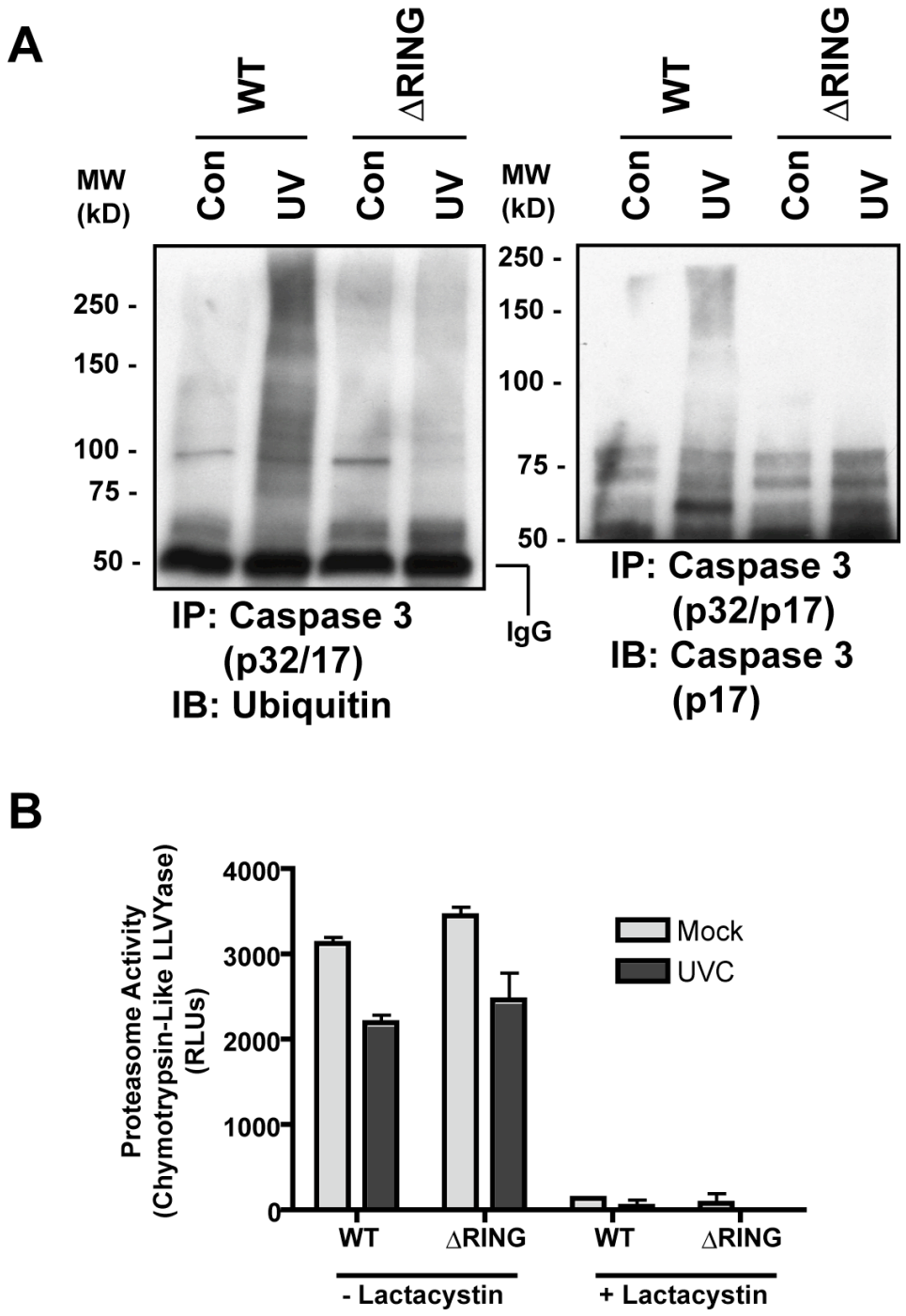


Figure 4.4 Impaired caspase-3 ubiquitination during apoptosis in *ΔRING* MEFs. (A) Caspase-3 was immunoprecipitated from MEFs 7h after UVC or mock irradiation and immunoblotted with ubiquitin (left) or active caspase-3 antibody (right). Cells were treated with 20 μM MG-132 for the last 30 minutes. **(B)** Chymotrypsin-like activity of the proteasome after 7h UVC treatment with and without lactacystin.

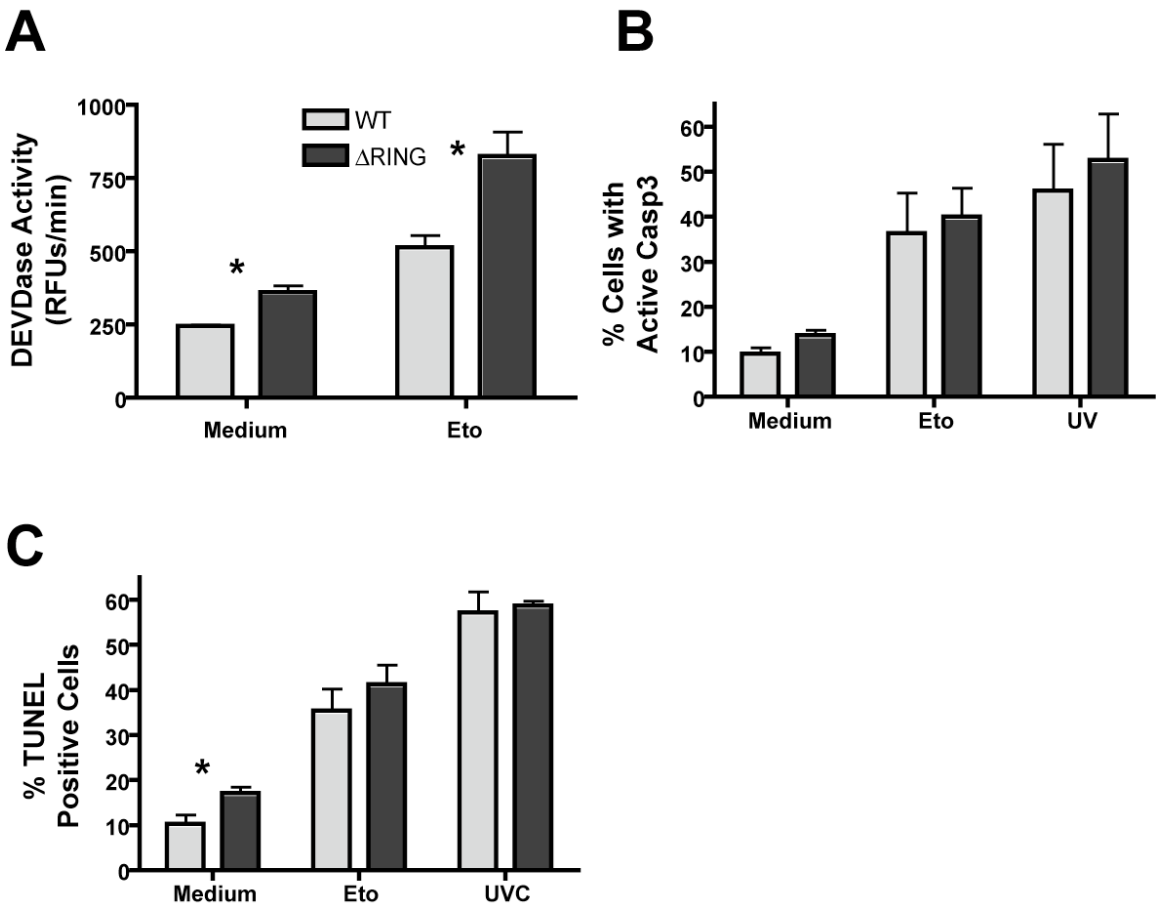


Figure 4.5 Apoptosis in Ras/E1A-transformed MEFs. (A) Ras/E1A MEFs were treated with etoposide or left untreated for 5 hours before assaying DEVDase assay in bulk. Ras/E1A MEFs were treated with etoposide, UVC irradiation, or left untreated for 5 hours before assaying caspase-3 activation by indirect immunofluorescence **(B)** and TUNEL **(C).** (*): $P < 0.05$, by paired two-tailed Student's t-test.

4.4.4 *Apoptosis in oncogenically transformed MEFs*

MEFs are primed to undergo apoptosis following oncogenic transformation (Harrington et al., 1994; White, 2001). We generated polyclonal pools of transformed *WT* and *ΔRING* MEFs by retroviral transduction of RasV12 and E1A to see if the transformed state might sensitize *ΔRING* MEFs to apoptosis. Different stimuli that impinge on mitochondria caused elevated caspase activity in transformed *ΔRING* cells without increasing apoptosis, albeit at lower drug concentrations and earlier times than with primary cells (Figure 4.5). Similar to primary MEFs, the transformed MEFs did not show any enhanced apoptosis in the absence of the XIAP RING. Intriguingly, spontaneous apoptosis in the culture medium was subtly elevated in untreated, transformed *ΔRING* cells.

4.5 DISCUSSION

This chapter describes genetic evidence for a role of XIAP ubiquitin-ligase activity in regulating caspase activity in *ΔRING* MEFs and ES cells. Genetic deletion of the XIAP RING domain increased caspase-3 activity in response to stimuli that activate apoptosis principally through factors associated with mitochondria. Removing XIAP RING function did not affect caspase-3 activation, however. This argues that the domain did not

play a significant upstream role in inhibiting caspase-9, the major activator of caspase-3 in MEFs. Surprisingly, MEFs and ES cells could tolerate higher effector caspase activity without any increased death.

The RING appears to exert its effects on caspase-3 once the caspase becomes activated. Indeed, we observed defective caspase-3 ubiquitination in apoptotic *ΔRING* cells. This is in line with a prior report showing a role for XIAP RING directed ubiquitination of caspase-3 (Suzuki et al., 2001). Defective caspase ubiquitination likely explains the increased caspase activity seen during apoptosis in *ΔRING* cells. It is surprising that the expression of the subunits was not elevated appreciably, because polyubiquitination generally leads to degradation by the proteasome. Additionally, genetic inactivation of the *Drosophila* DIAP1 RING leads to increased DRONC immunostaining that probably reflects increased protein expression. There are some precedents for this unexpected finding, however, especially with regard to caspase ubiquitination. For example, polyubiquitinated DRONC persists and is not degraded in cultured *Drosophila* cells in one account (Wilson et al., 2002). The proteasome itself is inactivated during apoptosis activated through the mitochondrial pathway. Caspases can cleave key subunits of the 19S regulatory complex that recognizes ubiquitinated substrates, thus inactivating the proteasome and

leading to an accumulation of polyubiquitinated proteins during apoptosis (Sun et al., 2004). To test this possibility in our system, we measured the chymotrypsin-like activity of the proteasome in UVC-irradiated MEFs and found that proteasome activity did indeed decrease during apoptosis, although it was not abolished completely (Figure 4.4B). The formation of a K48-linked polyubiquitination chain is a potent signal for the proteasome in most cases, but it is possible that XIAP can assemble polyubiquitination linkages with different topologies. The polyubiquitination chain assembled on the large subunit of caspase-3 awaits a more thorough analysis, perhaps by a direct determination of the ubiquitin linkages through mass-spectrometry. In principal, the polyubiquitin chain assembled on active caspase-3 could impair its ability to form product dimers through steric hindrance. This may explain the lower activity of the caspase in cells that retain XIAP E3 ubiquitin-ligase activity. Collectively, the experiments with irradiated fibroblasts imply that deletion of the RING elevated caspase activity without strongly affecting the levels of activated caspases, probably by interfering with RING-directed ubiquitination that occurs on active caspase-3 in normal apoptotic cells.

Comparisons between apoptotic thymocytes and embryonic cells reveal some distinctions in XIAP RING functions between these two cell

types. It is likely that the targets of the XIAP E3-ubiquitin ligase are distinct between these cell types: XIAP becomes labile during thymocyte apoptosis while caspases are unaffected by a RING deletion (Figure 3.1), while XIAP is quite stable and caspases become targets for ubiquitination during the death of embryonic cells (Figures 4.3, 4.4A). The molecular basis for this distinction remains unclear. The proteasome has an uncommonly prominent role in enabling apoptosis in thymocytes (Grimm et al., 1996). Perhaps the heightened importance of the proteasome in this cell type enhances the natural tendency of RING-containing IAPs to turn over (Silke et al., 2005), while the enhanced stability of IAPs in embryonic cells allows them to persist and participate in caspase ubiquitination.

5 ΔRING FIBROBLASTS ARE SENSITIZED TO TNF-α APOPTOSIS

5.1 SUMMARY

This chapter describes a novel role for XIAP as a negative regulator of TNF-α-dependent apoptosis. The elevated caspase-3 activity in ΔRING cells treated with TNF-α in the absence of NF-κB survival signaling recalled the results from Chapter 4, which described similar findings during apoptosis through mitochondrial stimuli. Unlike mitochondrial apoptosis, however, apoptosis was genuinely accelerated in *ΔRING* MEFs during TNF-α–dependent apoptosis because we observed faster assembly of the cytoplasmic complex that activates caspases and initiates apoptosis. Cleaved caspases-8 and -3 were detectable in more *ΔRING* cells over time, and TUNEL revealed markedly elevated apoptosis. We made identical observations in *XIAP*-null cells. The accelerated TNF-α-dependent apoptosis occurred independently or downstream of the JNK signaling pathway that is required for initiating apoptosis. We also obtained similar data with transformed *ΔRING* MEFs undergoing TRAIL-dependent apoptosis. Our genetic data implicate XIAP as a genuine negative regulator of the apoptotic program initiated by members of the TNF-α superfamily of cytokines.

5.2 INTRODUCTION

5.2.1 *JNK and NF-κB pathways are antagonistic during TNF-α signaling*

The TNF-α cytokine mediates a wide array of functions, including cell proliferation, inflammatory cytokine production, and programmed cell death. In MEFs, hepatocytes and many other cell types, soluble TNF-α has no cytotoxic effects after ligating its principal receptor, TNF-RI. This occurs because TNF-α signaling simultaneously activates nuclear factor kappa-B (NF-κB) pathways and c-Jun-N-terminal kinase (JNK), and these two pathways mutually antagonize each other (Bubici et al., 2004). Knockout mice have provided most of the evidence that JNK and NF-κB pathways act antagonistically during TNF-α signaling. Mice deficient for key components of the NF-κB pathway die in midgestation because of massive liver apoptosis; this phenotype is rescued by genetic deletion of *TNF-α* or its cognate receptor, *TNF-R1* (Karin and Lin, 2002). Additionally, knockout studies show that the *JNK1* isoform is necessary for TNF-α-dependent apoptosis (Liu et al., 2004). Much of the TNF-α signaling pathway remains unclear, however, and it is still unknown how signaling through both JNK and NF-κB is modulated to promote cell survival or death in different cell types.

The principal role for NF-κB signaling in cells such as MEFs and hepatocytes treated with TNF-α appears to involve transcriptional activation of factors that actively antagonize the JNK pathway. MEFs lacking either the IκB kinase β (*IKKβ*), an essential activator of the NF-κB pathway, or *RelA*, a major NF-κB transcription factor, show sustained JNK activity by TNF-α (Tang et al., 2001). This phenomenon has a functional consequence: cells lacking either gene die in response to TNF-α alone, whereas *WT* cells remain viable. *RelA*-deficient MEFs provide additional evidence that JNK signaling has pro-apoptotic functions. A dominant-negative form of JNK kinase (JNKK) prevents JNK activity and inhibits cell death, while a constitutively active JNKK-JNK fusion protein promotes cell death with a strong apoptotic component (Tang et al., 2001).

The NF-κB family transcribes a wide range of anti-apoptotic genes after TNF-α treatment, including *XIAP*, *cIAP1*, *BCL-2*, *BCL-X_L*, and *c-$FLIP_L$* (Tang et al., 2001). It is currently unclear which factors are the most important antagonists of JNK apoptotic signaling in MEFs treated with TNF-α. Intriguingly, XIAP is the only known NF-κB target that can inhibit both JNK signaling and apoptosis when overexpressed (Tang et al., 2001), although JNK activation is normal in *XIAP*-null cells (Harlin et al., 2001). The precise mechanism how JNK promotes apoptosis is still unclear, but one

candidate molecule in the pathway is the E3 ubiquitin-ligase, Itch (Chang et al., 2006). JNK phosphorylates and activates Itch, which in turn directs the assembly of a polyubiquitin chain on c-FLIP$_L$ that leads to its degradation by the proteasome. This facilitates cell death because c-FLIP$_L$ is a NF-κB survival target that interferes with caspase-8 activation and prevents the initiation of the TNF-α-dependent cell death program (Irmler et al., 1997).

5.2.2 *TNF-α-dependent apoptosis requires sequential complexes*

TNF-α triggers apoptosis in MEFs only when the NF-κB pathway is inhibited by blocking global protein synthesis (e.g., with cycloheximide) or by inactivating specific components of the pathway (Beg and Baltimore, 1996). TNF-α-dependent apoptosis proceeds through two sequential signaling complexes (Micheau and Tschopp, 2003). The first, "Complex I", is assembled proximal to the cell membrane immediately after receptor ligation. Complex I consists of the receptor, TNFR-1; the receptor interacting protein 1 (RIP1) kinase; the TNFR-associated death domain (TRADD) adaptor protein; and the TNFR-associated factor 2 (TRAF2), a RING ubiquitin-ligase. TRAF2 assembles a K63-linked ubiquitin chain on RIP1, which in turn recruits IKK to initiate NF-κB signaling. Intriguingly, cIAP1 is efficiently recruited to Complex I only when NF-κB signaling is intact. TNFR-1, RIP, and TRADD undergo complex post-translational

modifications in Complex I that include ubiquitination (Micheau and Tschopp, 2003). Caspase-8 and the FADD adaptor protein are conspicuously absent from Complex I regardless of NF-κB status (Micheau and Tschopp, 2003). In contrast, these proteins are immediately detected in complex with the Fas receptor when cells are treated with agonistic Fas antibody (Kischkel et al., 1995). This finding highlights the added complexity of TNF-α-dependent apoptosis.

A second complex, Complex II, is detectable in the cytoplasm starting several hours after Complex I forms at the membrane (Micheau and Tschopp, 2003). In the absence of NF-κB signaling, Complex II contains TRADD, RIP1, caspase-8, and FADD, and c-FLIP. Recruitment of TRADD and RIP1 to Complex II coincides with their dissociation from Complex I, while caspase-8 recruitment requires homotypic DED-DED interactions with FADD. Complex II is the activating platform for caspase-8; its assembly leads to caspase-8 cleavage and apoptosis. When NF-κB signaling is intact, however, Complex II contains much greater levels of c-FLIP$_L$ because *c-FLIP$_L$* is a transcriptional target of NF-κB. The presence of c-FLIP$_L$ antagonizes caspase-8 activation to promote cell survival (Micheau and Tschopp, 2003). In fact, the levels of c-FLIP seem to determine if caspase-8 is activated during TNF-α signaling: downregulating c-FLIP through the

ubiquitin system (Chang et al., 2006), or by preventing its synthesis (Kreuz et al., 2001), correlates tightly with caspase activation. Additionally, knockdown of c-FLIP by short interfering RNAs (siRNAs) substitutes for cycloheximide in promoting apoptosis through TNF-α (Wang et al., 2008).

5.2.3 *Involvement of IAPs in TNF-α apoptosis*

Recent studies using small-molecule IAP antagonists have revealed IAPs to be central negative regulators of TNF-α-dependent apoptosis. Even though IAP antagonists were designed using structural studies of the Smac/XIAP-BIR3 interaction, IAP antagonists also bind other IAPs with high affinity (Vucic and Fairbrother, 2007). IAP antagonists can displace caspase-3 from XIAP (Li et al., 2004), promote cIAP1/2 autoubiquitination and degradation by the proteasome (Varfolomeev et al., 2007; Vince et al., 2007), while probably exerting other effects. IAP antagonists synergize with TNF-α and a related death ligand, TRAIL (TNF-related apoptosis inducing ligand) to induce apoptosis in a variety of cancer cell lines, while exerting no cytotoxic effects in primary skin fibroblasts (Li et al., 2004). Downregulation of cIAP1/2 by IAP antagonists can initiate apoptosis in an NF-κB-dependent manner by transcriptionally upregulating TNF-α, which then appears to kill cells in an autocrine fashion (Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007). Upregulation of TNF-α

occurs in part through a non-canonical (e.g., RIP1-independent) NF-κB pathway that is dependent on NIK1 (NF-κB-interacting kinase) as an upstream activator. NIK1 is usually undetectable in cells because cIAP1/2 RING-dependent ubiquitination targets it for degradation in the proteasome; however, depletion of cIAP1/2 by IAP antagonists stabilizes NIK to activate NF-κB signaling (Varfolomeev et al., 2007; Vince et al., 2007).

This model of IAP antagonist activity invokes a pro-apoptotic role for NF-κB in upregulating TNF-α, which is surprising because NF-κB has pro-survival functions in virtually every other context. A novel mechanism for activating caspase-8 during TNF-α-dependent apoptosis was uncovered recently by studying IAP antagonists. Depleting cIAP1/2 by IAP antagonists (or siRNA silencing) liberates RIP1 from Complex I, allowing it to form a new complex in the cytosol with FADD that promotes caspase-8 activation. This complex is distinct from Complex II because it is not inhibited by c-FLIP; instead, cIAP1/2 control its formation.

5.2.4 *Pro-apoptotic role of cIAP1 RING in signaling through TNF-R2*

The cIAP1 RING has a pro-apoptotic role in the TNF-α-dependent apoptotic program signalled through TNF-RII, the second major TNF receptor (Li et al., 2002). Unlike ubiquitous TNF-R1, expression of TNF-RII is restricted to certain cell types including myeloid cells, T- and B-cells.

Ligation of TNF-RII potentiates pro-apoptotic signaling through TNF-R1, although it does not appear to directly activate caspase-8 (Zheng et al., 1995). TNF-RII lacks death domains that can recruit TRADD; instead, it binds TRAF2 directly to initiate signaling. TRAF2 protects cells from TNF-α-dependent apoptosis at least in part by activating NF-κB survival signaling, and *TRAF2*-null animals die from rampant TNF-α-dependent apoptosis (Yeh et al., 1997). Specifically ligating the TNF-RII recruits TRAF2 and cIAP1/2 to the receptor, leading to downregulation of TRAF2. This occurs because the cIAP1 RING is a ubiquitin-ligase that assembles a polyubiquitin chain on TRAF2 that marks it for degradation by the proteasome (Li et al., 2002). cIAP1 RING mutants function as dominant-negative proteins that prevent TRAF2 downregulation and delay TNF-α apoptosis. The cIAP1 RING also targets ASK1, a MAPK kinase kinase in the JNK pathway, for polyubiquitination and degradation (Zhao et al., 2007). Normally, ASK1 degradation would attenuate JNK signaling during TNF-α signaling; however, *cIAP1*-null B-cells show stabilized ASK1 after TNFR-II ligation and persistent JNK signaling (Zhao et al., 2007). It is unclear if this phenomenon has a pro-apoptotic consequence. Thus, IAP RINGs can act pro-apoptotically in some circumstances, by targeting anti-apoptotic molecules such as TRAF2 for degradation.

5.3 EXPERIMENTAL PROCEDURES

5.3.1 *Induction of death receptor apoptosis*

Primary MEFs were treated with 100 ng/mL recombinant murine TNF-α (Peprotech) and 1 μg/mL cycloheximide ("CHX", Sigma). Transformed MEFs were treated with 100 ng/mL TNF-α with and without 1 μg/mL CHX for 3 hours, or 50 ng/mL recombinant murine "SuperKiller" TRAIL (Alexis) for 10 hours.

5.3.2 *Immunoprecipitation of the caspase-8 activating complex*

To immunoprecipitate caspase-8 complexes, primary MEFs were treated with TNF-α/CHX and harvested by scraping detached and adherent cells from two 10-cm plates per treatment. Cell lysates were prepared in lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 0.2% Nonidet P40, 10% glycerol) supplemented with protease inhibitors, and then snap-frozen in liquid nitrogen. Lysates were thawed in ice water, clarified by centrifugation and normalized for total protein content (600 μg per sample) before incubating overnight with a FADD (Santa Cruz M-19 goat polyclonal, 2 μg) or RIP1 (BD Biosciences, 1:100 dilution) antibody. Immunocomplexes were collected as described in Chapter 4, except using protein G-magnetic beads (New England Biolabs).

5.3.3 *Stable knockdown of RelA by shRNA*

Ras/E1A-transformed MEFs were transduced with knockdown retroviruses (pMLP-empty or pMLP-RelA-shRNA; GFP produced by IRES) produced by the Phoenix ecotropic packaging line. Transduced cells were collected by sorting GFP-positive cells. Transduced cells were seeded in 60-mm plates and treated with 100 ng/mL TNF-α alone for 3 hours, before assaying DEVDase activity.

5.3.4 *Rescue experiment*

Primary MEFs were transduced with retroviruses produced by packaging pBabe Puro N-myc XIAP WT, XIAP ΔRING, or empty vector in the Phoenix ecotropic packaging line. After 24 hours, transduced cells were selected in 1 μg/mL puromycin for three days. Cells were treated with TNF-α/CHX for 8 hours and assayed for caspase-3 activity as described above.

5.3.5 *Transient JNK assay*

Primary MEFs were seeded in 6-well plates and treated with 100 ng/mL TNF-α alone. Cells were collected by scraping directly into microcentrifuge tubes, which were flash frozen in liquid nitrogen and used for immunoblotting as described above.

5.3.6 *NF-κB luciferase assay*

WT and *ΔRING* MEFs were transiently transfected with the pNF-κB Luc reporter plasmid (Clontech) using the Nucleofector MEF Kit 2 (Amaxa; program A-23). 24 hours later, MEFs were treated for 4 hours with 100 ng/mL TNF-α; 100 ng/mL TNF-α and 1 μg/mL CHX; or left untreated in fresh medium. Cells were harvested and lysed in DEVDase lysis buffer as described above. Cell lysates (30 μg/30 μL) were incubated with equal volumes of luciferase substrate solution (Stratagene), before measuring luciferase activity in a plate reader set to luminometer mode.

5.4 RESULTS

5.4.1 *Deletion of XIAP RING sensitizes primary MEFs to TNF-α apoptosis*

We treated *WT* and *ΔRING* MEFs with TNF-α and cycloheximide (CHX) to activate death receptor apoptosis, and found mutant cells to be much more responsive to death. This was evident by the increased number of cells with apoptotic morphology, or that had detached from the dish (Figure 5.1). Additionally, we found significantly elevated caspase-3 activity

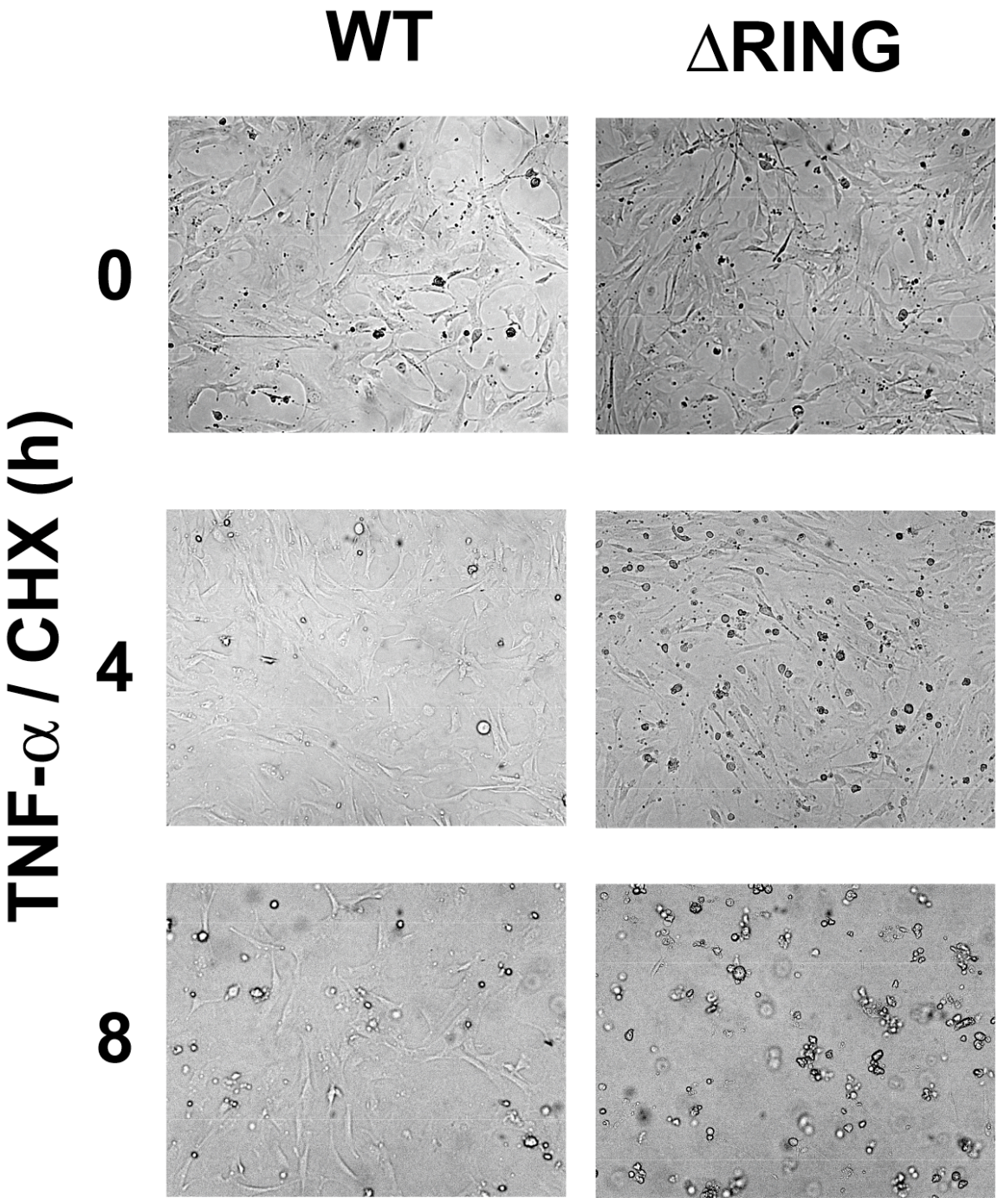


Figure 5.1 Sensitivity to TNF-α-dependent death in *ΔRING* MEFs. Brightfield micrographs of WT and ΔRING MEFs treated with TNF-α/CHX for the indicated times.

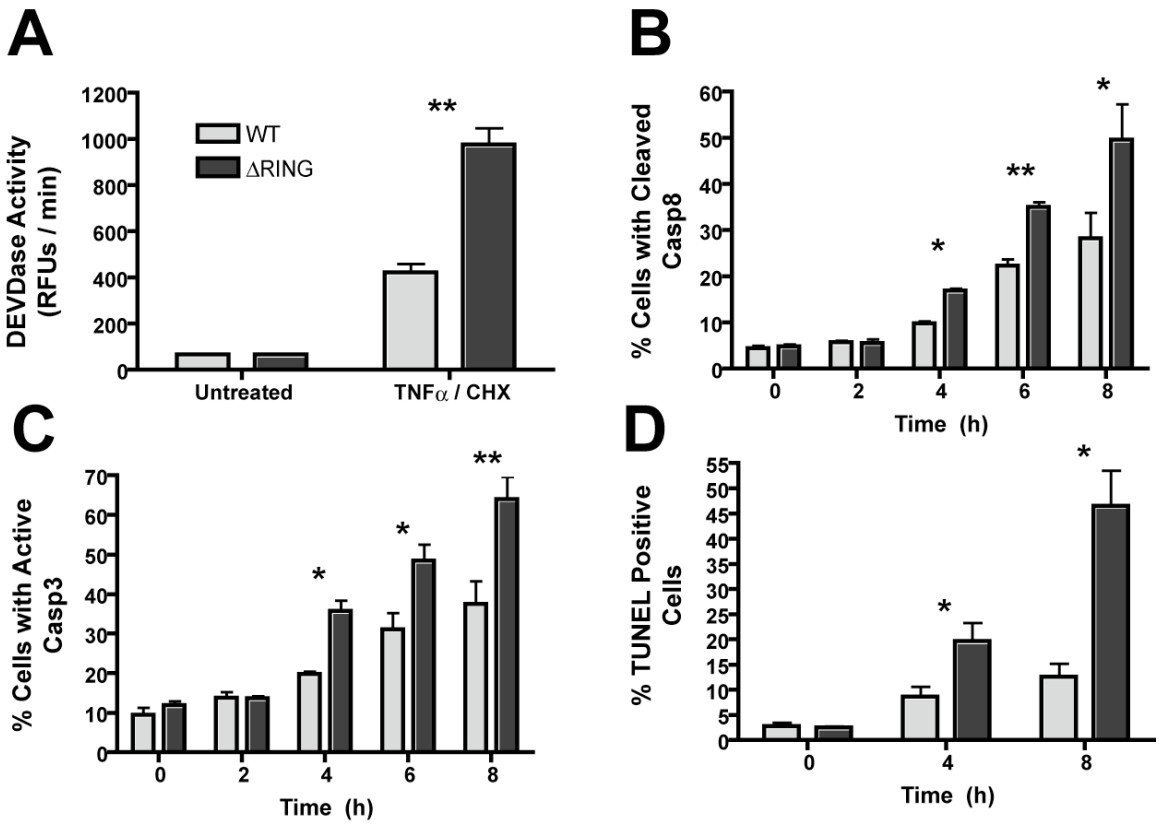


Figure 5.2 TNF-α-dependent apoptosis was accelerated in *ΔRING* MEFs. (A) Caspase-3-like enzyme activity in bulk cellular lysates after 8h TNF-α/CHX treatment. **(B)** Percentages of cells with cleaved caspase-8 as determined by indirect immunofluorescence over time. **(C)** Caspase-3 activation measured by indirect immunofluorescence. **(D)** Apoptosis measured by TUNEL staining. (*): $P < 0.05$; (**): $P < 0.01$, by two-tailed paired Student's t-test.

in *ΔRING* cells (Figure 5.2A). This result recalled the greater caspase activity described in Chapter 3, when apoptosis was triggered by stimuli with a strong mitochondrial signaling component. Unlike mitochondrial apoptosis, however, treatment with TNF-α/CHX accelerated different markers of apoptosis in *ΔRING* MEFs. Cleaved initiator caspase-8 was detected by indirect immunofluorescence in greater percentages of *ΔRING* cells over time (Figure 5.2B). Caspase-3 activation was accelerated, as evidenced by the increased number of cells stained by the CM1 antibody that recognizes the cleaved large subunit of caspase-3 (Figure 5.2C). Finally, TUNEL revealed that *ΔRING* MEFs had undergone more apoptosis (Figure 5.2D).

It is unclear if caspase-8 must undergo intrachain proteolysis before it can cleave and activate caspase-3 during death receptor apoptosis. To verify that the enhanced cleavage of caspase-8 detected by immunofluorescence reflected genuinely accelerated activation, we decided to immunoprecipitate the cytoplasmic complex (Complex II) that recruits and activates caspase-8 after TNF-α/CHX treatment (Micheau and Tschopp, 2003). We used FADD, a requisite cofactor for caspase-8 activation in this cell type, to immunoprecipitate the complex to study recruitment of key components

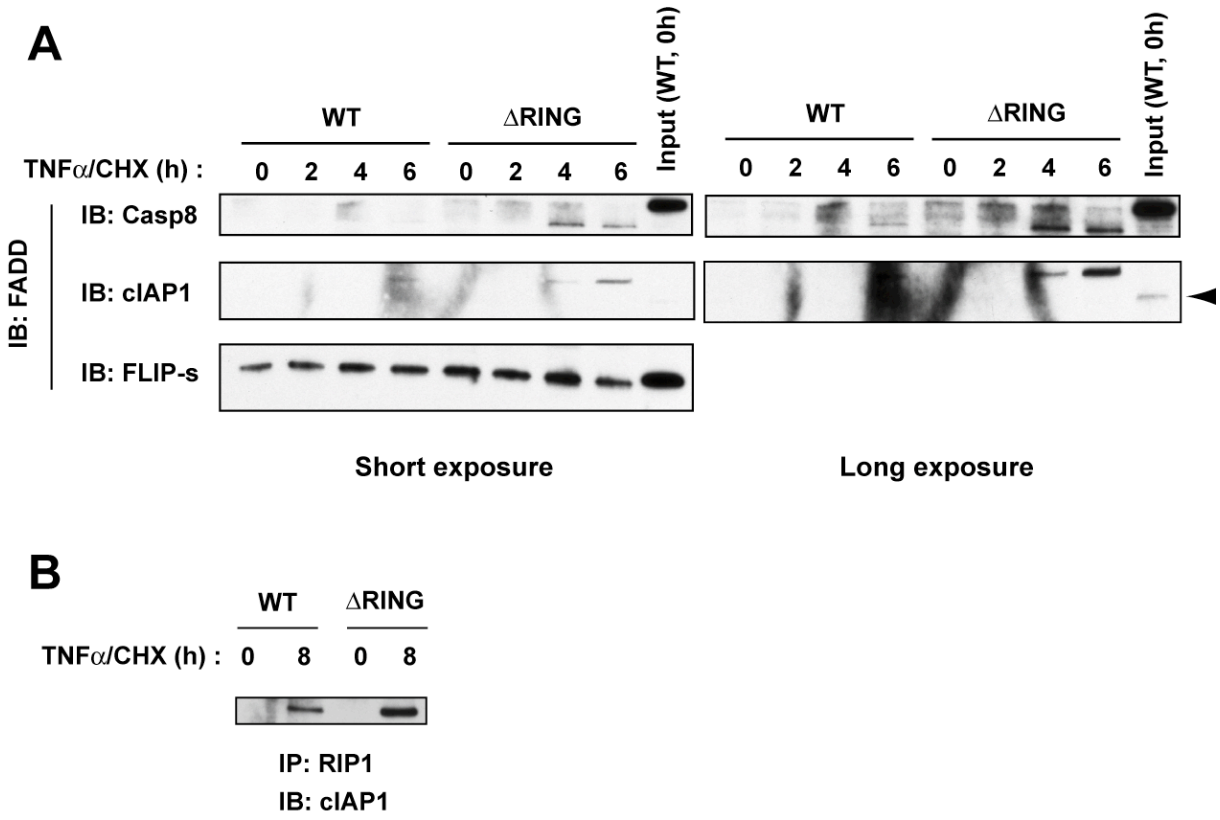


Figure 5.3 Faster assembly of caspase-8 activating complex in *ΔRING* MEFs. WT and ΔRING MEFs were treated with TNF-α/CHX for the indicated times, and the assembly of the complex that activates caspase-8 was studied by coimmunoprecipitation using a FADD **(A)** or RIP1 antibody **(B).** The native form of cIAP1 is indicated by an arrowhead.

because it is unique to Complex II. Both full-length and cleaved caspase-8 were readily detected in complex with FADD after 4 hours in *ΔRING* cells; this was evident after 6 hours in *WT* cells upon longer exposure of the immunoblot (Figure 5.3A). Likewise, a higher molecular weight form of cIAP1 was recruited to the complex more quickly in *ΔRING* cells. Intriguingly, this form of cIAP1 appeared with comparable kinetics in cells of both genotypes (Figures 3.1A; 5.7), but was recruited more quickly to the caspase-8 activating complex in *ΔRING* cells. The short form of c-FLIP (c-$FLIP_s$) was bound at all times to FADD in cells of both genotypes. Similarly, RIP1 pulled down greater amounts of cIAP1 in ΔRING MEFs after TNF-α/CHX treatment (Figure 5.3B). Taken together with the immunofluorescence data, these results argue that the increased apoptosis in *ΔRING* cells indeed reflected an accelerated activation of cell death signaling pathways.

5.4.2 *Transformed ΔRING MEFs are sensitized to TNF-α apoptosis after RelA-knockdown*

Inhibiting all translation with cycloheximide probably has far-reaching effects in a cell beyond preventing the synthesis of pro-survival NF-κB targets. To rule out off-target effects of CHX, we inactivated NF-κB

specifically by stably expressing a short hairpin interfering RNA against RelA, one of the principal members of the family of NF-κB transcription factors. We used Ras/E1A-transformed MEFs for this experiment. RelA knockdown mimicked CHX treatment, as evidenced by increased caspase-3 activity in *ΔRING* cells treated with TNF-α alone (Figure 5.4). This result suggests that *ΔRING* functioned specifically in TNF-α–dependent apoptosis and independently of any non-specific effects of CHX.

5.4.3 XIAP-*null MEFs were also sensitized to TNF-α-dependent apoptosis*

We reasoned that the hypersensitivity to TNF-α apoptosis would be observed in *XIAP*-null fibroblasts if the XIAP RING were a true negative regulator of apoptosis in this context. We isolated primary MEFs from a mouse engineered to lack XIAP expression by homology-based elimination of the first exon (Harlin et al., 2001). Immunoblotting verified the absence of XIAP protein in these cells (Figure 5.5E). Treatment with TNF-α/CHX revealed that the *XIAP*-null MEFs were as sensitized to apoptosis as cells lacking the RING alone. Caspase-3 enzyme activity increased (Figure 5.5A), cleaved caspases-8 and -3 were detected in more cells (Figure 5.5B, C), and TUNEL revealed heightened apoptosis (Figure 5.5D). Thus, XIAP was a genuine negative regulator of TNF-α-dependent on its RING domain.

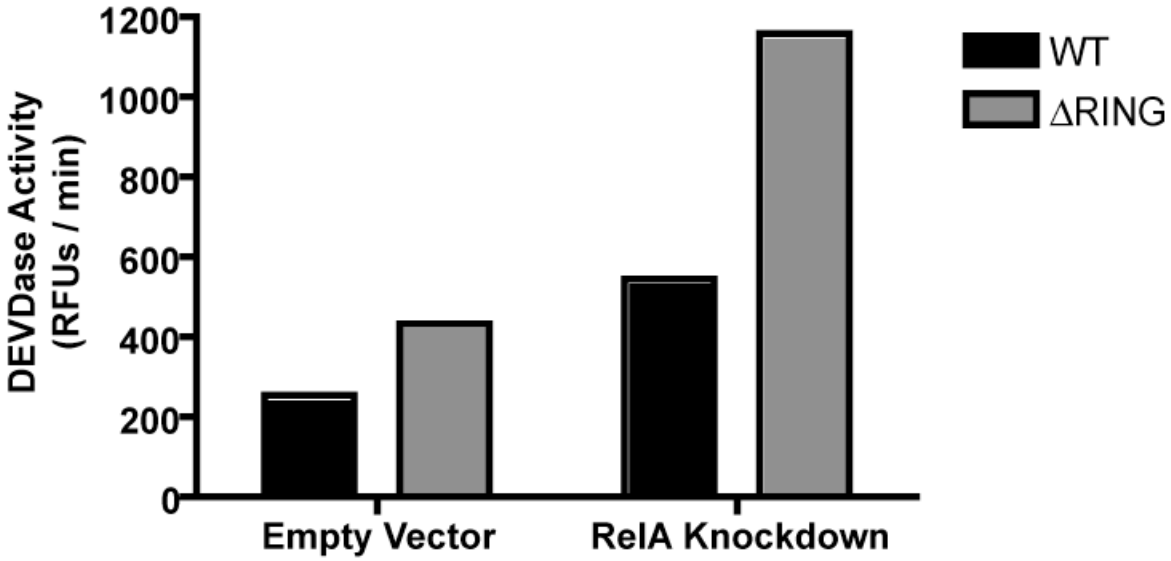


Figure 5.4 Greater TNF-α-dependent caspase-3 activity in *ΔRING* cells lacking RelA. Caspase-3-like activity measured from Ras/E1A-transformed MEFs expressing a shRNA against RelA and treated with TNF-α alone for 3 hours.

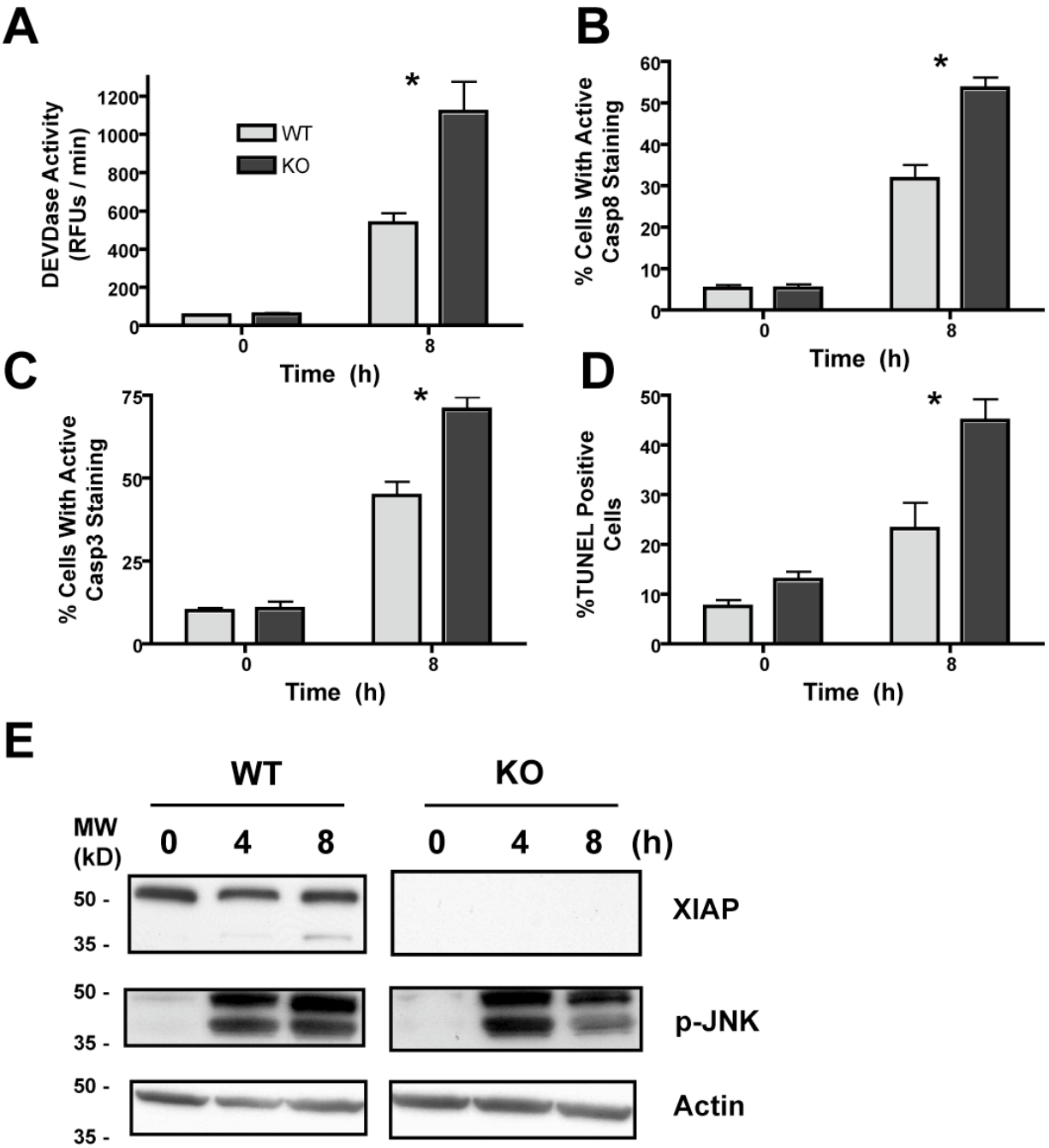


Figure 5.5 Sensitivity to TNF-α apoptosis in *XIAP*-null MEFs. (A) Caspase-3 activity measured in bulk lysates after 8h treatment with TNF-α/CHX. **(B)** Caspase-8 cleavage as assayed by indirect immunofluorescence. **(C)** Caspase-3 cleavage detected by indirect immunofluorescence. **(D)** Apoptosis as measured by the TUNEL assay. **(E)** Immunoblots of MEFs treated with TNF-α/CHX for the indicated times. (*): $P < 0.05$, two-tailed paired Student's t-test.

5.4.4 *The XIAP ΔRING mutation was not dominant in TNF-α apoptosis*

We used retroviral transduction to complement *ΔRING* MEFs with a full-length *XIAP* construct containing a functional RING domain, in an attempt to rescue the apoptosis phenotype in these cells. Reintroducing full-length *XIAP* to *ΔRING* cells reduced caspase-3 activity to the normal levels seen in *WT* cells during apoptosis (Figure 5.6). Ectopic ΔRING XIAP did not function as a dominant-negative inhibitor of the endogenous XIAP RING, because *WT* cells did not show elevated caspase-3 activity when we expressed a *ΔRING* construct. Ectopic expression of *XIAP ΔRING* also lowered caspase-3 activity in *ΔRING* cells, although not to the same extent as the XIAP WT construct. This may be explained by the greater abundance of ectopic ΔRING protein compared to ectopic full-length protein. XIAP has well-documented anti-apoptotic effects when overexpressed, and the decreased caspase-3 activity in *ΔRING*-overexpressing, *ΔRING* MEFs may reflect this phenomenon. The differences in expression may be an artifact from using strong retroviral promoters, since WT and ΔRING proteins are expressed at comparable basal levels when transcribed from the endogenous XIAP promoter in MEFs (Figure 5.7).

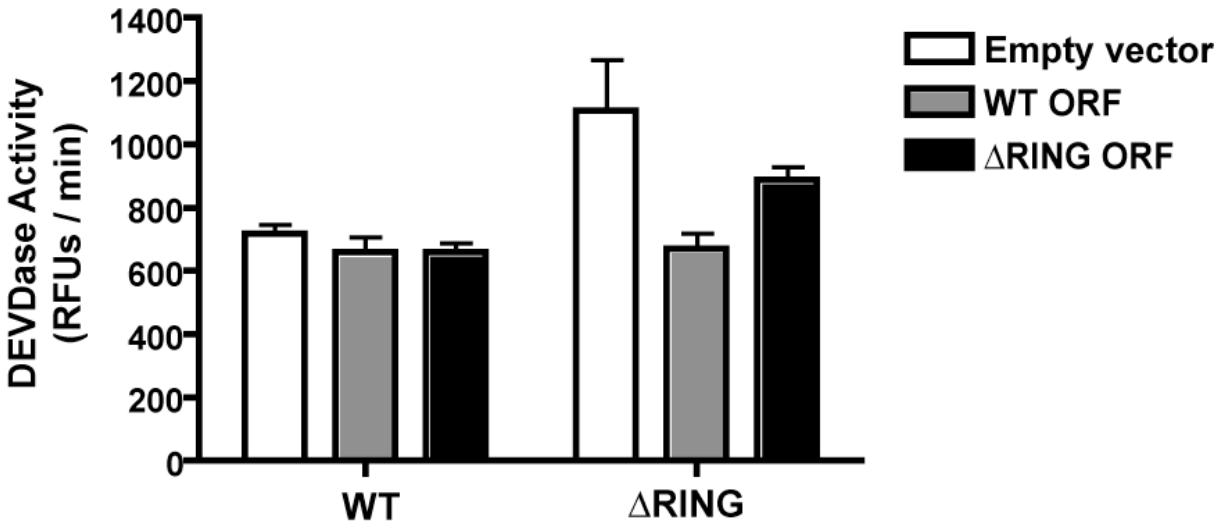


Figure 5.6 Rescue experiment. MEFs were retrovirally transduced with the indicated vectors, then treated with TNF-α/CHX for 8 hours before assaying caspase-3 activity.

5.4.5 *Normal expression of components of TNF-a apoptotic pathway*

We investigated the expression levels and behaviors of key downstream components of the TNF-α apoptotic program (Figure 5.7). The principal TNF receptor in MEFs, TNF-R1, was expressed comparably in cells of both genotypes. Other key cytoplasmic components of the receptor complex were as well, including TRAF2, FADD, and c-FLIP (the large form of which was degraded during apoptosis). RIP kinase plays a key role in promoting NF-κB signaling and inhibiting apoptosis in TNF-α treated cells, and it is inactivated by caspase-8 cleavage (Lin et al., 1999) and proteasomal turnover (Wertz et al., 2004). The basal levels of RIP1 were similar in WT and mutant cells, though it was completely cleaved in ΔRING MEFs at a time (8h) when most of the cells were apoptotic. The XIAP RING functions anti-apoptotically in primary hepatocytes treated with TGF-β by targeting TAK1 (a MAPK kinase kinase in the JNK pathway) for degradation and thereby attenuating apoptosis through the JNK pathway (Kaur et al., 2005). We investigated the expression of this protein in *ΔRING* cells, since elevated levels of TAK1 might sensitize cells to JNK activation and hence apoptosis in the absence of NF-κB signaling. However, there was no difference in TAK1 expression in the mutant cells (Figure 5.7). NIK was upregulated similarly in *WT* and *ΔRING* cells. This seems to argue against any role for

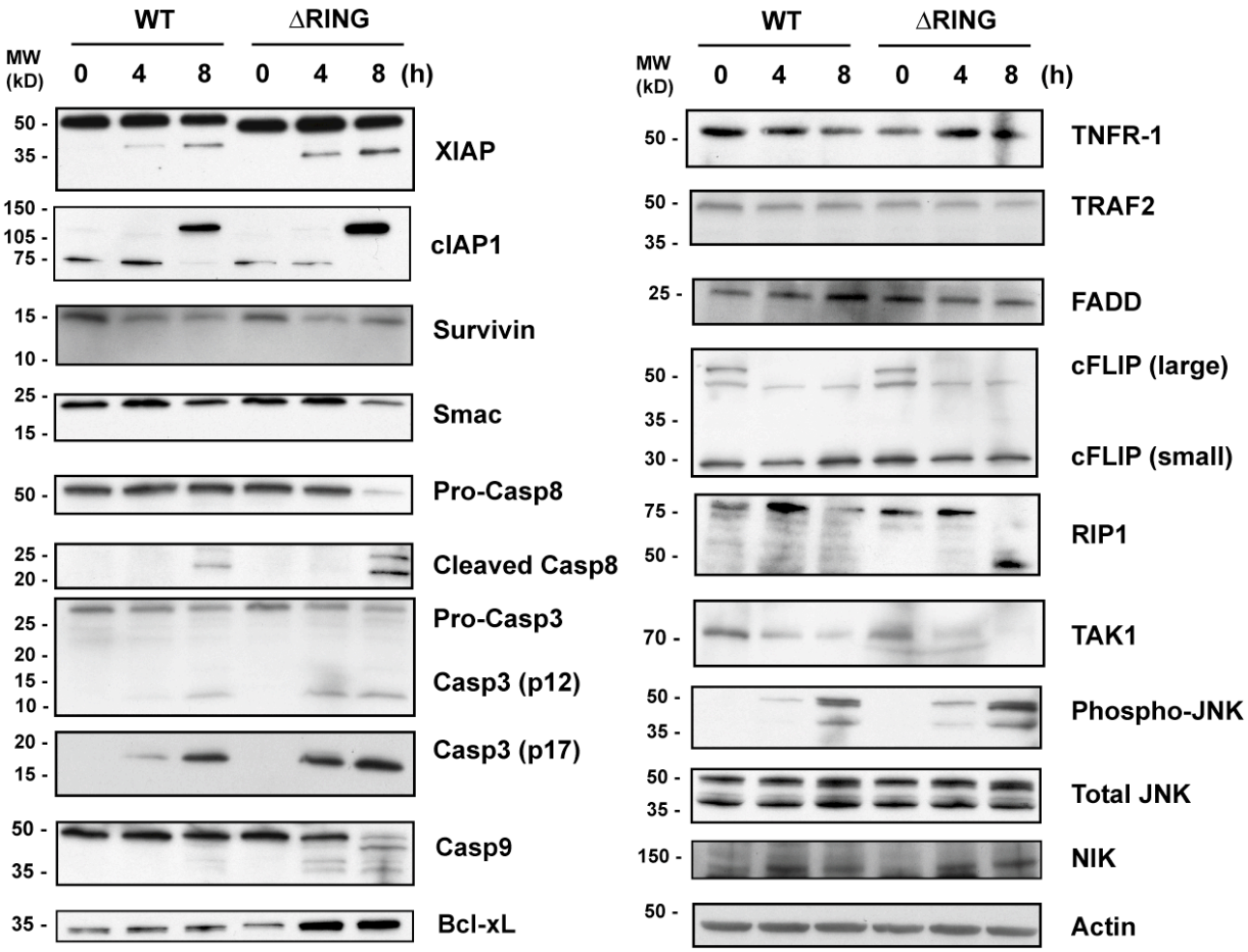


Figure 5.7 TNF-α apoptosis immunoblots. MEFs were treated with TNF-a/CHX for the indicated times before immunoblotting with as specified.

the *ΔRING* mutation in NIK-mediated upregulation of TNF-α and apoptosis seen after IAP antagonist treatment (Varfolomeev et al., 2007; Vince et al., 2007).

Both WT and ΔRING XIAP were cleaved during apoptosis, while cIAP1 and the BIR-containing protein Survivin were downregulated similarly in both genotypes (Figure 5.7). The expression of Smac/DIABLO was unchanged between *WT* and *ΔRING* cells. Immunoblotting confirmed the immunofluorescence data, by demonstrating enhanced caspase-3 cleavage that was evident after 4 hours of treatment with TNF-α/CHX. Additionally, the expression of the native form of caspase-8 strongly decreased after 8 hours in *ΔRING* cells (Figure 5.7). Anti-apoptotic BCL-X_L expression was elevated somewhat after 4 hours in *ΔRING* cells, but the significance is unclear because apoptosis was already underway.

5.4.6 *Normal JNK activation and NF-κB signaling in* ΔRING *MEFs*

Since the key components that activate or inhibit JNK were similarly expressed in *WT* and *ΔRING* MEFs, we predicted that JNK activation would occur normally in *ΔRING* MEFs. Indeed, the activating phosphorylations of different JNK isoforms appeared with identical kinetics in TNF-α/CHX treated *ΔRING* MEFs (Figure 5.7). Surprisingly, this was evident at a time

(4h) when there were already differences in apoptosis between the two genotypes. We also observed normal JNK phosphorylation in *XIAP*-null cells (Figure 5.5E). The phosphorylation status of JNK is transient when the NF-κB pathway is intact, and sustained in its absence (Tang et al., 2001). This occurs because NF-κB signaling prevents the accumulation of reactive oxygen species that would otherwise inactivate MAP kinase phosphatases (Kamata et al., 2005). Treatment with TNF-α alone elicited transient JNK phosphorylation in *ΔRING* MEFs that was indistinguishable from *WT* cells (Figure 5.8A). This result implies that NF-κB signaling remained intact in MEFs when the XIAP RING was genetically deleted, because transient JNK phosphorylation is a readout for NF-κB function.

We also measured NF-κB activity in MEFs using a reporter plasmid where the expression of luciferase was driven by optimal NF-κB responsive elements. Treating MEFs with TNF-α alone provoked a strong increase in the luciferase signal in cells of both genotypes, while TNF-α together with CHX yielded essentially the same signal as no treatment at all (Figure 5.8B). Taken together, these results suggest that there was no general propensity for *ΔRING* cells to activate JNK, or maintain activated JNK. The mechanism that promoted enhanced caspase activation in *ΔRING* and *XIAP*-null cells probably acted independently or downstream from signals that activated

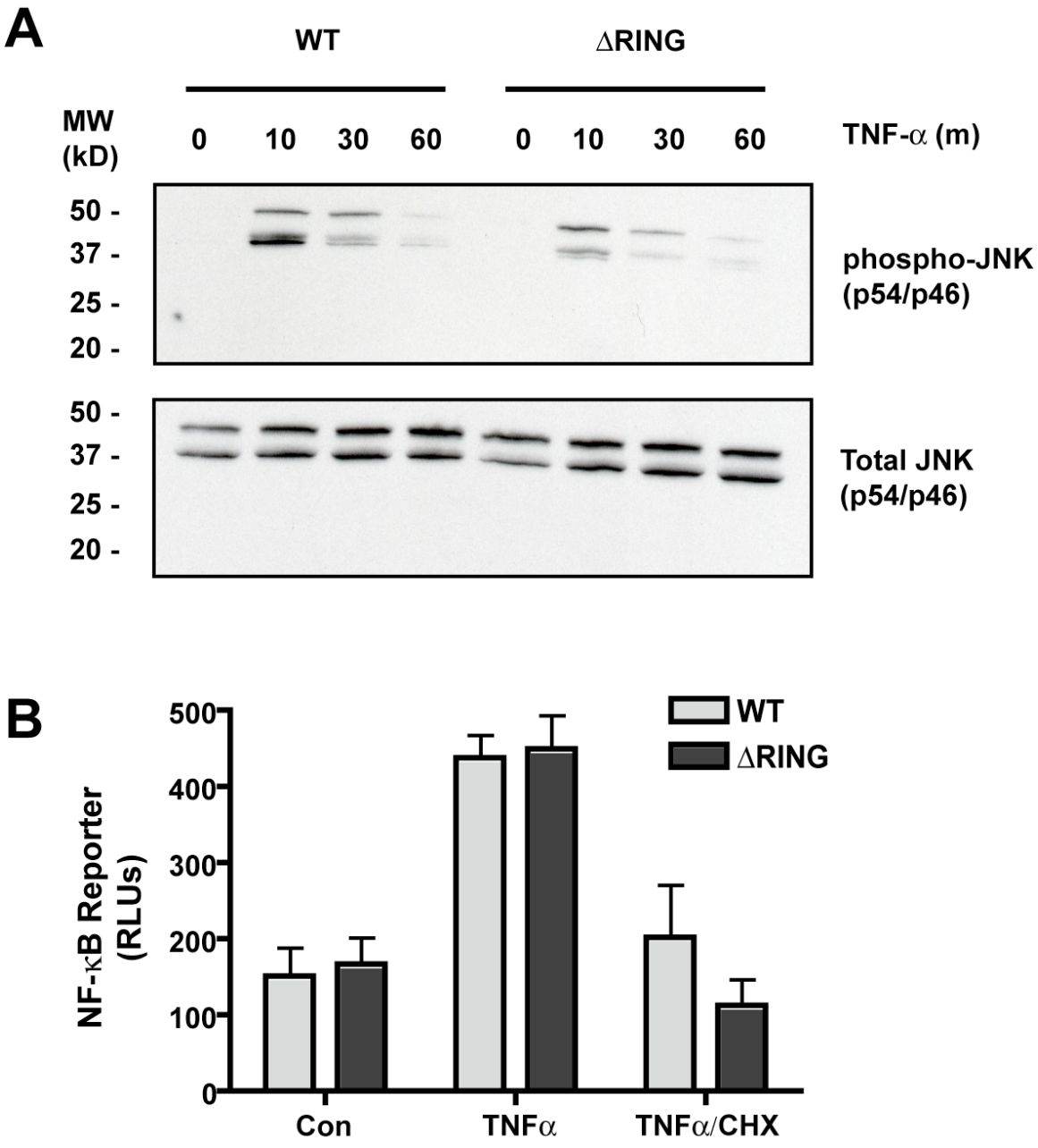


Figure 5.8 NF-κB signaling appeared intact in *ΔRING* MEFs. (A) *WT* and *ΔRING* MEFs were treated with TNF-α alone for the indicated times before immunoblotting. **(B)** MEFs were transfected with a NF-κB luciferase reporter 24 hours prior to assessing NF-κB activity 4 hours after treatment as indicated.

JNK. JNK activation is normal in TNF-α-treated MEFs from the *XIAP*-null mouse (Harlin et al., 2001), and we have now shown these cells to be sensitized to TNF-α apoptosis.

5.4.7 *Transformed* ΔRING *MEFs were sensitized to TRAIL apoptosis*

TRAIL is a member of the TNF-α superfamily of death receptor ligands with potential therapeutic applications, because it can preferentially trigger apoptosis in many transformed cell types while leaving normal cells unharmed. The reasons behind this phenomenon are unclear, although there are some clues. E1A, c-Myc and other factors that enforce cycling can prime mitochondria to activate caspase-3 further, by shifting BAK into a poised, activated state (Nieminen et al., 2007). Furthermore, E1A expression leads to transcriptional upregulation of caspases and thus sensitizes fibroblasts to apoptosis (Nahle et al., 2002). We used the Ras/E1A-transformed MEFs described in Chapter 3 to test whether genetic deletion of the XIAP RING can sensitize MEFs to TRAIL-mediated apoptosis. Recombinant TRAIL alone elicited apoptosis in both *WT* and *ΔRING* Ras/E1A MEFs, but *ΔRING* cells were significantly more responsive. Bulk caspase-3 activity increased (Figure 5.9A), and caspases-8 and -3 were detected in greater percentages of *ΔRING* cells (Figure 5.9B, C). Cell death was also elevated in mutant MEFs (Figure 5.9D). The data from

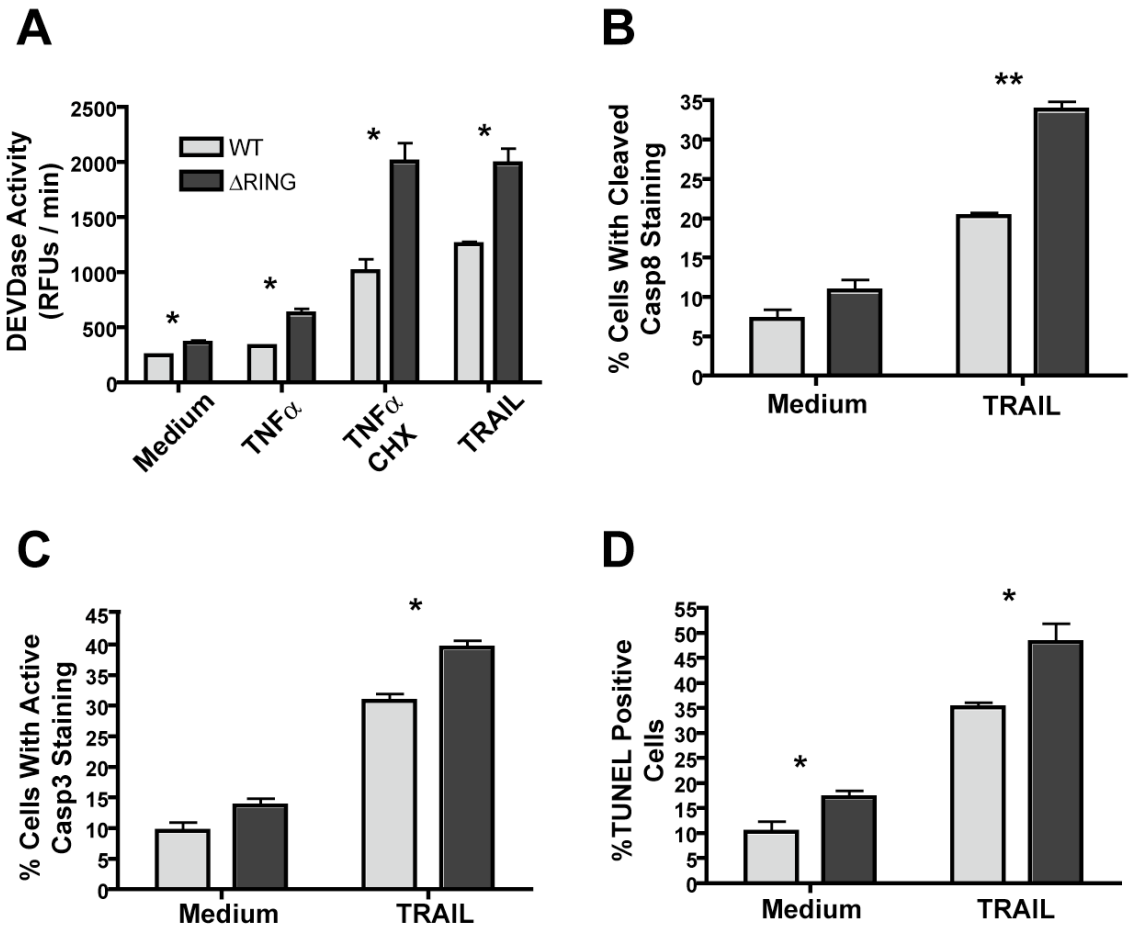


Figure 5.9 Hypersensitivity to TRAIL in transformed *ΔRING* MEFs. **(A)** Caspase-3 activity as measured in bulk. Indirect immunofluorescence was used to count the number of cells with cleaved caspase-8 **(B)** and caspase-3 **(C)**. **(D)** Apoptosis as measured by TUNEL. Cells were treated as described in Experimental Procedures. (*): $P < 0.05$; (**): $P < 0.01$, two-tailed paired Student's t-test.

RING-mutant cells imply that the RING negatively regulates TRAIL apoptosis, as well as TNF-α. Ras/E1A transformation also rendered MEFs more susceptible to TNF-α/CHX apoptosis. The differences in caspase-3 activity were still apparent between *WT* and *ΔRING* cells, but they were detectable at earlier times (Ras/E1A: 3 hours, primary: 8 hours). Intriguingly, mutant cells also showed a subtle but significant increase in caspase-3 activity in response to TNF-α alone. Similar observations were made using immortalized *cIAP1*-null MEFs (Vince et al., 2007). These results also accord with a genetic argument that that XIAP non-redundantly inhibits TRAIL apoptosis in human colon cancer cells (Cummins et al., 2004).

5.5 DISCUSSION

Our experiments with XIAP ΔRING and knockout cells implicate the XIAP RING as a novel negative regulator of TNF-α-dependent apoptosis in MEFs. This phenotype was not noted in the initial description of the XIAP-null mouse (Harlin et al., 2001). The published account describes normal JNK activation and concludes that TNF-α apoptosis is unaltered in *XIAP*-null cells. This finding is consistent with our observations, since the activating phosphorylations on JNK occurred with normal kinetics in cells

from the published *XIAP*-null mouse (Figure 5.5) and the *ΔRING* mouse (Figure 5.7). Furthermore, since loss of RING function mimics a complete null allele in TNF-α-dependent apoptosis, the *ΔRING* mutation constitutes a genuine loss-of-function.

We failed to identify any differentially expressed proteins in the TNF-α effector pathways that would readily explain the sensitivity to TNF-α apoptosis. All of the key players in the JNK pathway were present in equal amounts in unstimulated MEFs of both genotypes, and the only differences (e.g., RIP1 cleavage and TAK1 disappearance after 8h in *ΔRING* MEFs) were evident at a time when most of the mutant cells were dead. Previous work identified XIAP as a NF-κB target that specifically antagonized JNK activity (Tang et al., 2001). This conclusion is based on overexpression studies in MEFs. Although we did not do a JNK assay, the activation of JNK by upstream kinases occurred normally, as assessed by immunoblotting with a phosphorylation-specific antibody. Based on this result and other published findings, we conclude that the sensitivity to TNF-α-dependent apoptosis occurs downstream or independently of the signals that activate JNK.

It is unclear if caspases can account for the hypersensitivity to TNF-α apoptosis in *XIAP* loss-of-function MEFs. Caspase-3 ubiquitination is

probably impaired during TNF-α apoptosis, as we demonstrated with UVC irradiation (Figure 4.4A). There is evidence that caspase-8 can be activated by caspase-6 in a feedback mechanism (Murphy et al., 2004). This may account for some of the accelerated apoptosis if XIAP RING normally ubiquitinates and inhibits other effector caspases. It seems more likely that the differences in apoptosis arise at a point proximal to the receptor, because we noted that Complex II assembled more rapidly in *ΔRING* cells. It is also possible that the effects are indirect. XIAP and cIAP1 can heterodimerize through RING-RING interactions (Silke et al., 2005). If this interaction were necessary for cIAP1 localization or anti-apoptotic functions, such as those discussed in the chapter introduction, then removing the XIAP RING may sensitize cells to TNF-α-dependent apoptosis indirectly. However, XIAP does not associate with the TNF-R1 complex, and attempts to investigate the subcellular localization of endogenous cIAP1 in *ΔRING* cells were stymied by the lack of a suitable antibody for immunofluorescence. Perhaps this matter will be resolved as better tools are developed.

The sensitivity to death receptor apoptosis extended to TRAIL as well, as demonstrated by the experiments with oncogenically transformed MEFs in Figure 5.8. This is consistent with prior experiments showing that deletion of the *XIAP* locus by homologous recombination sensitizes colon

cancer cells to TRAIL (Cummins et al., 2004). Attempts to extend these findings to Fas death receptor signaling were unsuccessful, probably because Fas receptor is expressed at very low levels in fibroblasts. The susceptibility to TNF-α or TRAIL apoptosis did not extend to an experimental model of septic shock and liver failure triggered by systemic TNF-α administration (Figure 2.4). This difference may stem from the requirement for mitochondrial amplification of death receptor signaling in hepatocytes (Yin et al., 1999). Perhaps the sensitizing effects of *ΔRING XIAP* are not sufficient to overcome this threshold.

6 LOSS OF XIAP RING FUNCTION IMPROVES SURVIVAL IN THE *Eμ-Myc* LYMPHOMA MODEL

6.1 SUMMARY

This chapter describes how loss of RING function improves survival in the *Eμ-Myc* model of non-Hodgkin's lymphoma. *ΔRING* mice showed significantly longer lymphoma-free survival and lessened incidence of leukemia. There was a reduction in the number of large, proliferating B-cell precursors in the bone marrow and in the periphery. RING inactivation led to elevated apoptosis in the population of proliferating, pre-malignant B-cells in the bone marrow. Purified B-cells underwent apoptosis more readily when cultured without serum. The data strengthen the notion that the *ΔRING* mutation is an XIAP loss-of-function and show a novel role for RING function in tumorigenesis.

6.2 INTRODUCTION

6.2.1 *The* Eμ-Myc *lymphoma model*

The chromosomal translocations found in hematopoietic malignancies have offered a wealth of insights into the signaling pathways misregulated in human cancer. Many common lymphomas and leukemias arise when somatic B-cell receptor arrangement goes awry and juxtaposes enhancers in

the receptor locus with potent oncogenes (Cory, 1986). The *BCL-2* gene, for example, was first cloned at the breakpoint of the *t(14;18)* translocation found in follicular lymphoma, where its expression is harnessed to the *IgH* locus (Tsujimoto et al., 1984). A *t(8;14)* translocation is commonly found in Burkitt's lymphoma in humans, and the analogous translocation *t(12;15)* underlies experimentally induced plasmacytomas in BALB/c mice (Adams et al., 1983). This translocation brings the *Myc* coding sequence under the control of the μ intronic enhancer in the *IgH* locus. Since the *IgH* locus encodes the heavy chain of the B-cell receptor, the *Eμ-Myc* translocation leads to constitutive misexpression of *Myc* throughout the stages of B-cell development where the B-cell receptor is expressed (typically, the pro-B stage and onward) (Harris et al., 1988).

Transgenic mice expressing the *Eμ-Myc* fusion offer clear evidence that this translocation can cause lymphoma and leukemia (Adams et al., 1985). This mouse transgenically expresses an *Eμ-Myc* translocation cloned from a murine plasmacytoma, and it represents a technological triumph because it is one of the first transgenic mice ever created. Mice harboring the *Eμ-Myc* translocation develop lymphoma and lymphoblastic leukemia, but the neoplastic cells (principally, pre-B and B-cells) are distinct from the cells that arise in Burkitt's lymphoma, which affects mostly mature B-cells

(Adams et al., 1985). Better models of Burkitt's lymphoma have since been developed (Kovalchuk et al., 2000), but the *Eμ-Myc* mouse remains a widely used model of non-Hodgkin's lymphoma.

Lymphocytes from *Eμ-Myc* mice progress through three distinct stages (Sidman et al., 1993). Initially, B-cell precursors hyper-proliferate in the bone marrow starting before birth (Langdon et al., 1986). The expanded pool of lymphocytes is comprised principally of pre-B-cells, and some of them migrate to the periphery (Sidman et al., 1993). The leukocyte population then returns to near-normal levels, probably because of apoptosis in the bone marrow and periphery (Jacobsen et al., 1994). This occurs partly because pre-malignant *Eμ-Myc* B-cells remain dependent on limiting amounts of cytokines for survival signaling (Langdon et al., 1988). The length of this intermediary period varies among individuals. The final stage is marked by emergence of disseminated lymphoma and a pathological elevation in the numbers of large, circulating lymphoblasts as the mice develop leukemia (Sidman et al., 1993). Death usually ensues soon after.

Lymphoma latency varies among not only different mouse strains, but also genetically identical mice from the same inbred strain (Sidman et al., 1988). We can make several inferences from these observations. First, genetic modifiers unique to different mouse strains synergize with *Eμ-Myc*

to influence the latency of disease. *Eμ-Myc* mice on the C57BL/6 strain, for example, live considerably longer than *Eμ-Myc* BALB/c mice (Sidman et al., 1988). Additionally, the variable latency among inbred mice points to the involvement of somatic, tumorigenic mutations that occur stochastically. Indeed, cells from the pre-lymphoma stage in *Eμ-Myc* mice are not malignant and do not survive transplantation into compatible hosts (Langdon et al., 1986). Even cells from double transgenic *Eμ-Myc/BCL-2* mice are not transplantable at early stages, despite their markedly enhanced survival in the absence of growth factors (Strasser et al., 1990). These findings imply that three or more oncogenic "hits" may be required to transform lymphocytes. Remarkably, the likelihood that a given *Eμ-Myc* pre-B cell will become malignant is only about 1 in 10^{10} on a mixed genetic background (Harris et al., 1988), and the tumors that do arise are invariably of clonal origin (Adams et al., 1985; Harris et al., 1988).

6.2.2 *Apoptosis as a critical modulator of* Eμ-Myc *lymphoma*

There is a wealth of evidence implicating apoptosis as a key modulator of lymphoma development in the *Eμ-Myc* mouse. The observation that *BCL-2* synergizes with *Eμ-Myc* to accelerate lymphoma by increasing the survival of transgenic B-cells is a hallmark finding in the cell death field

(Strasser et al., 1990; Vaux et al., 1988). Many other factors related to apoptosis are now known to modulate the *Eμ-Myc* model. For instance, B-cells from *Eμ-Myc* mice undergo spontaneous p53-dependent apoptosis, and there is strong selective pressure to inactivate the ARF-Mdm2-p53 axis (Eischen et al., 1999) (Eischen et al., 2004; Schmitt et al., 1999). *p53* deficiency reduces survival of *Eμ-Myc* mice (Schmitt et al., 1999), while loss of *Mdm2* prolongs survival by facilitating p53-dependent apoptosis of pre-malignant cells (Alt et al., 2003). Expression of *BCL-2* or dominant-negative *caspase-9* phenocopies loss of *p53* function in the *Eμ-Myc* model; remarkably, expression of either genes relieves the selective pressure to inactive the *p53* locus *in vivo* (Schmitt et al., 2002). These findings imply that p53's chief tumor suppressor function in *Eμ-Myc* lymphoma is to activate apoptosis through mitochondrial factors and caspase-9. Furthermore, *BIM* appears to be the principal BH3-only factor that activates apoptosis in the *Eμ-Myc* model (Egle et al., 2004).

6.2.3 *XIAP in lymphoid malignancy*

Overexpression of XIAP can inhibit cell death in virtually every cell culture system (LaCasse et al., 1998), and elevated XIAP expression is found in many human tumors (Hunter et al., 2007). Studies like these

implicate XIAP gain-of-function mutations in the development of cancer, and led to the development of therapeutic compounds that antagonize IAPs to promote apoptosis in human tumors (Vucic and Fairbrother, 2007). There is little experimental evidence linking XIAP function to tumorigenesis, however, and much of the data remains circumstantial. Although transgenic mice overexpressing human *XIAP* in the thymus show increased numbers of developing T-cells and resistance to apoptosis (Conte et al., 2001), no published mouse models have shown that *XIAP* can promote tumor formation.

A positional cloning effort identified *XIAP* deficiency as a genetic basis for the human X-linked lymphoproliferative syndrome (Rigaud et al., 2006). Lymphocytes in afflicted patients are sensitized to TRAIL and Fas death receptor apoptosis and natural killer T-lymphocytes are depleted. How enhanced apoptosis leads to lymphoproliferation remains a mystery. This report provides the clearest link between *XIAP* loss-of-function and human disease. The data described in this chapter extend these findings to the *Eμ-Myc* mouse model of lymphoma, and provide genetic evidence implicating RING function in inhibiting apoptosis in tumorigenesis.

6.3 EXPERIMENTAL PROCEDURES

6.3.1 Eμ-Myc *Mouse Experiments*

ΔRING mice were backcrossed to C57BL/6 mice for at least six generations before mating with C57BL/6 *Eμ-Myc* mice purchased from Jackson Laboratory (strain B6.Cg-Tg(IghMyc)22Bri/J). Cohorts of mice were monitored for lymphoma-free survival over time before generating a Kaplan-Meier survival curve. Lymphoma was documented in every mouse scored as a fatality in the Kaplan-Meier analysis. Mice were censored from analysis when used for breeding or an experiment. Peripheral blood was sampled periodically by retro-orbital eye bleeds and analyzed by the Laboratory of Comparative Pathology at Memorial Sloan-Kettering Cancer Center. Analyses included complete counts of major lymphocyte populations and viewing of blood smears by pathologists.

6.3.2 *Bone marrow and cultured B-cell experiments*

For bone marrow analysis, cell suspensions were flushed from femurs and tibias, treated with erythrocyte lysis buffer (9 volumes of 150 mM NH_4Cl, 1 volume of 130 mM Tris-Cl, pH 7.65), and stained with B220-APC (1:50, BD Pharmingen) for 20 minutes on ice to label B-cells. Cells were fixed in 3% paraformaldehyde for 15 minutes at room temperature, permeabilized in 2% Triton X-100/PBS for 15 minutes at room temperature,

then assayed by TUNEL. Apoptosis was measured in proliferating B-cells by gating large-scattering B220+ cells.

Eμ-Myc B-cells were purified from spleen preparations by magnetic depletion of non-B-cells, as described in Chapter 3. An aliquot of cells was saved from each preparation to verify the purity of the depleted samples, as described in Chapter 3. Purified B-cells were cultured at a density of 10^6 cells/mL in B-cell medium (50% Iscove's MEM, 50% DMEM, 100 U/ml penicillin, 100 g/ml streptomycin, 4 mM l-glutamine and 25 μM 2-mercaptoethanol) in the absence of serum for 3.5 hours, prior to performing TUNEL as described in Chapter 3.

6.4 RESULTS

6.4.1 *The* ΔRING *mutation improved survival of the* Eμ-Myc *mouse lymphoma model*

Removing the RING domain rendered XIAP a worse caspase inhibitor in embryonic cells, which suggests that the *ΔRING* allele is a loss-of-function. We explored the effects of this mutation on a mouse lymphoma and leukemia model where apoptosis has an essential tumor suppression function. By crossing the *ΔRING* allele onto the *Eμ-Myc* background and following the survival of *WT* and *ΔRING* mice over time, we found that *ΔRING* mice showed improved survival on this sensitized background

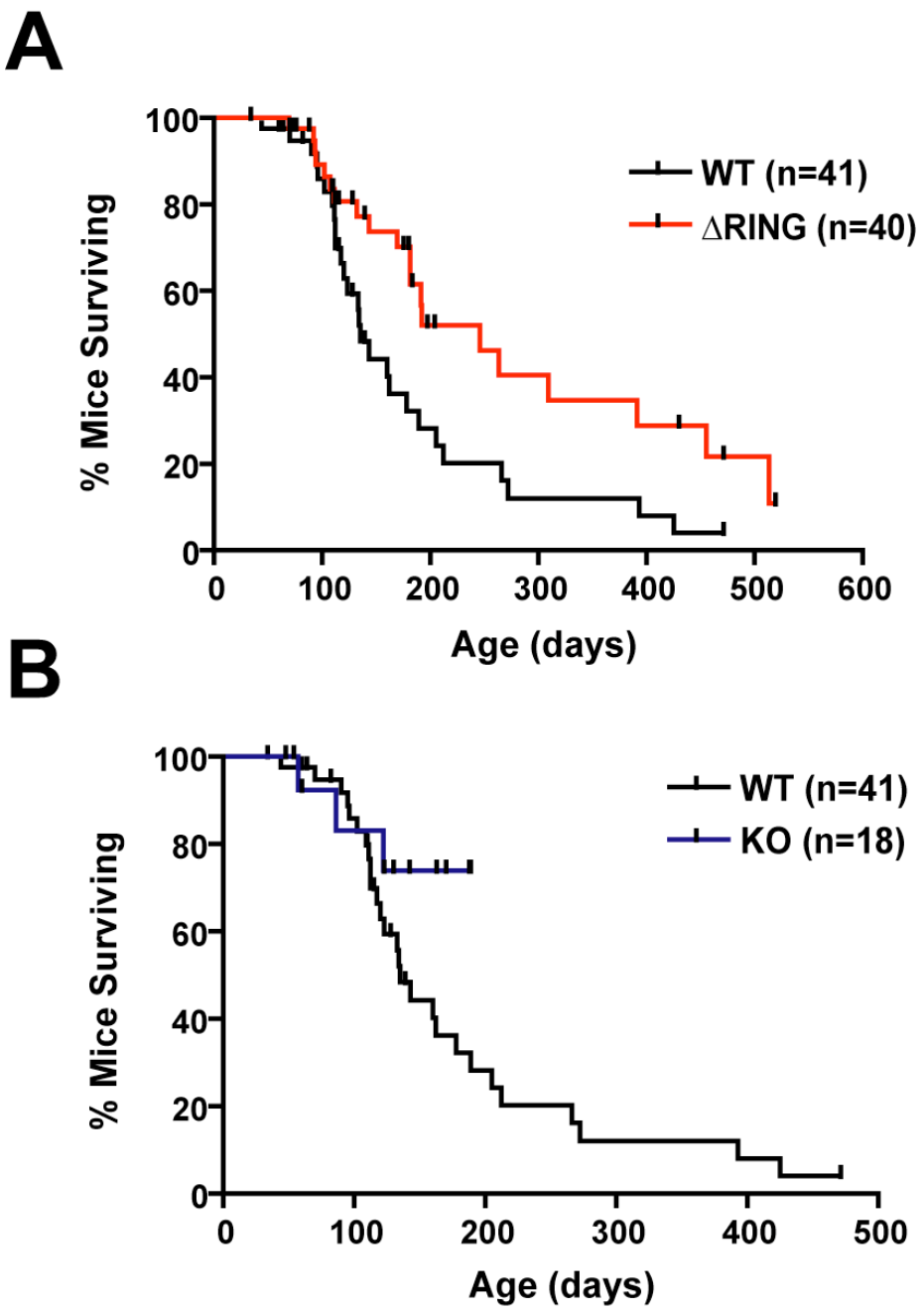


Figure 6.1 Genetic deletion of XIAP RING prolongs life in the *Eμ-Myc* lymphoma model. (A) Kaplan-Meier survival curve of *WT* and *ΔRING* mice on the *Eμ-Myc* background. **(B)** Kaplan-Meier survival curve of *WT* and *XIAP-null* mice on the *Eμ-Myc* background.

(Figure 6.1A; p = 0.010). The median survival time for WT mice was 135 days, whereas median survival time increased to 246 days for ΔRING mice on the *Eμ-Myc* genetic background. 32.0% of *ΔRING* mice were alive after 45 weeks, while 8.4% of *WT* mice survived that long; the number of *WT Eμ-Myc* mice still alive was consistent with published data on the comparatively resistant C57BL/6 strain (Sidman et al., 1988). Thus, the survival curve demonstrates that inhibiting RING function by genetic deletion can prolong survival of *Eμ-Myc* mice. Intriguingly, genetic deletion of *XIAP* may also prolong survival on the *Eμ-Myc* background; however, the significance of this observation awaits further monitoring of the colony (Figure 6.1B).

6.4.2 *Decreased incidence of leukemia in* ΔRING *E*μ-Myc *mice*

Peripheral white blood cells progress through characteristic stages in the course of *Eμ-Myc* lymphomagenesis (Sidman et al., 1993). We followed progression through these stages by sampling blood at regular intervals from *WT* and *ΔRING Eμ-Myc* mice. White blood cell (WBC) counts were comparably elevated in young mice of both genotypes (Figure 6.2A). The WBC counts were consistent with those of mice transitioning from the first to second stages of disease in the *Eμ-Myc* model (Sidman et al., 1993). WBC counts returned to lower physiological levels by seven weeks of age in

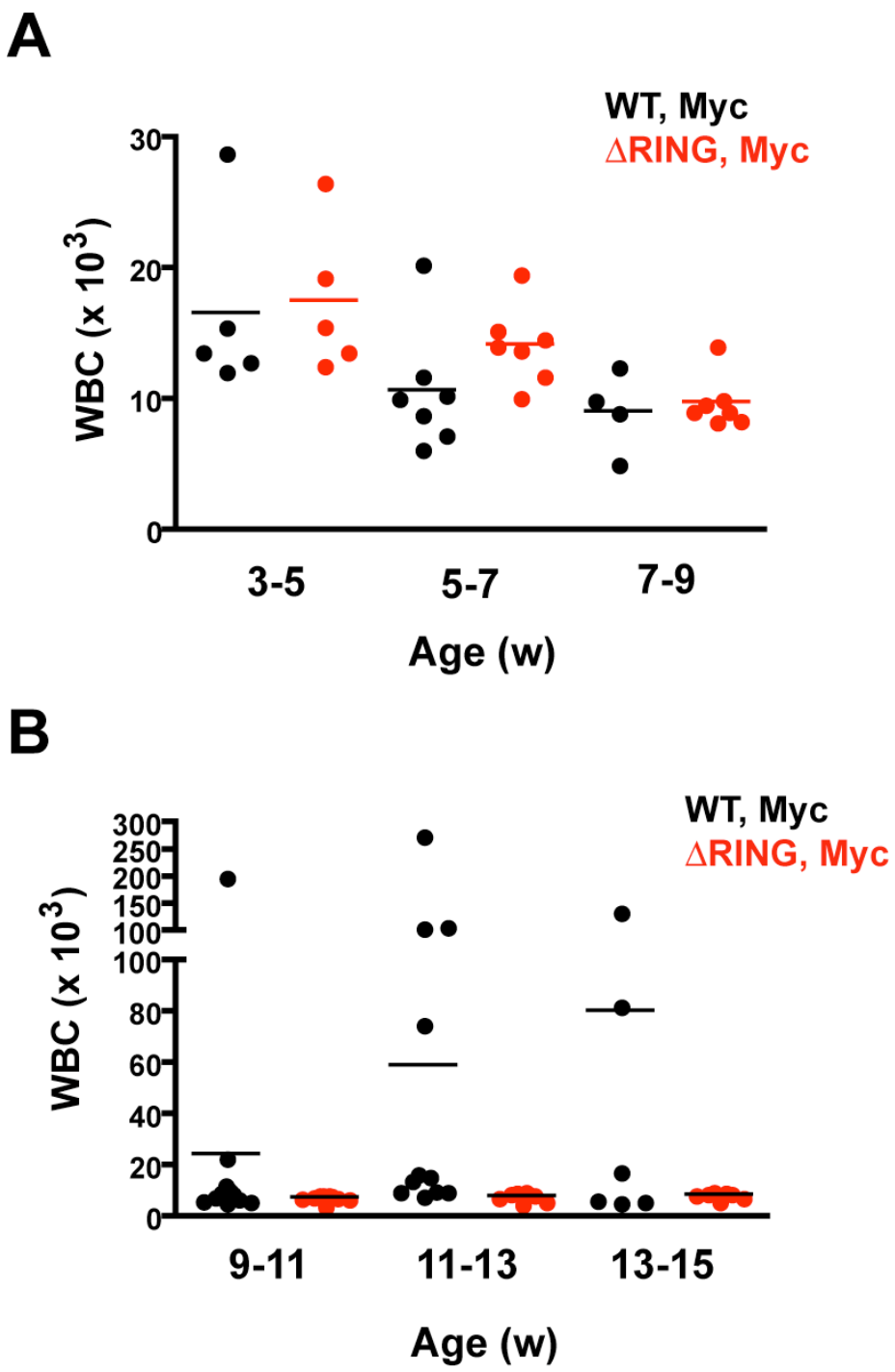


Figure 6.2 Lessened incidence of leukemia in *ΔRING Eμ-Myc* mice. White blood cell counts in peripheral blood from mice of indicated ages. Bars indicate mean counts.

all mice. The following weeks saw some *WT* mice begin to develop pathological WBC counts characteristic of leukemia, whereas *ΔRING* mice maintained consistently low counts over the same period (Figure 6.2B). Large leukocytes typical of lymphoblastic leukemia were noted in peripheral blood from all mice with pathologically elevated WBC counts. Therefore, loss of the XIAP RING curtailed leukemia on the *Eμ-Myc* background.

6.4.3 ΔRING Eμ-Myc *B-cells were sensitized to apoptosis in the bone marrow*

Proliferating B-cells in *Eμ-Myc* mice can be distinguished based on their increased size (Langdon et al., 1986; Sidman et al., 1993). This population of cells co-labels with BrdU, and represents a significantly larger percentage of the *Eμ-Myc* bone marrow compared to nontransgenic mice (Langdon et al., 1986). In fact, the larger size of *Eμ-Myc* lymphocytes can be used to genotype transgenic mice unambiguously (Harris et al., 1988). We isolated bone marrow from *Eμ-Myc* mice and found decreased numbers of large proliferating B-cells in the bone marrow of *ΔRING* transgenic mice of different ages (Figure 6.3A). The percentages of large B-cells in the bone marrow increased with time in mice of both genotypes, but *ΔRING* mice always had fewer large cells. The fraction of TUNEL-positive, apoptotic cells was elevated in the large B-cell population in *ΔRING Eμ-Myc* mice

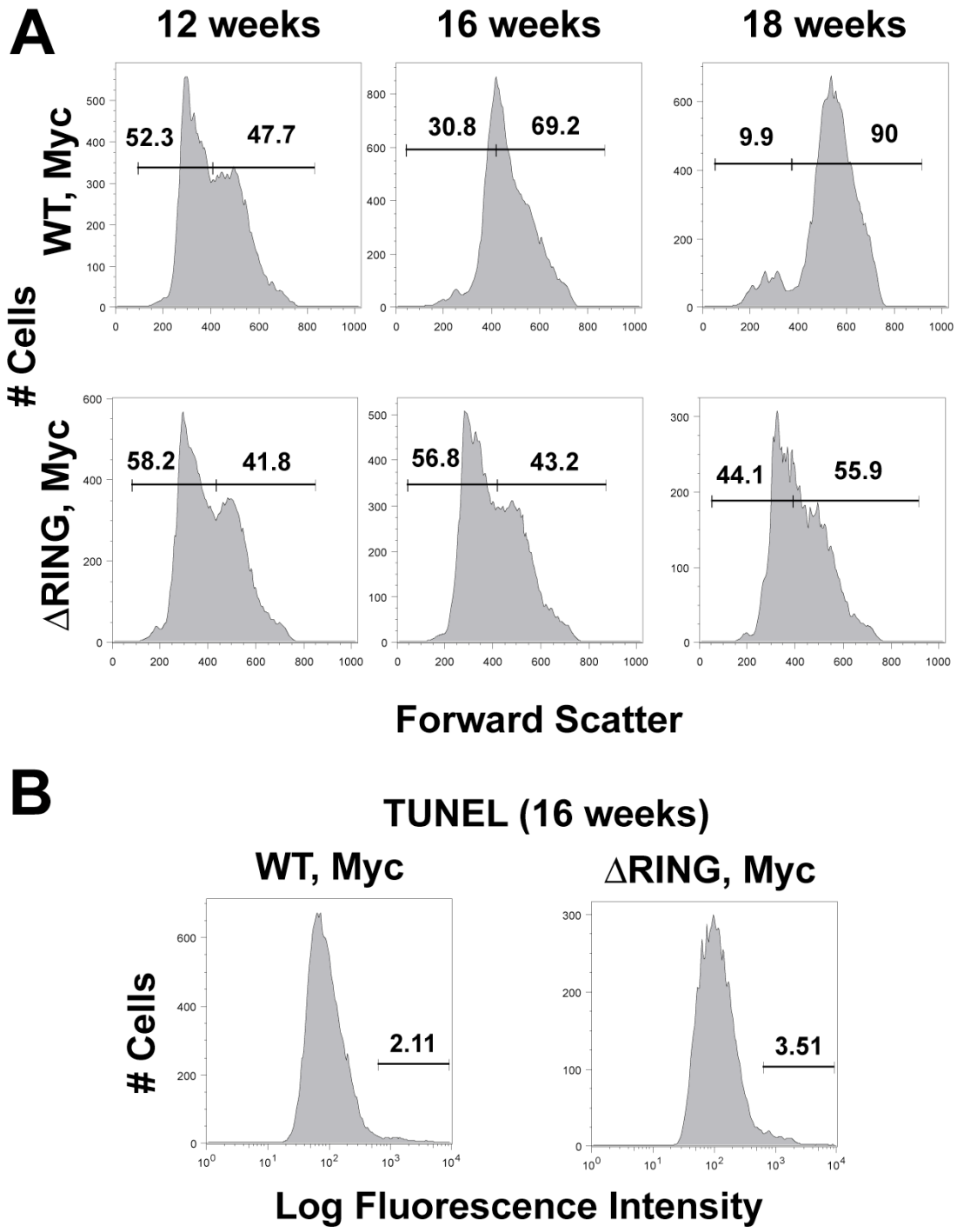


Figure 6.3 Decreased abundance and increased apoptosis of proliferating B-cells in *ΔRING Eμ-Myc* bone marrow. Histograms showing: **(A)** the percentages of small (resting) and large (proliferating) B220+ cells in the bone marrow of age-matched *Eμ-Myc* mice. **(B)** TUNEL in the large-scattering B220+ cell population.

(Figure 6.3B). These findings support the notion that the *ΔRING* mutation constitutes a loss-of-function allele that can promote apoptosis *in vivo*.

6.4.4 *Loss of XIAP RING facilitated apoptosis of cultured* Eμ-Myc *B-cells*

Apoptotic *Eμ-Myc* cells are rapidly cleared by phagocytes *in situ* (Jacobsen et al., 1994). This observation may explain why TUNEL-positive cells were scarce even in the proliferating population of B-cells that undergoes high levels of *Eμ-Myc*-driven apoptosis in the bone marrow (Figure 6.3B). We isolated pure populations of B-cells from the spleens of *Eμ-Myc* mice to characterize apoptosis under cell culture conditions in the absence of phagocytes. Before isolation, the major cell populations in the spleens of *WT* and *ΔRING Eμ-Myc* mice were distributed similarly (Figure 6.4A, B) and consistent with a prior account (Langdon et al., 1986). After purifying B-cells, the isolated cells were nearly pure populations of early B-cells (>95% B220+, CD43-, IgM-) as assessed by flow cytometry. We deprived the purified *Eμ-Myc* B-cells of serum because B-cells that misexpress *Myc* are strongly sensitized to apoptosis caused by growth factor withdrawal (Cherney et al., 1994; Milner et al., 1993). B-cells isolated from *ΔRING* mice showed significantly greater apoptosis after 3.5 hours of serum deprivation in culture (Figure 6.4C; $p < 0.01$ by two-tailed Student's t-test).

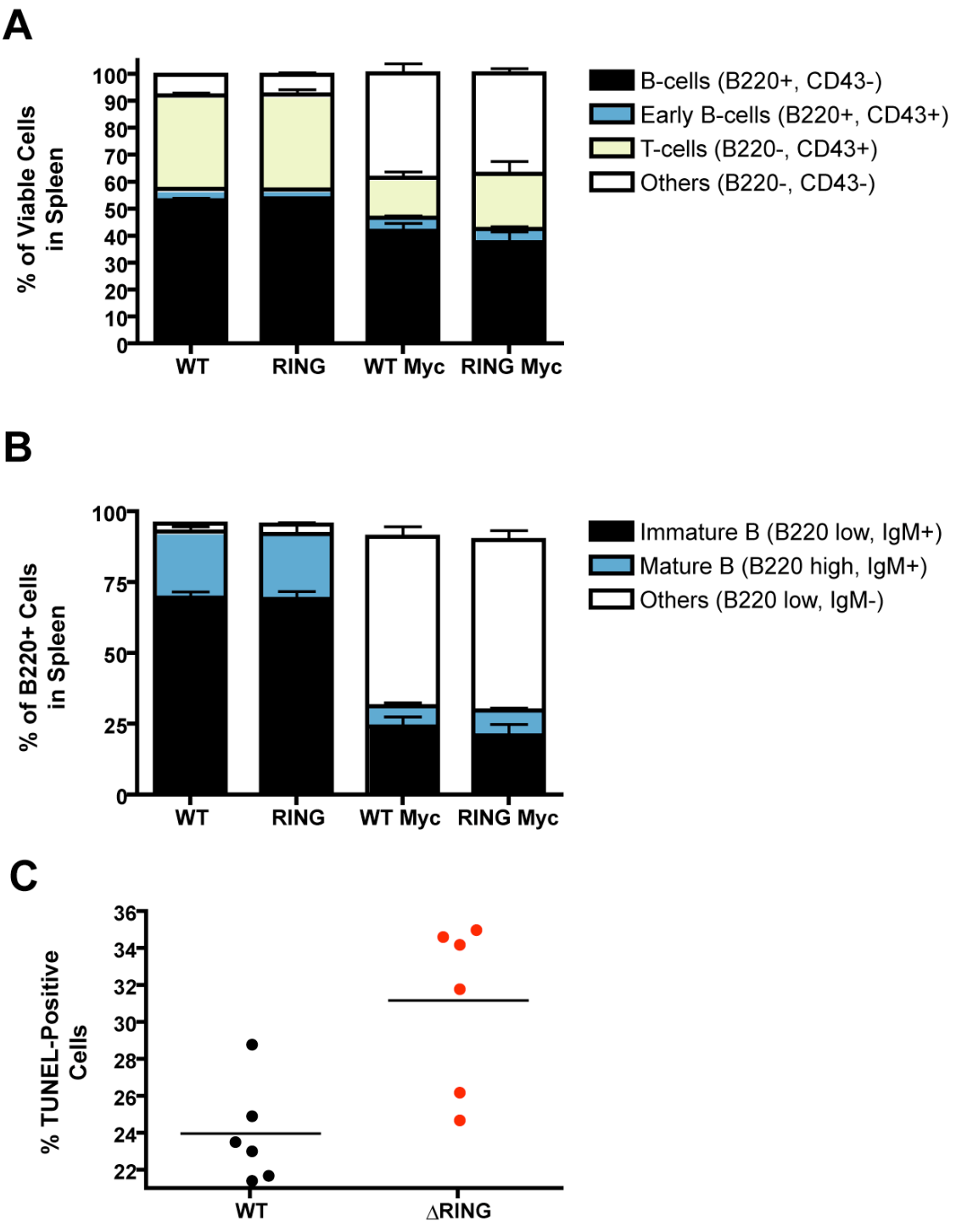


Figure 6.4 *ΔRING Eμ-Myc* B-cells were sensitized to serum withdrawal. **(A)** Compositions of *WT* and *ΔRING Eμ-Myc* spleens as assessed by flow cytometry. **(B)** Distributions of B220+ cells in spleens. **(C)** B-cells purified from spleens were cultured without serum for 3.5 hours before performing TUNEL. Black bars denote mean values.

This result is in line with the elevated apoptosis seen in freshly isolated bone marrow, and it further supports the notion that loss of XIAP RING function sensitizes pre-malignant B-cells to apoptosis.

6.5 DISCUSSION

XIAP has been implicated in human tumorigenesis and lymphoproliferative diseases (Nakagawa et al., 2005; Rigaud et al., 2006; Tamm et al., 2000), and inhibiting XIAP and other IAPs with Smac/DIABLO-derived peptides (Fulda et al., 2002) or small molecule antagonists (Petersen et al., 2007) can reduce tumor xenografts in mice. To our knowledge, there are no reports showing a causative role for XIAP in tumor models. We provide evidence that loss of XIAP RING function synergized with *Eμ-Myc* to enhance survival in a model of lymphoma and leukemia. A prevailing notion holds that *XIAP* overexpression enhances tumor cell survival. Since deleting the RING rendered XIAP a worse inhibitor of caspases, our findings with the *Eμ-Myc* model are consistent with the notion that the *ΔRING* mutation is a loss-of-function allele.

The sensitized *Eμ-Myc* background was necessary to reveal that loss of XIAP RING function has a pro-survival effect in lymphocytes. The increased sensitivity to apoptosis when *Myc* is misexpressed lowers the threshold to caspase activation, in part because of *Myc*-dependent activation

of *ARF* (Eischen et al., 1999). Indeed, improved survival on this background is also seen when *Mdm2* expression is reduced by genetic deletion (Alt et al., 2003). This study also identified decreased numbers of peripheral lymphocytes and increased spontaneous apoptosis in cells cultured without survival factors. *XIAP ΔRING* did not afford as much protection as *Mdm2* deficiency. This is not surprising perhaps because specific p53-dependent apoptosis underlies the death of *Eμ-Myc* cells (Eischen et al., 1999; Schmitt et al., 1999). Presumably, loss of anti-apoptotic *BCL-2* genes would also stimulate apoptosis and improve survival on this background. To our knowledge, this experiment has not been done yet. This would probably require adoptive transfer of bone marrow or lymphoma cells because knockouts of anti-apoptotic *BCL-2* genes result in lethality.

The molecular basis for our phenotype is unclear. The mechanism may arise from decreased caspase ubiquitination during p53-mediated death of *Eμ-Myc* B-cells. This hypothesis is testable, but we were unable to obtain enough input material from our existing *Eμ-Myc* colony to investigate caspase-3 ubiquitination (the caspase-3 immunoprecipitation described in Chapter 4 required 1 mg of input lysate). Enhanced sensitivity to death-receptor apoptosis, seen in *ΔRING* MEFs, may offer some clues into the mechanism of prolonged survival. *Myc* expression sensitizes cells to TRAIL

(Ricci et al., 2007), and loss of the TRAIL (Finnberg et al., 2008) or Fas (Zornig et al., 1995) receptors worsens prognosis on a *Myc* background. Unfortunately, we were unable to test this hypothesis because treatment with TRAIL, Fas, or TNF-α (all with and without cycloheximide) did not raise the levels of apoptosis beyond background levels, even when serum was removed. Others have noted that *Eμ-Myc* cells do not die readily in response to these treatments as well (Finnberg et al., 2008). It remains unclear how death receptors modulate the phenotype of *Eμ-Myc* mice.

It is also possible that the XIAP RING has in indirect role in this context, through RING-dependent interactions with cIAP1. cIAP1 was recently identified as a ubiquitin-ligase that targets Mad1, an inhibitor of Myc, for degradation, thereby enhancing proliferation and colony formation (Xu et al., 2007). This idea is speculative, but perhaps RING-RING interactions normally promote the formation of a XIAP/cIAP1 heterodimer that enhances cIAP1-dependent ubiquitination of Mad1. The absence of XIAP RING could then inhibit cIAP1-mediated turnover of Mad1 and dampen Myc-dependent proliferative signals. This hypothesis is testable by immunoblotting.

7 CONCLUDING DISCUSSION AND IMPLICATIONS FOR FUTURE RESEARCH

7.1 Perspective

Previous efforts to study the regulation of apoptosis by XIAP have largely focused on how BIR domains bind and inhibit caspases, often by relying on overexpression in cancer cell lines and on the biochemical and structural analyses of isolated domains (Hunter et al., 2007). These approaches have offered valuable insights into the molecular mechanisms by which XIAP may regulate caspases, and they also guided the design of small-molecule therapeutics that disrupt BIR/caspase complexes to promote apoptosis in tumors (Carter et al., 2005; Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007). The results from this study emphasize the importance of the RING domain, which bestows E3-ubiqutin ligase activity on XIAP, for the negative regulation of caspases, cell death and tumor suppression by XIAP *in vivo*. The RING may be a promising target for therapeutic intervention in some cancers because genetic removal of this domain limited the abundance of proliferating, pre-malignant B cells and prolonged survival in a mouse model of lymphoma, without adversely affecting lymphoid cells or causing any other overt negative consequences in control mice. Since we also observed elevated spontaneous apoptosis in

transformed *ΔRING* MEFs, these observations may extend to other transformed cells.

7.2 Comparisons between DIAP1 and XIAP RING function

Studies on the DIAP1 RING in *Drosophila* show that cells can use RING ubiquitin-ligase activity to exert both pro- and anti-apoptotic effects. An antiapoptotic role for DIAP1 RING function in healthy cells was revealed using mutant animals: point mutations that inactivate key structural residues in the DIAP1 RING are lethal as advanced embryos (Lisi et al., 2000; Wilson et al., 2002), and a ΔRING truncation causes virtually every embryonic cell to die by apoptosis (Goyal et al., 2000). Point mutations that inactivate RING ubiquitin-ligase function do not preclude DIAP1 from binding the initiator caspase DRONC, but they do prevent polyubiquitination of Dronc (Wilson et al., 2002). The failure to ubiquitinate DRONC has catastrophic consequences in tissues like the wing imaginal disc: DRONC immunostaining increases dramatically, probably through enhanced stability of the protein; effector caspases are activated; and cells die by apoptosis (Ryoo et al., 2002; Ryoo et al., 2004). These experiments describe an essential anti-apoptotic function for the DIAP1 RING in the developing embryo by ubiquitinating DRONC and preventing it from triggering apoptosis in cells that should normally live.

An opposing pro-apoptotic function for the RING has emerged from studies on the DIAP1 antagonist Reaper, which is transcribed only in dying cells. Expression of Reaper depletes DIAP1 through RING-dependent autoubiquitination, and this leads to apoptosis (Ryoo et al., 2002). The case for a pro-apoptotic DIAP1 RING function is further strengthened by genetic interaction studies that show a requirement for Ubcd1, the cognate E2 ubiquitin conjugating enzyme for DIAP1, in Reaper-dependent apoptosis (Ryoo et al., 2002). Collectively, these experiments imply that the outcome and targets of RING-dependent ubiquitination depends on the state of the cell: healthy cells can employ the RING to keep caspases in check, and dying cells engage it to permanently lower the threshold against cell death.

How RING ubiquitin-ligase activity regulates apoptosis in mammalian cells was largely unknown until now. There are certain parallels between *Drosophila* and mammalian IAP RING functions, especially with regard to ubiquitination substrates. The first links between the ubiquitin system and IAP RING function were described in mouse thymocytes (Yang et al., 2000). RING-dependent autoubiquitination and downregulation of XIAP and cIAP1 was proposed as a mechanism explaining why the proteasome is required for thymocyte apoptosis (Yang and Li, 2000). Depletion of IAPs through the ubiquitin system does not

seem to be a universal phenomenon, however, and some cells still die when IAPs are abundantly expressed (ES cells, for example). In thymocytes, increasing the abundance of XIAP by removing the RING did not affect apoptosis, however, so it remains unclear why IAPs are degraded so rapidly by the proteasome in these cells. It is possible that the heightened importance of the ubiquitin system in thymocytes enhances the natural tendency of RING domain IAPs to degrade themselves (Silke et al., 2005). The degradation of other IAPs may be sufficient to lower the inhibitory threshold against cell death in spite of XIAP persistence. Moreover, the factor(s) that actively promotes IAP turnover during thymocyte remain elusive.

Specific inhibition of XIAP is required for cytochrome c-mediated apoptosis in mouse sympathetic neurons, and this occurs by post-translational downregulation in dying cells (Potts et al., 2003). Primary neuronal cultures from *XIAP*-null mice die in response to cytochrome c injection alone, while WT cells are resistant. Remarkably, cultured ΔRING neurons are as sensitive to cytochrome c injection as knockout cells (M. Deshmukh, personal communication). This strengthens the notion that the XIAP RING deletion is a loss-of-function allele, while casting further doubt

on the role of ubiquitin-mediated turnover in promoting apoptosis in primary cells.

In addition to autoubiquitination, XIAP can function as a ubiquitin ligase for most of its binding partners, at least in overexpression and *in vitro* assays. The effects can be both pro- and anti-apoptotic. Ectopically expressed XIAP can promote polyubiquitination of caspases-3, -7 and -9, and this effect can have anti-apoptotic consequences by antagonizing caspase function (Creagh et al., 2004; Morizane et al., 2005; Suzuki et al., 2001). We did not observe appreciable increases in the expression levels of these caspases, however. The XIAP RING functions anti-apoptotically in primary hepatocytes treated with TGF-β by targeting TAK1 for degradation and thereby attenuating apoptosis through the JNK pathway (Kaur et al., 2005). We did not see any altered expression of TAK1 in MEFs during TNF-α/CHX apoptosis, and it will be worthwhile investigating the consequences of the RING mutation in hepatocyte cultures especially since cIAP1 overexpression contributes to hepatocellular carcinoma (Zender et al., 2006). Anti-apoptotic functions for the RING were inferred from the observation that XIAP can polyubiquitinate its antagonist ARTS and target it for degradation (Lotan et al., 2005), though it is unclear if Smac/DIABLO is a ubiquitination substrate as well (Creagh et al., 2004; MacFarlane et al.,

2002). XIAP also acts independently of cell death pathways to influence intracellular copper levels through ubiquitin-dependent regulation of the copper regulatory protein MURR1, and *XIAP*-null fibroblasts show modestly elevated MURR1 protein expression (Burstein et al., 2004).

Since the RING can exert pro- and anti-apoptotic effects, we engineered a *ΔRING* allele to genetically investigate the relative contributions and outcomes of RING activity in primary cells and *in vivo*. Inactivation of the mouse XIAP RING by gene targeting revealed parallels with fruit fly DIAP1 RING function and differences as well. While *DIAP1 RING* mutants die as advanced embryos, *ΔRING* mice are viable on mixed and congenic C57BL/6 backgrounds. This may reflect different regulatory strategies utilized by *Drosophila* and mice. *Drosophila* seems to rely on DIAP1 as a central control point in controlling apoptosis: RING-mediated suppression of DRONC as a key regulatory step to prevent accidental caspase activation (Wilson et al., 2002), and IAP antagonists are absolutely required for apoptosis (White et al., 1994). Many mammalian cells seem to control apoptosis initiation more stringently upstream of mitochondria (Lindsten et al., 2000; Wei et al., 2001) while particular IAPs (XIAP, cIAP1, cIAP2) and IAP antagonists (Smac/DIABLO, Omi/HtrA2, ARTS) are dispensable individually for regulating apoptosis during development. The

lack of strong phenotypes in mice deficient for individual negative regulators is perhaps not surprising, as cell death pathways are almost certainly redundant in comparatively long-lived animals. Furthermore, it is very likely that there are additional negative regulators of apoptosis apart from known caspase inhibitors like IAPs. This is an area of research that merits further investigation.

Nonetheless, we have shown that XIAP RING function influences caspase functions in embryonic cells. Caspase-3 enzyme activity was elevated in *ΔRING* cells in response to diverse apoptotic stimuli and irradiated MEFs were defective in caspase subunit ubiquitination. Unlike *DIAP1 RING* mutant cells, caspase subunit abundance was not dramatically altered in apoptotic *ΔRING* cells, which leaves open the possibility that polyubiquitination may not lead to degradation of caspases. In principle the ubiquitination of active caspase-3 by a functional XIAP RING could impair the enzymatic activity of a caspase or its ability to form functional dimers, although ΔRING cells could tolerate higher caspase activity without increased death when cells were killed through the mitochondrial pathway of apoptosis. WT XIAP was not cleaved much during thymocyte apoptosis, while cleavage products were detected during TNF-α-dependent apoptosis in MEFs. The presence or absence of a polyubiquitin chain may determine if

XIAP can be cleaved by caspases, because XIAP was cleaved by caspases only in situations when it was not very labile.

7.3 IAPs in death receptor signaling

IAPs have diverse regulatory roles in death receptor signaling pathways that have become known recently (Li et al., 2004; Varfolomeev et al., 2007; Vince et al., 2007). Much of this evidence comes from studies with IAP antagonist compounds that can liberate caspase-3 from XIAP and trigger cIAP1/2 autoubiquitination and degradation. IAP antagonists synergize with apoptotic stimuli to induce apoptosis in most human cancer cell lines studied so far, and some cell lines are killed by the compound alone (Petersen et al., 2007). Our findings may offer some important mechanistic insights into how IAP antagonists induce apoptosis. cIAP1/2 are degraded within minutes after IAP antagonist treatment, while XIAP remains relatively stable (Varfolomeev et al., 2007; Vince et al., 2007). The authors describe a mechanism where TNF-α is transcribed through a NF-κB-dependent mechanism (normally inhibited by cIAP1/2), and induces apoptosis through autocrine signaling. While IAP antagonists do not strongly promote XIAP degradation, they do bind XIAP very tightly and prevent it from binding caspase-3 (Li et al., 2004; Vucic and Fairbrother,

2007). This effect may functionally substitute for genetic deletion of *XIAP*, which we have shown to sensitize cells to TNF-α-dependent apoptosis.

The mechanism for TNF-α and TRAIL sensitivity remains elusive. RING ubiquitin-ligases usually function to regulate the abundance of their binding partners, but the differential sensitivity between *WT* and *ΔRING* cells was not obvious from the protein expression levels of key components in the TNF effector pathways. A proteomics effort may be useful to identify proteins that are upregulated in mutant cells during the earliest stages of TNF-α apoptosis signaling. This method may identify misregulated factors, normally kept in lower abundance by XIAP RING-mediated turnover, which may underlie sensitization. Alternatively, the mechanism may be independent from ubiquitination, and may instead arise instead if factors involved in death receptor signaling were mislocalized in the cell. In this regard, a comprehensive examination of the subcellular localizations of key signaling components may offer some insights.

The sensitivity to death receptor apoptosis was not common to all ΔRING cell types, however: hepatocellular apoptosis occurred normally *in vivo* following intravenous administration of agonistic Fas antibody, or TNF-α together with the liver-selective transcriptional inhibitor GalN. A key difference between death receptor apoptosis in MEFs and hepatocytes is

the obligate requirement for mitochondrial factors in hepatocyte apoptosis (Yin et al., 1999), and it is possible that one of these proteins modulates the effect of the *ΔRING* mutation. This may also account for the otherwise normal cell death that occurs in embryonic cells killed by stimuli that act through the mitochondrial pathway of apoptosis.

7.4 Implications for XIAP in tumor suppression and disease

Although *ΔRING* mice appeared as healthy as *WT* littermates, we found an anti-apoptotic role for RING function after crossing mutant mice onto the *Eμ-Myc* transgenic background. The importance of apoptosis in modulating cancer progression in *Eμ-Myc* mice is evident from animals carrying mutations in cell death genes (Schmitt et al., 2002; Strasser et al., 1990). We found small but reproducible increases in apoptosis in the population of proliferating, pre-malignant *ΔRING* B-cells in the bone marrow and in culture, and a general decrease in their relative abundance. This genetic evidence supports the long-standing notion that XIAP antagonism can lead to the death of cancer cells (Hunter et al., 2007).

In principal, it is possible to mimic the effects of the *XIAP ΔRING* mutation by using a small-molecule inhibitor of the RING (Sun, 2003). Our results suggest that such an inhibitor may have therapeutic benefits in

lymphoma and leukemia, and possibly other malignancies. A screen for XIAP RING inhibitors could use methods developed for identifying small-molecule antagonists of the RING domain of Hdm2, the ubiquitin-ligase that suppresses p53 expression in human cells (Davydov et al., 2004; Murray et al., 2007). Hdm2 RING antagonists repress autoubiquitination *in vitro*, increase the abundance of p53 in cells, and can induce apoptosis (Yang et al., 2005). The inhibitors identified to date are neither very specific nor potent; however, the field is in its infancy, and optimized techniques will undoubtedly yield better inhibitors. Another concern about using an XIAP RING inhibitor is raised by the observation that heritable mutations in XIAP underlie a human X-linked lymphoproliferative syndrome, characterized by increased TRAIL and Fas death receptor apoptosis and depletion of natural killer T-lymphocytes (Rigaud et al., 2006). XIAP RING inhibitors may have a therapeutic window similar to bortezomib, a proteasome inhibitor currently in use as a treatment for multiple myeloma; multiple myeloma cells can be killed by apoptosis even by a modest decrease in proteasome activity (Chauhan et al., 2008).

XIAP deficiency does not significantly alter tumor development in a mouse model of prostate cancer (Hwang et al., 2008). This finding suggests that XIAP may not play a key role in the development of all tumors.

However, emerging data implicate IAPs in regulating hematopoietic malignancies, melanoma and hepatocellular carcinoma, among other cancers. The results presented here complement recent findings that genetic deletion of the *Sept4* locus, which encodes the IAP antagonist *ARTS*, dramatically accelerates lymphomagenesis in the *Eμ-Myc* model (M. Garcia-Fernandez, unpublished data). The findings using mouse knockouts are entirely consistent with the hypothesis that XIAP has an anti-apoptotic role in lymphoid malignancy that can be inhibited by antagonists like ARTS. It is very likely that more roles for IAPs will be uncovered in different malignancies. For example, melanocytes upregulate ML-IAP during melanoma development and small-molecule IAP antagonists have entered clinical trials for treatment of this disease.

Complementary roles for IAPs and IAP antagonists are observed in liver cancer as well. cIAP1 gain-of-function has a causative role in hepatocellular carcinoma (Zender et al., 2006), while loss of the ARTS IAP antagonist dramatically predisposes mice to chemically induced carcinogenesis in the liver (C. Pham, unpublished data). It is unclear if XIAP is the principal ARTS target in the liver, but it will be worthwhile investigating the roles of *XIAP* loss-of-function alleles, such as *ΔRING*, in mouse models of liver cancer.

The dramatic sensitivity to TNF-α and TRAIL apoptosis seen in *ΔRING* embryonic fibroblasts may also apply to other tissues and function in the response to inflammation or infection. Although *ΔRING* hepatocytes activated caspase-3 with normal kinetics in response to TNF-α administration, other mouse models of infection or endotoxic shock may reveal sensitivity to apoptotic cytokine responses. TNF-α has a causative role in inflammatory diseases like rheumatoid arthritis, where the inflammatory cytokines originate from TNF-α-stimulated synovial fibroblasts (Fox, 2000). Perhaps the local inhibition of XIAP function in synovial joints by IAP antagonists could terminate the TNF-α signal by inducing apoptosis in fibroblasts, and subvert the long-term inflammatory effects. It will also be worthwhile investigating apoptosis in diverse post-mitotic cell types, since *XIAP*-deficiency or the *ΔRING* mutation predispose sympathetic neurons to apoptosis under culture conditions. Collectively, our data demonstrate that XIAP, and RING function in particular, plays a far greater role in regulating apoptosis *in vivo* than was previously recognized.

8 APPENDICES

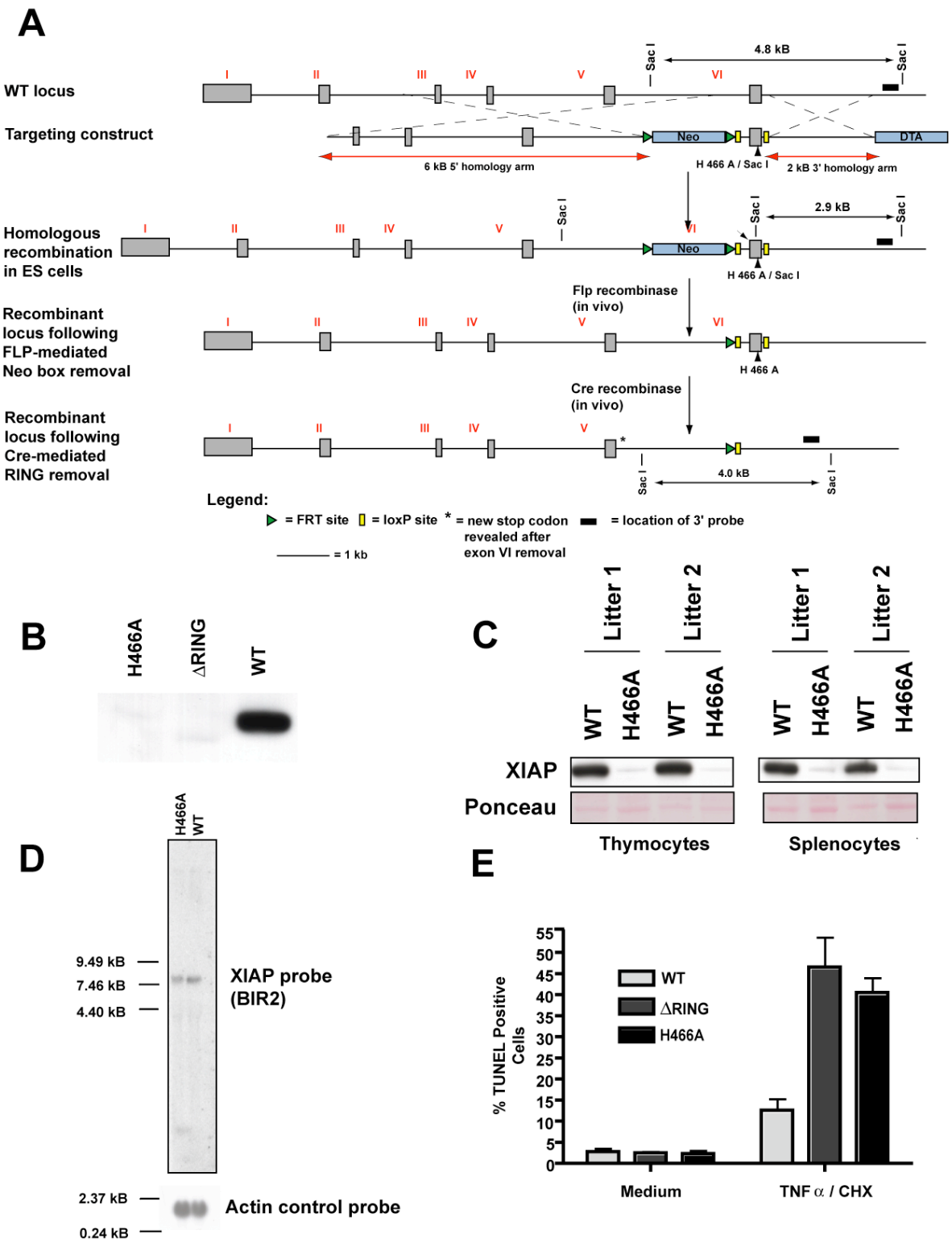
8.1 Antibodies used for immunoblotting

Antibody	Manufacturer / order #	Dilution
Actin	Sigma AC-15	1:20000
Bcl-XL	Cell Signaling 2764	1:1000
Caspase-3 (p17)	Cell Signaling 9661	1:1000
Caspase-3 (p32/17)	Cell Signaling 8G10	1:1000
Caspase-3 (p32/p12)	Gift from Joe Rodriguez	1:3000
Caspase-7	Gift from Joe Rodriguez	1:1000
Caspase-8	Cell Signaling 4927	1:1000
Caspase-8 (cleaved)	Cell Signaling 18C8	1:1000
Caspase-9	Cell Signaling 9504	1:1000
c-FLIP	Cell Signaling 3210	1:1000
cIAP1	R&D AF818	1:2000
FADD	Santa Cruz M-19	1:500
NIK	Santa Cruz H-248	1:500
PARP	BD clone C-2-10	1:2000
Phospho-JNK	Cell Signaling 9251	1:1000
RIP1	Cell Signaling 4926	1:1000
Smac/DIABLO	BD monoclonal #56	1:1000
Survivin	R&D AF886	1:2000
TAK1	Cell Signaling 4505	1:1000
TNF-R1	Santa Cruz H-5	1:500
Total JNK	Santa Cruz sc-571	1:2000
TRAF2	Cell Signaling 4712	1:1000
Ubiquitin	Sigma U5379	1:500
XIAP	BD monoclonal #28	1:1000

8.2 The curious case of the *XIAP H466A/ΔRING* mouse

The *XIAP ΔRING* mouse was our second attempt to make a RING-mutant allele. Our initial approach was to knock-in a single amino acid substitution (H466A) in the RING, thereby removing a residue essential for RING function. We also included the option to remove the domain altogether by flanking the sixth exon (encoding the RING) with *lox*P sites for excision by the Cre recombinase. We positioned *lox*P sites in the final intron and 3' UTR, so that Cre would excise the final exon and the splicing machinery would retain the final intron in the spliced mRNA (Figure A). Excising exon 6 would generate a truncated protein because of a fortuitous in-frame stop codon in the former final intron. All steps of gene targeting were carried out successfully and chimeric mice transmitted the mutant allele through the germline. We did some preliminary experiments with ES cells similar to those described above. We were surprised to find that the XIAP H466A protein was virtually undetectable in ES cells after transient FLPe expression to delete the *Neo* box (Figure B). We also deleted the final exon by expressing Cre recombinase using a self-excising, "hit and run" Cre recombinase retrovirus. Deleting the final exon did in fact generate a truncated protein, but this too was expressed at nearly undetectable levels. Moreover, low XIAP H466A expression was seen in other cells (Figure C).

We obtained identical results after lysing cells in 8 M urea before immunoblotting. Furthermore, XIAP H466A expression was insensitive to proteasome or lysosome inhibitors. We found that the mRNA was expressed normally in MEFs after doing a Northern blot (Figure D). We concluded that the *lox*P site in the 3' UTR was inhibiting translation of the XIAP H466A message, perhaps by interrupting an element necessary for translation or by creating a micro-RNA binding site by accident. Furthermore, the *XIAP H466A* knockdown phenocopies a complete *XIAP*-null in TNF-α-dependent apoptosis (Figure E).



Appendix Figure (A) Gene targeting strategy. Immunoblot of XIAP expression in H466A and ΔRING ES cells **(B)** and thymocytes/splenocytes **(C)**. **(D)** MEF Northern blots. **(E)** TUNEL in MEFs after 8h TNF-α/CHX.

9 REFERENCES

Abraham, M. C., Lu, Y., and Shaham, S. (2007). A morphologically conserved nonapoptotic program promotes linker cell death in Caenorhabditis elegans. Dev Cell *12*, 73-86.

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.

Adams, J. M., Gerondakis, S., Webb, E., Corcoran, L. M., and Cory, S. (1983). Cellular myc oncogene is altered by chromosome translocation to an immunoglobulin locus in murine plasmacytomas and is rearranged similarly in human Burkitt lymphomas. Proc Natl Acad Sci U S A *80*, 1982-1986.

Adams, J. M., Harris, A. W., Pinkert, C. A., Corcoran, L. M., Alexander, W. S., Cory, S., Palmiter, R. D., and Brinster, R. L. (1985). The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. Nature *318*, 533-538.

Alberola-Ila, J., Hogquist, K. A., Swan, K. A., Bevan, M. J., and Perlmutter, R. M. (1996). Positive and negative selection invoke distinct signaling pathways. J Exp Med *184*, 9-18.

Alt, J. R., Greiner, T. C., Cleveland, J. L., and Eischen, C. M. (2003). Mdm2 haplo-insufficiency profoundly inhibits Myc-induced lymphomagenesis. Embo J *22*, 1442-1450.

Ambrosini, G., Adida, C., and Altieri, D. C. (1997). A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med *3*, 917-921.

Arama, E., Agapite, J., and Steller, H. (2003). Caspase activity and a specific cytochrome C are required for sperm differentiation in Drosophila. Dev Cell *4*, 687-697.

Ashwell, J. D., Lu, F. W., and Vacchio, M. S. (2000). Glucocorticoids in T cell development and function*. Annu Rev Immunol *18*, 309-345.

Baens, M., Maes, B., Steyls, A., Geboes, K., Marynen, P., and De Wolf-Peeters, C. (2000). The product of the t(11;18), an API2-MLT fusion, marks

nearly half of gastric MALT type lymphomas without large cell proliferation. Am J Pathol *156*, 1433-1439.

Baird, A. M., Gerstein, R. M., and Berg, L. J. (1999). The role of cytokine receptor signaling in lymphocyte development. Curr Opin Immunol *11*, 157-166.

Beg, A. A., and Baltimore, D. (1996). An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. Science *274*, 782-784.

Bergmann, A., Agapite, J., McCall, K., and Steller, H. (1998). The Drosophila gene hid is a direct molecular target of Ras-dependent survival signaling. Cell *95*, 331-341.

Birnbaum, M. J., Clem, R. J., and Miller, L. K. (1994). An apoptosis-inhibiting gene from a nuclear polyhedrosis virus encoding a polypeptide with Cys/His sequence motifs. J Virol *68*, 2521-2528.

Bloss, T. A., Witze, E. S., and Rothman, J. H. (2003). Suppression of CED-3-independent apoptosis by mitochondrial betaNAC in Caenorhabditis elegans. Nature *424*, 1066-1071.

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

Bouillet, P., Metcalf, D., Huang, D. C., Tarlinton, D. M., Kay, T. W., Kontgen, F., Adams, J. M., and Strasser, A. (1999). Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. Science *286*, 1735-1738.

Bouillet, P., Purton, J. F., Godfrey, D. I., Zhang, L. C., Coultas, L., Puthalakath, H., Pellegrini, M., Cory, S., Adams, J. M., and Strasser, A. (2002). BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. Nature *415*, 922-926.

Brancolini, C., Lazarevic, D., Rodriguez, J., and Schneider, C. (1997). Dismantling cell-cell contacts during apoptosis is coupled to a caspase-dependent proteolytic cleavage of beta-catenin. J Cell Biol *139*, 759-771.

Bratton, S. B., Walker, G., Srinivasula, S. M., Sun, X. M., Butterworth, M., Alnemri, E. S., and Cohen, G. M. (2001). Recruitment, activation and retention of caspases-9 and -3 by Apaf-1 apoptosome and associated XIAP complexes. Embo J *20*, 998-1009.

Brodsky, M. H., Nordstrom, W., Tsang, G., Kwan, E., Rubin, G. M., and Abrams, J. M. (2000). Drosophila p53 binds a damage response element at the reaper locus. Cell *101*, 103-113.

Bubici, C., Papa, S., Pham, C. G., Zazzeroni, F., and Franzoso, G. (2004). NF-kappaB and JNK: an intricate affair. Cell Cycle *3*, 1524-1529.

Burstein, E., Ganesh, L., Dick, R. D., van De Sluis, B., Wilkinson, J. C., Klomp, L. W., Wijmenga, C., Brewer, G. J., Nabel, G. J., and Duckett, C. S. (2004). A novel role for XIAP in copper homeostasis through regulation of MURR1. Embo J *23*, 244-254.

Carlyle, J. R., and Zuniga-Pflucker, J. C. (1998). Requirement for the thymus in alphabeta T lymphocyte lineage commitment. Immunity *9*, 187-197.

Carter, B. Z., Gronda, M., Wang, Z., Welsh, K., Pinilla, C., Andreeff, M., Schober, W. D., Nefzi, A., Pond, G. R., Mawji, I. A.*, et al.* (2005). Small-molecule XIAP inhibitors derepress downstream effector caspases and induce apoptosis of acute myeloid leukemia cells. Blood *105*, 4043-4050.

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. (2001a). Structural basis of caspase-7 inhibition by XIAP. Cell *104*, 769-780.

Chai, J., Wu, Q., Shiozaki, E., Srinivasula, S. M., Alnemri, E. S., and Shi, Y. (2001b). Crystal structure of a procaspase-7 zymogen: mechanisms of activation and substrate binding. Cell *107*, 399-407.

Chai, J., Yan, N., Huh, J. R., Wu, J. W., Li, W., Hay, B. A., and Shi, Y. (2003). Molecular mechanism of Reaper-Grim-Hid-mediated suppression of DIAP1-dependent Dronc ubiquitination. Nat Struct Biol *10*, 892-898.

Chang, L., Kamata, H., Solinas, G., Luo, J. L., Maeda, S., Venuprasad, K., Liu, Y. C., and Karin, M. (2006). The E3 ubiquitin ligase itch couples JNK activation to TNFalpha-induced cell death by inducing c-FLIP(L) turnover. Cell *124*, 601-613.

Chauhan, D., Hideshima, T., and Anderson, K. C. (2008). Targeting proteasomes as therapy in multiple myeloma. Adv Exp Med Biol *615*, 251-260.

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.

Chen, Z., Naito, M., Hori, S., Mashima, T., Yamori, T., and Tsuruo, T. (1999). A human IAP-family gene, apollon, expressed in human brain cancer cells. Biochem Biophys Res Commun *264*, 847-854.

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.

Cherney, B. W., Bhatia, K., and Tosato, G. (1994). A role for deregulated c-Myc expression in apoptosis of Epstein-Barr virus-immortalized B cells. Proc Natl Acad Sci U S A *91*, 12967-12971.

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

Clarke, P. G., and Clarke, S. (1996). Nineteenth century research on naturally occurring cell death and related phenomena. Anat Embryol (Berl) *193*, 81-99.

Clem, R. J., and Miller, L. K. (1994). Control of programmed cell death by the baculovirus genes p35 and iap. Mol Cell Biol *14*, 5212-5222.

Cohen, S., and Levi-Montalcini, R. (1957). Purification and properties of a nerve growth-promoting factor isolated from mouse sarcoma 180. Cancer Res *17*, 15-20.

Coleman, M. L., Sahai, E. A., Yeo, M., Bosch, M., Dewar, A., and Olson, M. F. (2001). Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. Nat Cell Biol *3*, 339-345.

Conte, D., Holcik, M., Lefebvre, C. A., Lacasse, E., Picketts, D. J., Wright, K. E., and Korneluk, R. G. (2006). Inhibitor of apoptosis protein cIAP2 is essential for lipopolysaccharide-induced macrophage survival. Mol Cell Biol *26*, 699-708.

Conte, D., Liston, P., Wong, J. W., Wright, K. E., and Korneluk, R. G. (2001). Thymocyte-targeted overexpression of xiap transgene disrupts T lymphoid apoptosis and maturation. Proc Natl Acad Sci U S A *98*, 5049-5054.

Conze, D. B., Albert, L., Ferrick, D. A., Goeddel, D. V., Yeh, W. C., Mak, T., and Ashwell, J. D. (2005). Posttranscriptional downregulation of c-IAP2 by the ubiquitin protein ligase c-IAP1 in vivo. Mol Cell Biol *25*, 3348-3356.

Cory, S. (1986). Activation of cellular oncogenes in hemopoietic cells by chromosome translocation. Adv Cancer Res *47*, 189-234.

Creagh, E. M., Murphy, B. M., Duriez, P. J., Duckett, C. S., and Martin, S. J. (2004). Smac/Diablo antagonizes ubiquitin ligase activity of inhibitor of apoptosis proteins. J Biol Chem *279*, 26906-26914.

Crocker, S. J., Liston, P., Anisman, H., Lee, C. J., Smith, P. D., Earl, N., Thompson, C. S., Park, D. S., Korneluk, R. G., and Robertson, G. S. (2003). Attenuation of MPTP-induced neurotoxicity and behavioural impairment in NSE-XIAP transgenic mice. Neurobiol Dis *12*, 150-161.

Croft, D. R., Coleman, M. L., Li, S., Robertson, D., Sullivan, T., Stewart, C. L., and Olson, M. F. (2005). Actin-myosin-based contraction is responsible for apoptotic nuclear disintegration. J Cell Biol *168*, 245-255.

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.

Cummings, J., Ranson, M., Lacasse, E., Ganganagari, J. R., St-Jean, M., Jayson, G., Durkin, J., and Dive, C. (2006). Method validation and preliminary qualification of pharmacodynamic biomarkers employed to evaluate the clinical efficacy of an antisense compound (AEG35156)

targeted to the X-linked inhibitor of apoptosis protein XIAP. Br J Cancer *95*, 42-48.

Cummins, J. M., Kohli, M., Rago, C., Kinzler, K. W., Vogelstein, B., and Bunz, F. (2004). X-linked inhibitor of apoptosis protein (XIAP) is a nonredundant modulator of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis in human cancer cells. Cancer Res *64*, 3006-3008.

Dai, Z., Zhu, W. G., Morrison, C. D., Brena, R. M., Smiraglia, D. J., Raval, A., Wu, Y. Z., Rush, L. J., Ross, P., Molina, J. R.*, et al.* (2003). A comprehensive search for DNA amplification in lung cancer identifies inhibitors of apoptosis cIAP1 and cIAP2 as candidate oncogenes. Hum Mol Genet *12*, 791-801.

Davydov, I. V., Woods, D., Safiran, Y. J., Oberoi, P., Fearnhead, H. O., Fang, S., Jensen, J. P., Weissman, A. M., Kenten, J. H., and Vousden, K. H. (2004). Assay for ubiquitin ligase activity: high-throughput screen for inhibitors of HDM2. J Biomol Screen *9*, 695-703.

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. J Cell Biol *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.

Donepudi, M., Mac Sweeney, A., Briand, C., and Grutter, M. G. (2003). Insights into the regulatory mechanism for caspase-8 activation. Mol Cell *11*, 543-549.

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.

Duckett, C. S., Nava, V. E., Gedrich, R. W., Clem, R. J., Van Dongen, J. L., Gilfillan, M. C., Shiels, H., Hardwick, J. M., and Thompson, C. B. (1996). A conserved family of cellular genes related to the baculovirus iap gene and encoding apoptosis inhibitors. Embo J *15*, 2685-2694.

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

Egle, A., Harris, A. W., Bouillet, P., and Cory, S. (2004). Bim is a suppressor of Myc-induced mouse B cell leukemia. Proc Natl Acad Sci U S A *101*, 6164-6169.

Eischen, C. M., Alt, J. R., and Wang, P. (2004). Loss of one allele of ARF rescues Mdm2 haploinsufficiency effects on apoptosis and lymphoma development. Oncogene *23*, 8931-8940.

Eischen, C. M., Weber, J. D., Roussel, M. F., Sherr, C. J., and Cleveland, J. L. (1999). Disruption of the ARF-Mdm2-p53 tumor suppressor pathway in Myc-induced lymphomagenesis. Genes Dev *13*, 2658-2669.

Ellis, H. M., and Horvitz, H. R. (1986). Genetic control of programmed cell death in the nematode C. elegans. Cell *44*, 817-829.

Emamaullee, J. A., Rajotte, R. V., Liston, P., Korneluk, R. G., Lakey, J. R., Shapiro, A. M., and Elliott, J. F. (2005). XIAP overexpression in human islets prevents early posttransplant apoptosis and reduces the islet mass needed to treat diabetes. Diabetes *54*, 2541-2548.

Evan, G. I., Wyllie, A. H., Gilbert, C. S., Littlewood, T. D., Land, H., Brooks, M., Waters, C. M., Penn, L. Z., and Hancock, D. C. (1992). Induction of apoptosis in fibroblasts by c-myc protein. Cell *69*, 119-128.

Farley, F. W., Soriano, P., Steffen, L. S., and Dymecki, S. M. (2000). Widespread recombinase expression using FLPeR (flipper) mice. Genesis *28*, 106-110.

Finnberg, N., Klein-Szanto, A. J., and El-Deiry, W. S. (2008). TRAIL-R deficiency in mice promotes susceptibility to chronic inflammation and tumorigenesis. J Clin Invest *118*, 111-123.

Fox, D. A. (2000). Cytokine blockade as a new strategy to treat rheumatoid arthritis: inhibition of tumor necrosis factor. Arch Intern Med *160*, 437-444.

Fulda, S. (2007). Inhibitor of apoptosis proteins as targets for anticancer therapy. Expert Rev Anticancer Ther *7*, 1255-1264.

Fulda, S., Wick, W., Weller, M., and Debatin, K. M. (2002). Smac agonists sensitize for Apo2L/TRAIL- or anticancer drug-induced apoptosis and induce regression of malignant glioma in vivo. Nat Med *8*, 808-815.

Gaither, A., Porter, D., Yao, Y., Borawski, J., Yang, G., Donovan, J., Sage, D., Slisz, J., Tran, M., Straub, C.*, et al.* (2007). A Smac mimetic rescue screen reveals roles for inhibitor of apoptosis proteins in tumor necrosis factor-alpha signaling. Cancer Res *67*, 11493-11498.

Gao, Z., Tian, Y., Wang, J., Yin, Q., Wu, H., Li, Y. M., and Jiang, X. (2007). A dimeric Smac/diablo peptide directly relieves caspase-3 inhibition by XIAP. Dynamic and cooperative regulation of XIAP by Smac/Diablo. J Biol Chem *282*, 30718-30727.

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

Glickman, M. H., and Ciechanover, A. (2002). The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev *82*, 373-428.

Glucksmann, A. (1951). Cell Deaths in Normal Vertebrate Ontogeny. Biological Reviews *26*, 59-86.

Goldknopf, I. L., and Busch, H. (1975). Remarkable similarities of peptide fingerprints of histone 2A and nonhistone chromosomal protein A24. Biochem Biophys Res Commun *65*, 951-960.

Gottfried, Y., Rotem, A., Lotan, R., Steller, H., and Larisch, S. (2004). The mitochondrial ARTS protein promotes apoptosis through targeting XIAP. Embo J *23*, 1627-1635.

Goyal, L., McCall, K., Agapite, J., Hartwieg, E., and Steller, H. (2000). Induction of apoptosis by Drosophila reaper, hid and grim through inhibition of IAP function. Embo J *19*, 589-597.

Grimm, L. M., Goldberg, A. L., Poirier, G. G., Schwartz, L. M., and Osborne, B. A. (1996). Proteasomes play an essential role in thymocyte apoptosis. Embo J *15*, 3835-3844.

Haining, W. N., Carboy-Newcomb, C., Wei, C. L., and Steller, H. (1999). The proapoptotic function of Drosophila Hid is conserved in mammalian cells. Proc Natl Acad Sci U S A *96*, 4936-4941.

Hakem, R., Hakem, A., Duncan, G. S., Henderson, J. T., Woo, M., Soengas, M. S., Elia, A., de la Pompa, J. L., Kagi, D., Khoo, W.*, et al.* (1998). Differential requirement for caspase 9 in apoptotic pathways in vivo. Cell *94*, 339-352.

Hamburger, V., and Levi-Montalcini, R. (1949). Proliferation, differentiation and degeneration in the spinal ganglia of the chick embryo under normal and experimental conditions. J Exp Zool *111*, 457-501.

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

Hao, Y., Sekine, K., Kawabata, A., Nakamura, H., Ishioka, T., Ohata, H., Katayama, R., Hashimoto, C., Zhang, X., Noda, T.*, et al.* (2004). Apollon ubiquitinates SMAC and caspase-9, and has an essential cytoprotection function. Nat Cell Biol *6*, 849-860.

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.

Hardy, K., Handyside, A. H., and Winston, R. M. (1989). The human blastocyst: cell number, death and allocation during late preimplantation development in vitro. Development *107*, 597-604.

Harlin, H., Reffey, S. B., Duckett, C. S., Lindsten, T., and Thompson, C. B. (2001). Characterization of XIAP-deficient mice. Mol Cell Biol *21*, 3604-3608.

Harrington, E. A., Fanidi, A., and Evan, G. I. (1994). Oncogenes and cell death. Curr Opin Genet Dev *4*, 120-129.

Harris, A. W., Pinkert, C. A., Crawford, M., Langdon, W. Y., Brinster, R. L., and Adams, J. M. (1988). The E mu-myc transgenic mouse. A model for high-incidence spontaneous lymphoma and leukemia of early B cells. J Exp Med *167*, 353-371.

Hartley, S. B., Cooke, M. P., Fulcher, D. A., Harris, A. W., Cory, S., Basten, A., and Goodnow, C. C. (1993). Elimination of self-reactive B lymphocytes proceeds in two stages: arrested development and cell death. Cell *72*, 325-335.

Hengartner, M. O., Ellis, R. E., and Horvitz, H. R. (1992). Caenorhabditis elegans gene ced-9 protects cells from programmed cell death. Nature *356*, 494-499.

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

Hetz, C. A., Torres, V., and Quest, A. F. (2005). Beyond apoptosis: nonapoptotic cell death in physiology and disease. Biochem Cell Biol *83*, 579-588.

Ho, A. T., Li, Q. H., Hakem, R., Mak, T. W., and Zacksenhaus, E. (2004). Coupling of caspase-9 to Apaf1 in response to loss of pRb or cytotoxic drugs is cell-type-specific. Embo J *23*, 460-472.

Ho, A. T., Li, Q. H., Okada, H., Mak, T. W., and Zacksenhaus, E. (2007). XIAP activity dictates Apaf-1 dependency for caspase 9 activation. Mol Cell Biol *27*, 5673-5685.

Hochstrasser, M. (2006). Lingering mysteries of ubiquitin-chain assembly. Cell *124*, 27-34.

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

Holley, C. L., Olson, M. R., Colon-Ramos, D. A., and Kornbluth, S. (2002). Reaper eliminates IAP proteins through stimulated IAP degradation and generalized translational inhibition. Nat Cell Biol *4*, 439-444.

Huesmann, M., Scott, B., Kisielow, P., and von Boehmer, H. (1991). Kinetics and efficacy of positive selection in the thymus of normal and T cell receptor transgenic mice. Cell *66*, 533-540.

Hughes, P. D., Belz, G. T., Fortner, K. A., Budd, R. C., Strasser, A., and Bouillet, P. (2008). Apoptosis regulators Fas and Bim cooperate in

shutdown of chronic immune responses and prevention of autoimmunity. Immunity *28*, 197-205.

Hunter, A. M., LaCasse, E. C., and Korneluk, R. G. (2007). The inhibitors of apoptosis (IAPs) as cancer targets. Apoptosis *12*, 1543-1568.

Hutcheson, J., Scatizzi, J. C., Siddiqui, A. M., Haines, G. K., 3rd, Wu, T., Li, Q. Z., Davis, L. S., Mohan, C., and Perlman, H. (2008). Combined deficiency of proapoptotic regulators Bim and Fas results in the early onset of systemic autoimmunity. Immunity *28*, 206-217.

Hwang, C., Oetjen, K. A., Kosoff, D., Wojno, K. J., Albertelli, M. A., Dunn, R. L., Robins, D. M., Cooney, K. A., and Duckett, C. S. (2008). X-linked inhibitor of apoptosis deficiency in the TRAMP mouse prostate cancer model. Cell Death Differ *15*, 831-840.

Imoto, I., Tsuda, H., Hirasawa, A., Miura, M., Sakamoto, M., Hirohashi, S., and Inazawa, J. (2002). Expression of cIAP1, a target for 11q22 amplification, correlates with resistance of cervical cancers to radiotherapy. Cancer Res *62*, 4860-4866.

Irmler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, K., Steiner, V., Bodmer, J. L., Schroter, M., Burns, K., Mattmann, C.*, et al.* (1997). Inhibition of death receptor signals by cellular FLIP. Nature *388*, 190-195.

Ishizaki, Y., Jacobson, M. D., and Raff, M. C. (1998). A role for caspases in lens fiber differentiation. J Cell Biol *140*, 153-158.

Jacobsen, K. A., Prasad, V. S., Sidman, C. L., and Osmond, D. G. (1994). Apoptosis and macrophage-mediated deletion of precursor B cells in the bone marrow of E mu-myc transgenic mice. Blood *84*, 2784-2794.

Jiang, C., Lamblin, A. F., Steller, H., and Thummel, C. S. (2000). A steroid-triggered transcriptional hierarchy controls salivary gland cell death during Drosophila metamorphosis. Mol Cell *5*, 445-455.

Joazeiro, C. A., and Weissman, A. M. (2000). RING finger proteins: mediators of ubiquitin ligase activity. Cell *102*, 549-552.

Jones, J. M., Datta, P., Srinivasula, S. M., Ji, W., Gupta, S., Zhang, Z., Davies, E., Hajnoczky, G., Saunders, T. L., Van Keuren, M. L.*, et al.* (2003).

Loss of Omi mitochondrial protease activity causes the neuromuscular disorder of mnd2 mutant mice. Nature *425*, 721-727.

Jotereau, F., Heuze, F., Salomon-Vie, V., and Gascan, H. (1987). Cell kinetics in the fetal mouse thymus: precursor cell input, proliferation, and emigration. J Immunol *138*, 1026-1030.

Kamata, H., Honda, S., Maeda, S., Chang, L., Hirata, H., and Karin, M. (2005). Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. Cell *120*, 649-661.

Kappler, J. W., Roehm, N., and Marrack, P. (1987). T cell tolerance by clonal elimination in the thymus. Cell *49*, 273-280.

Karin, M., and Lin, A. (2002). NF-kappaB at the crossroads of life and death. Nat Immunol *3*, 221-227.

Kaur, S., Wang, F., Venkatraman, M., and Arsura, M. (2005). X-linked inhibitor of apoptosis (XIAP) inhibits c-Jun N-terminal kinase 1 (JNK1) activation by transforming growth factor beta1 (TGF-beta1) through ubiquitin-mediated proteosomal degradation of the TGF-beta1-activated kinase 1 (TAK1). J Biol Chem *280*, 38599-38608.

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.

Kiefer, M. C., Brauer, M. J., Powers, V. C., Wu, J. J., Umansky, S. R., Tomei, L. D., and Barr, P. J. (1995). Modulation of apoptosis by the widely distributed Bcl-2 homologue Bak. Nature *374*, 736-739.

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.

Kissel, H., Georgescu, M. M., Larisch, S., Manova, K., Hunnicutt, G. R., and Steller, H. (2005). The Sept4 septin locus is required for sperm terminal differentiation in mice. Dev Cell *8*, 353-364.

Korsmeyer, S. J., Wei, M. C., Saito, M., Weiler, S., Oh, K. J., and Schlesinger, P. H. (2000). Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome c. Cell Death Differ *7*, 1166-1173.

Kovalchuk, A. L., Qi, C. F., Torrey, T. A., Taddesse-Heath, L., Feigenbaum, L., Park, S. S., Gerbitz, A., Klobeck, G., Hoertnagel, K., Polack, A.*, et al.* (2000). Burkitt lymphoma in the mouse. J Exp Med *192*, 1183-1190.

Kozopas, K. M., Yang, T., Buchan, H. L., Zhou, P., and Craig, R. W. (1993). MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. Proc Natl Acad Sci U S A *90*, 3516-3520.

Krammer, P. H. (2000). CD95's deadly mission in the immune system. Nature *407*, 789-795.

Kreuz, S., Siegmund, D., Scheurich, P., and Wajant, H. (2001). NF-kappaB inducers upregulate cFLIP, a cycloheximide-sensitive inhibitor of death receptor signaling. Mol Cell Biol *21*, 3964-3973.

Kuida, K., Haydar, T. F., Kuan, C. Y., Gu, Y., Taya, C., Karasuyama, H., Su, M. S., Rakic, P., and Flavell, R. A. (1998). Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. Cell *94*, 325-337.

Kuida, K., Zheng, T. S., Na, S., Kuan, C., Yang, D., Karasuyama, H., Rakic, P., and Flavell, R. A. (1996). Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature *384*, 368-372.

Kurada, P., and White, K. (1998). Ras promotes cell survival in Drosophila by downregulating hid expression. Cell *95*, 319-329.

LaCasse, E. C., Baird, S., Korneluk, R. G., and MacKenzie, A. E. (1998). The inhibitors of apoptosis (IAPs) and their emerging role in cancer. Oncogene *17*, 3247-3259.

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.

Langdon, W. Y., Harris, A. W., and Cory, S. (1988). Growth of E mu-myc transgenic B-lymphoid cells in vitro and their evolution toward autonomy. Oncogene Res *3*, 271-279.

Langdon, W. Y., Harris, A. W., Cory, S., and Adams, J. M. (1986). The c-myc oncogene perturbs B lymphocyte development in E-mu-myc transgenic mice. Cell *47*, 11-18.

Larisch, S., Yi, Y., Lotan, R., Kerner, H., Eimerl, S., Tony Parks, W., Gottfried, Y., Birkey Reffey, S., de Caestecker, M. P., Danielpour, D.*, et al.* (2000). A novel mitochondrial septin-like protein, ARTS, mediates apoptosis dependent on its P-loop motif. Nat Cell Biol *2*, 915-921.

Lavrik, I., Krueger, A., Schmitz, I., Baumann, S., Weyd, H., Krammer, P. H., and Kirchhoff, S. (2003). The active caspase-8 heterotetramer is formed at the CD95 DISC. Cell Death Differ *10*, 144-145.

Leist, M., Gantner, F., Bohlinger, I., Germann, P. G., Tiegs, G., and Wendel, A. (1994). Murine hepatocyte apoptosis induced in vitro and in vivo by TNF-alpha requires transcriptional arrest. J Immunol *153*, 1778-1788.

Lens, S. M., Vader, G., and Medema, R. H. (2006). The case for Survivin as mitotic regulator. Curr Opin Cell Biol *18*, 616-622.

Leonard, J. R., Klocke, B. J., D'Sa, C., Flavell, R. A., and Roth, K. A. (2002). Strain-dependent neurodevelopmental abnormalities in caspase-3-deficient mice. J Neuropathol Exp Neurol *61*, 673-677.

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.

Levi-Montalcini, R., and Hamburger, V. (1951). Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. J Exp Zool *116*, 321-361.

Lewis, J., Burstein, E., Reffey, S. B., Bratton, S. B., Roberts, A. B., and Duckett, C. S. (2004). Uncoupling of the signaling and caspase-inhibitory properties of X-linked inhibitor of apoptosis. J Biol Chem *279*, 9023-9029.

Lewis, S. M., and Holcik, M. (2005). IRES in distress: translational regulation of the inhibitor of apoptosis proteins XIAP and HIAP2 during cell stress. Cell Death Differ *12*, 547-553.

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, P., Allen, H., Banerjee, S., Franklin, S., Herzog, L., Johnston, C., McDowell, J., Paskind, M., Rodman, L., Salfeld, J., and et al. (1995). Mice deficient in IL-1 beta-converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock. Cell *80*, 401-411.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell *91*, 479-489.

Li, X., Yang, Y., and Ashwell, J. D. (2002). TNF-RII and c-IAP1 mediate ubiquitination and degradation of TRAF2. Nature *416*, 345-347.

Lin, Y., Devin, A., Rodriguez, Y., and Liu, Z. G. (1999). Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. Genes Dev *13*, 2514-2526.

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.

Lisi, S., Mazzon, I., and White, K. (2000). Diverse domains of THREAD/DIAP1 are required to inhibit apoptosis induced by REAPER and HID in Drosophila. Genetics *154*, 669-678.

Liu, J., Minemoto, Y., and Lin, A. (2004). c-Jun N-terminal protein kinase 1 (JNK1), but not JNK2, is essential for tumor necrosis factor alpha-induced c-Jun kinase activation and apoptosis. Mol Cell Biol *24*, 10844-10856.

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.

Liu, X., Zou, H., Slaughter, C., and Wang, X. (1997). DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell *89*, 175-184.

Liu, Z., Sun, C., Olejniczak, E. T., Meadows, R. P., Betz, S. F., Oost, T., Herrmann, J., Wu, J. C., and Fesik, S. W. (2000). Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain. Nature *408*, 1004-1008.

Lockshin, R. A., and Williams, C. M. (1965). Programmed cell death. IV. The influence of drugs on the breakdown of the intersegmental muscles of silkmoths. J Insect Physiol *11*, 803-809.

Lohmann, I., McGinnis, N., Bodmer, M., and McGinnis, W. (2002). The Drosophila Hox gene deformed sculpts head morphology via direct regulation of the apoptosis activator reaper. Cell *110*, 457-466.

Lotan, R., Rotem, A., Gonen, H., Finberg, J. P., Kemeny, S., Steller, H., Ciechanover, A., and Larisch, S. (2005). Regulation of the proapoptotic ARTS protein by ubiquitin-mediated degradation. J Biol Chem *280*, 25802-25810.

Lotz, K., Pyrowolakis, G., and Jentsch, S. (2004). BRUCE, a giant E2/E3 ubiquitin ligase and inhibitor of apoptosis protein of the trans-Golgi network, is required for normal placenta development and mouse survival. Mol Cell Biol *24*, 9339-9350.

Lu, M., Lin, S. C., Huang, Y., Kang, Y. J., Rich, R., Lo, Y. C., Myszka, D., Han, J., and Wu, H. (2007). XIAP induces NF-kappaB activation via the BIR1/TAB1 interaction and BIR1 dimerization. Mol Cell *26*, 689-702.

Luthi, A. U., and Martin, S. J. (2007). The CASBAH: a searchable database of caspase substrates. Cell Death Differ *14*, 641-650.

MacFarlane, M., Merrison, W., Bratton, S. B., and Cohen, G. M. (2002). Proteasome-mediated degradation of Smac during apoptosis: XIAP promotes Smac ubiquitination in vitro. J Biol Chem *277*, 36611-36616.

Maraskovsky, E., O'Reilly, L. A., Teepe, M., Corcoran, L. M., Peschon, J. J., and Strasser, A. (1997). Bcl-2 can rescue T lymphocyte development in interleukin-7 receptor-deficient mice but not in mutant rag-1-/- mice. Cell *89*, 1011-1019.

Martins, L. M., Morrison, A., Klupsch, K., Fedele, V., Moisoi, N., Teismann, P., Abuin, A., Grau, E., Geppert, M., Livi, G. P.*, et al.* (2004). Neuroprotective role of the Reaper-related serine protease HtrA2/Omi revealed by targeted deletion in mice. Mol Cell Biol *24*, 9848-9862.

Mattson, M. P. (2000). Apoptosis in neurodegenerative disorders. Nat Rev Mol Cell Biol *1*, 120-129.

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

McDonnell, T. J., and Korsmeyer, S. J. (1991). Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14; 18). Nature *349*, 254-256.

Medema, J. P., Scaffidi, C., Kischkel, F. C., Shevchenko, A., Mann, M., Krammer, P. H., and Peter, M. E. (1997). FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). Embo J *16*, 2794-2804.

Melchers, F., ten Boekel, E., Seidl, T., Kong, X. C., Yamagami, T., Onishi, K., Shimizu, T., Rolink, A. G., and Andersson, J. (2000). Repertoire selection by pre-B-cell receptors and B-cell receptors, and genetic control of B-cell development from immature to mature B cells. Immunol Rev *175*, 33-46.

Merkenschlager, M., Graf, D., Lovatt, M., Bommhardt, U., Zamoyska, R., and Fisher, A. G. (1997). How many thymocytes audition for selection? J Exp Med *186*, 1149-1158.

Metzstein, M. M., Stanfield, G. M., and Horvitz, H. R. (1998). Genetics of programmed cell death in C. elegans: past, present and future. Trends Genet *14*, 410-416.

Micheau, O., and Tschopp, J. (2003). Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell *114*, 181-190.

Miller, L. K. (1999). An exegesis of IAPs: salvation and surprises from BIR motifs. Trends Cell Biol *9*, 323-328.

Milner, A. E., Grand, R. J., Waters, C. M., and Gregory, C. D. (1993). Apoptosis in Burkitt lymphoma cells is driven by c-myc. Oncogene *8*, 3385-3391.

Morizane, Y., Honda, R., Fukami, K., and Yasuda, H. (2005). X-linked inhibitor of apoptosis functions as ubiquitin ligase toward mature caspase-9 and cytosolic Smac/DIABLO. J Biochem *137*, 125-132.

Murphy, B. M., Creagh, E. M., and Martin, S. J. (2004). Interchain proteolysis, in the absence of a dimerization stimulus, can initiate apoptosis-associated caspase-8 activation. J Biol Chem *279*, 36916-36922.

Murray, M. F., Jurewicz, A. J., Martin, J. D., Ho, T. F., Zhang, H., Johanson, K. O., Kirkpatrick, R. B., Ma, J., Lor, L. A., Thrall, S. H., and Schwartz, B. (2007). A high-throughput screen measuring ubiquitination of p53 by human mdm2. J Biomol Screen *12*, 1050-1058.

Nagy, A. (2003). Manipulating the Mouse Embryo: Cold Spring Harbor Laboratory Press).

Nahle, Z., Polakoff, J., Davuluri, R. V., McCurrach, M. E., Jacobson, M. D., Narita, M., Zhang, M. Q., Lazebnik, Y., Bar-Sagi, D., and Lowe, S. W. (2002). Direct coupling of the cell cycle and cell death machinery by E2F. Nat Cell Biol *4*, 859-864.

Nakagawa, Y., Hasegawa, M., Kurata, M., Yamamoto, K., Abe, S., Inoue, M., Takemura, T., Hirokawa, K., Suzuki, K., and Kitagawa, M. (2005). Expression of IAP-family proteins in adult acute mixed lineage leukemia (AMLL). Am J Hematol *78*, 173-180.

Nakano, K., and Vousden, K. H. (2001). PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell *7*, 683-694.

Nicholson, D. W. (1999). Caspase structure, proteolytic substrates, and function during apoptotic cell death. Cell Death Differ *6*, 1028-1042.

Nieminen, A. I., Partanen, J. I., Hau, A., and Klefstrom, J. (2007). c-Myc primed mitochondria determine cellular sensitivity to TRAIL-induced apoptosis. Embo J *26*, 1055-1067.

Nijhawan, D., Fang, M., Traer, E., Zhong, Q., Gao, W., Du, F., and Wang, X. (2003). Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation. Genes Dev *17*, 1475-1486.

Nordstrom, W., Chen, P., Steller, H., and Abrams, J. M. (1996). Activation of the reaper gene during ectopic cell killing in Drosophila. Dev Biol *180*, 213-226.

Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T., and Tanaka, N. (2000). Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science *288*, 1053-1058.

Ogasawara, J., Watanabe-Fukunaga, R., Adachi, M., Matsuzawa, A., Kasugai, T., Kitamura, Y., Itoh, N., Suda, T., and Nagata, S. (1993). Lethal effect of the anti-Fas antibody in mice. Nature *364*, 806-809.

Okada, H., Suh, W. K., Jin, J., Woo, M., Du, C., Elia, A., Duncan, G. S., Wakeham, A., Itie, A., Lowe, S. W.*, et al.* (2002). Generation and characterization of Smac/DIABLO-deficient mice. Mol Cell Biol *22*, 3509-3517.

Olayioye, M. A., Kaufmann, H., Pakusch, M., Vaux, D. L., Lindeman, G. J., and Visvader, J. E. (2005). XIAP-deficiency leads to delayed lobuloalveolar development in the mammary gland. Cell Death Differ *12*, 87-90.

Oltvai, Z. N., Milliman, C. L., and Korsmeyer, S. J. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell *74*, 609-619.

Opferman, J. T. (2007). Life and death during hematopoietic differentiation. Curr Opin Immunol *19*, 497-502.

Opferman, J. T., Letai, A., Beard, C., Sorcinelli, M. D., Ong, C. C., and Korsmeyer, S. J. (2003). Development and maintenance of B and T lymphocytes requires antiapoptotic MCL-1. Nature *426*, 671-676.

Overholtzer, M., Mailleux, A. A., Mouneimne, G., Normand, G., Schnitt, S. J., King, R. W., Cibas, E. S., and Brugge, J. S. (2007). A nonapoptotic cell death process, entosis, that occurs by cell-in-cell invasion. Cell *131*, 966-979.

Perrelet, D., Ferri, A., Liston, P., Muzzin, P., Korneluk, R. G., and Kato, A. C. (2002). IAPs are essential for GDNF-mediated neuroprotective effects in injured motor neurons in vivo. Nat Cell Biol *4*, 175-179.

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.

Pickart, C. M., and Eddins, M. J. (2004). Ubiquitin: structures, functions, mechanisms. Biochim Biophys Acta *1695*, 55-72.

Pohl, C., and Jentsch, S. (2008). Final stages of cytokinesis and midbody ring formation are controlled by BRUCE. Cell *132*, 832-845.

Potts, M. B., Vaughn, A. E., McDonough, H., Patterson, C., and Deshmukh, M. (2005). Reduced Apaf-1 levels in cardiomyocytes engage strict regulation of apoptosis by endogenous XIAP. J Cell Biol *171*, 925-930.

Potts, P. R., Singh, S., Knezek, M., Thompson, C. B., and Deshmukh, M. (2003). Critical function of endogenous XIAP in regulating caspase activation during sympathetic neuronal apoptosis. J Cell Biol *163*, 789-799.

Puthalakath, H., Villunger, A., O'Reilly, L. A., Beaumont, J. G., Coultas, L., Cheney, R. E., Huang, D. C., and Strasser, A. (2001). Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. Science *293*, 1829-1832.

Rathmell, J. C., and Thompson, C. B. (2002). Pathways of apoptosis in lymphocyte development, homeostasis, and disease. Cell *109 Suppl*, S97-107.

Ren, J., Shi, M., Liu, R., Yang, Q. H., Johnson, T., Skarnes, W. C., and Du, C. (2005). The Birc6 (Bruce) gene regulates p53 and the mitochondrial pathway of apoptosis and is essential for mouse embryonic development. Proc Natl Acad Sci U S A *102*, 565-570.

Ricci, M. S., Kim, S. H., Ogi, K., Plastaras, J. P., Ling, J., Wang, W., Jin, Z., Liu, Y. Y., Dicker, D. T., Chiao, P. J.*, et al.* (2007). Reduction of TRAIL-induced Mcl-1 and cIAP2 by c-Myc or sorafenib sensitizes resistant human cancer cells to TRAIL-induced death. Cancer Cell *12*, 66-80.

Richter, B. W., Mir, S. S., Eiben, L. J., Lewis, J., Reffey, S. B., Frattini, A., Tian, L., Frank, S., Youle, R. J., Nelson, D. L.*, et al.* (2001). Molecular cloning of ILP-2, a novel member of the inhibitor of apoptosis protein family. Mol Cell Biol *21*, 4292-4301.

Riedl, S. J., Li, W., Chao, Y., Schwarzenbacher, R., and Shi, Y. (2005). Structure of the apoptotic protease-activating factor 1 bound to ADP. Nature *434*, 926-933.

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

Rigaud, S., Fondaneche, M. C., Lambert, N., Pasquier, B., Mateo, V., Soulas, P., Galicier, L., Le Deist, F., Rieux-Laucat, F., Revy, P.*, et al.* (2006). XIAP deficiency in humans causes an X-linked lymphoproliferative syndrome. Nature *444*, 110-114.

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

Rothe, M., Pan, M. G., Henzel, W. J., Ayres, T. M., and Goeddel, D. V. (1995). The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. Cell *83*, 1243-1252.

Rothstein, T. L., Wang, J. K., Panka, D. J., Foote, L. C., Wang, Z., Stanger, B., Cui, H., Ju, S. T., and Marshak-Rothstein, A. (1995). Protection against Fas-dependent Th1-mediated apoptosis by antigen receptor engagement in B cells. Nature *374*, 163-165.

Ryoo, H. D., Bergmann, A., Gonen, H., Ciechanover, A., and Steller, H. (2002). Regulation of Drosophila IAP1 degradation and apoptosis by reaper and ubcD1. Nat Cell Biol *4*, 432-438.

Ryoo, H. D., Gorenc, T., and Steller, H. (2004). Apoptotic cells can induce compensatory cell proliferation through the JNK and the Wingless signaling pathways. Dev Cell *7*, 491-501.

Sakahira, H., Enari, M., and Nagata, S. (1998). Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature *391*, 96-99.

Salmena, L., and Hakem, R. (2005). Caspase-8 deficiency in T cells leads to a lethal lymphoinfiltrative immune disorder. J Exp Med *202*, 727-732.

Salvesen, G. S., and Duckett, C. S. (2002). IAP proteins: blocking the road to death's door. Nat Rev Mol Cell Biol *3*, 401-410.

Sattler, M., Liang, H., Nettesheim, D., Meadows, R. P., Harlan, J. E., Eberstadt, M., Yoon, H. S., Shuker, S. B., Chang, B. S., Minn, A. J.*, et al.* (1997). Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. Science *275*, 983-986.

Saunders, J. W., Jr. (1966). Death in embryonic systems. Science *154*, 604-612.

Schmitt, C. A., Fridman, J. S., Yang, M., Baranov, E., Hoffman, R. M., and Lowe, S. W. (2002). Dissecting p53 tumor suppressor functions in vivo. Cancer Cell *1*, 289-298.

Schmitt, C. A., McCurrach, M. E., de Stanchina, E., Wallace-Brodeur, R. R., and Lowe, S. W. (1999). INK4a/ARF mutations accelerate lymphomagenesis and promote chemoresistance by disabling p53. Genes Dev *13*, 2670-2677.

Schumacher, B., Schertel, C., Wittenburg, N., Tuck, S., Mitani, S., Gartner, A., Conradt, B., and Shaham, S. (2005). C. elegans ced-13 can promote apoptosis and is induced in response to DNA damage. Cell Death Differ *12*, 153-161.

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.

Seshagiri, S., and Miller, L. K. (1997a). Baculovirus inhibitors of apoptosis (IAPs) block activation of Sf-caspase-1. Proc Natl Acad Sci U S A *94*, 13606-13611.

Seshagiri, S., and Miller, L. K. (1997b). Caenorhabditis elegans CED-4 stimulates CED-3 processing and CED-3-induced apoptosis. Curr Biol *7*, 455-460.

Shaham, S., and Horvitz, H. R. (1996a). An alternatively spliced C. elegans ced-4 RNA encodes a novel cell death inhibitor. Cell *86*, 201-208.

Shaham, S., and Horvitz, H. R. (1996b). Developing Caenorhabditis elegans neurons may contain both cell-death protective and killer activities. Genes Dev *10*, 578-591.

Shi, Y. (2002a). A conserved tetrapeptide motif: potentiating apoptosis through IAP-binding. Cell Death Differ *9*, 93-95.

Shi, Y. (2002b). Mechanisms of caspase activation and inhibition during apoptosis. Mol Cell *9*, 459-470.

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.

Shortman, K., Egerton, M., Spangrude, G. J., and Scollay, R. (1990). The generation and fate of thymocytes. Semin Immunol *2*, 3-12.

Sidman, C. L., Marshall, J. D., and Harris, A. W. (1988). Genetic studies on Emu-myc transgenic mice. Curr Top Microbiol Immunol *141*, 94-99.

Sidman, C. L., Shaffer, D. J., Jacobsen, K., Vargas, S. R., and Osmond, D. G. (1993). Cell populations during tumorigenesis in Eu-myc transgenic mice. Leukemia *7*, 887-895.

Siegel, R. M., Frederiksen, J. K., Zacharias, D. A., Chan, F. K., Johnson, M., Lynch, D., Tsien, R. Y., and Lenardo, M. J. (2000). Fas preassociation required for apoptosis signaling and dominant inhibition by pathogenic mutations. Science *288*, 2354-2357.

Silke, J., Hawkins, C. J., Ekert, P. G., Chew, J., Day, C. L., Pakusch, M., Verhagen, A. M., and Vaux, D. L. (2002). The anti-apoptotic activity of XIAP is retained upon mutation of both the caspase 3- and caspase 9-interacting sites. J Cell Biol *157*, 115-124.

Silke, J., Kratina, T., Chu, D., Ekert, P. G., Day, C. L., Pakusch, M., Huang, D. C., and Vaux, D. L. (2005). Determination of cell survival by RING-

mediated regulation of inhibitor of apoptosis (IAP) protein abundance. Proc Natl Acad Sci U S A *102*, 16182-16187.

Silke, J., Kratina, T., Ekert, P. G., Pakusch, M., and Vaux, D. L. (2004). Unlike Diablo/smac, Grim promotes global ubiquitination and specific degradation of X chromosome-linked inhibitor of apoptosis (XIAP) and neither cause apoptosis. J Biol Chem *279*, 4313-4321.

Sordet, O., Rebe, C., Plenchette, S., Zermati, Y., Hermine, O., Vainchenker, W., Garrido, C., Solary, E., and Dubrez-Daloz, L. (2002). Specific involvement of caspases in the differentiation of monocytes into macrophages. Blood *100*, 4446-4453.

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

Steller, H., Abrams, J. M., Grether, M. E., and White, K. (1994). Programmed cell death in Drosophila. Philos Trans R Soc Lond B Biol Sci *345*, 247-250.

Strasser, A., Harris, A. W., Bath, M. L., and Cory, S. (1990). Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. Nature *348*, 331-333.

Strasser, A., Harris, A. W., von Boehmer, H., and Cory, S. (1994). Positive and negative selection of T cells in T-cell receptor transgenic mice expressing a bcl-2 transgene. Proc Natl Acad Sci U S A *91*, 1376-1380.

Suda, T., Takahashi, T., Golstein, P., and Nagata, S. (1993). Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. Cell *75*, 1169-1178.

Sulston, J. E., and Horvitz, H. R. (1977). Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. Dev Biol *56*, 110-156.

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

Sun, H., Nikolovska-Coleska, Z., Lu, J., Qiu, S., Yang, C. Y., Gao, W., Meagher, J., Stuckey, J., and Wang, S. (2006). Design, synthesis, and evaluation of a potent, cell-permeable, conformationally constrained second mitochondria derived activator of caspase (Smac) mimetic. J Med Chem *49*, 7916-7920.

Sun, X. M., Butterworth, M., MacFarlane, M., Dubiel, W., Ciechanover, A., and Cohen, G. M. (2004). Caspase activation inhibits proteasome function during apoptosis. Mol Cell *14*, 81-93.

Sun, Y. (2003). Targeting E3 ubiquitin ligases for cancer therapy. Cancer Biol Ther *2*, 623-629.

Surh, C. D., and Sprent, J. (1994). T-cell apoptosis detected in situ during positive and negative selection in the thymus. Nature *372*, 100-103.

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., Nakabayashi, Y., and Takahashi, R. (2001). Ubiquitin-protein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase-3 and enhances its anti-apoptotic effect in Fas-induced cell death. Proc Natl Acad Sci U S A *98*, 8662-8667.

Takahashi, A., Musy, P. Y., Martins, L. M., Poirier, G. G., Moyer, R. W., and Earnshaw, W. C. (1996). CrmA/SPI-2 inhibition of an endogenous ICE-related protease responsible for lamin A cleavage and apoptotic nuclear fragmentation. J Biol Chem *271*, 32487-32490.

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. J Biol Chem *273*, 7787-7790.

Takeuchi, O., Fisher, J., Suh, H., Harada, H., Malynn, B. A., and Korsmeyer, S. J. (2005). Essential role of BAX,BAK in B cell homeostasis and prevention of autoimmune disease. Proc Natl Acad Sci U S A *102*, 11272-11277.

Tamm, I., Kornblau, S. M., Segall, H., Krajewski, S., Welsh, K., Kitada, S., Scudiero, D. A., Tudor, G., Qui, Y. H., Monks, A.*, et al.* (2000). Expression

and prognostic significance of IAP-family genes in human cancers and myeloid leukemias. Clin Cancer Res *6*, 1796-1803.

Tamm, I., Richter, S., Oltersdorf, D., Creutzig, U., Harbott, J., Scholz, F., Karawajew, L., Ludwig, W. D., and Wuchter, C. (2004). High expression levels of x-linked inhibitor of apoptosis protein and survivin correlate with poor overall survival in childhood de novo acute myeloid leukemia. Clin Cancer Res *10*, 3737-3744.

Tang, E. D., Wang, C. Y., Xiong, Y., and Guan, K. L. (2003). A role for NF-kappaB essential modifier/IkappaB kinase-gamma (NEMO/IKKgamma) ubiquitination in the activation of the IkappaB kinase complex by tumor necrosis factor-alpha. J Biol Chem *278*, 37297-37305.

Tang, G., Minemoto, Y., Dibling, B., Purcell, N. H., Li, Z., Karin, M., and Lin, A. (2001). Inhibition of JNK activation through NF-kappaB target genes. Nature *414*, 313-317.

Tansley, K., Spear, F. G., and Glucksmann, A. (1937). The Effect of Gamma Rays on Cell Division in the Developing Rat Retina. Br J Ophthalmol *21*, 273-298.

Tata, J. R. (1966). Requirement for RNA and protein synthesis for induced regression of the tadpole tail in organ culture. Dev Biol *13*, 77-94.

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

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

Thrower, J. S., Hoffman, L., Rechsteiner, M., and Pickart, C. M. (2000). Recognition of the polyubiquitin proteolytic signal. Embo J *19*, 94-102.

Trauth, B. C., Klas, C., Peters, A. M., Matzku, S., Moller, P., Falk, W., Debatin, K. M., and Krammer, P. H. (1989). Monoclonal antibody-mediated tumor regression by induction of apoptosis. Science *245*, 301-305.

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.

Uren, A. G., O'Rourke, K., Aravind, L. A., Pisabarro, M. T., Seshagiri, S., Koonin, E. V., and Dixit, V. M. (2000). Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. Mol Cell *6*, 961-967.

van der Weyden, L., Adams, D. J., and Bradley, A. (2002). Tools for targeted manipulation of the mouse genome. Physiol Genomics *11*, 133-164.

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.

Varfolomeev, E. E., Schuchmann, M., Luria, V., Chiannilkulchai, N., Beckmann, J. S., Mett, I. L., Rebrikov, D., Brodianski, V. M., Kemper, O. C., Kollet, O.*, et al.* (1998). Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. Immunity *9*, 267-276.

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.

Vaux, D. L., and Silke, J. (2003). Mammalian mitochondrial IAP binding proteins. Biochem Biophys Res Commun *304*, 499-504.

Vaux, D. L., and Silke, J. (2005). IAPs, RINGs and ubiquitylation. Nat Rev Mol Cell Biol *6*, 287-297.

Vaux, D. L., Weissman, I. L., and Kim, S. K. (1992). Prevention of programmed cell death in Caenorhabditis elegans by human bcl-2. Science *258*, 1955-1957.

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., Kratina, T. K., Hawkins, C. J., Silke, J., Ekert, P. G., and Vaux, D. L. (2007). Identification of mammalian mitochondrial proteins that

interact with IAPs via N-terminal IAP binding motifs. Cell Death Differ *14*, 348-357.

Vernooy, S. Y., Chow, V., Su, J., Verbrugghe, K., Yang, J., Cole, S., Olson, M. R., and Hay, B. A. (2002). Drosophila Bruce can potently suppress Rpr- and Grim-dependent but not Hid-dependent cell death. Curr Biol *12*, 1164-1168.

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.

von Freeden-Jeffry, U., Solvason, N., Howard, M., and Murray, R. (1997). The earliest T lineage-committed cells depend on IL-7 for Bcl-2 expression and normal cell cycle progression. Immunity *7*, 147-154.

Vucic, D., Deshayes, K., Ackerly, H., Pisabarro, M. T., Kadkhodayan, S., Fairbrother, W. J., and Dixit, V. M. (2002). SMAC negatively regulates the anti-apoptotic activity of melanoma inhibitor of apoptosis (ML-IAP). J Biol Chem *277*, 12275-12279.

Vucic, D., and Fairbrother, W. J. (2007). The inhibitor of apoptosis proteins as therapeutic targets in cancer. Clin Cancer Res *13*, 5995-6000.

Vucic, D., Stennicke, H. R., Pisabarro, M. T., Salvesen, G. S., and Dixit, V. M. (2000). ML-IAP, a novel inhibitor of apoptosis that is preferentially expressed in human melanomas. Curr Biol *10*, 1359-1366.

Vyas, S., Juin, P., Hancock, D., Suzuki, Y., Takahashi, R., Triller, A., and Evan, G. (2004). Differentiation-dependent sensitivity to apoptogenic factors in PC12 cells. J Biol Chem *279*, 30983-30993.

Wang, K., Yin, X. M., Chao, D. T., Milliman, C. L., and Korsmeyer, S. J. (1996). BID: a novel BH3 domain-only death agonist. Genes Dev *10*, 2859-2869.

Wang, L., Du, F., and Wang, X. (2008). TNF-alpha induces two distinct caspase-8 activation pathways. Cell *133*, 693-703.

Watanabe, D., Suda, T., and Nagata, S. (1995). Expression of Fas in B cells of the mouse germinal center and Fas-dependent killing of activated B cells. Int Immunol *7*, 1949-1956.

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.

Wertz, I. E., O'Rourke, K. M., Zhou, H., Eby, M., Aravind, L., Seshagiri, S., Wu, P., Wiesmann, C., Baker, R., Boone, D. L.*, et al.* (2004). De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. Nature *430*, 694-699.

Westphal, C. H., Hoyes, K. P., Canman, C. E., Huang, X., Kastan, M. B., Hendry, J. H., and Leder, P. (1998). Loss of atm radiosensitizes multiple p53 null tissues. Cancer Res *58*, 5637-5639.

White, E. (2001). Regulation of the cell cycle and apoptosis by the oncogenes of adenovirus. Oncogene *20*, 7836-7846.

White, K., Grether, M. E., Abrams, J. M., Young, L., Farrell, K., and Steller, H. (1994). Genetic control of programmed cell death in Drosophila. Science *264*, 677-683.

Willis, S. N., Chen, L., Dewson, G., Wei, A., Naik, E., Fletcher, J. I., Adams, J. M., and Huang, D. C. (2005). Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Genes Dev *19*, 1294-1305.

Wilson, R., Goyal, L., Ditzel, M., Zachariou, A., Baker, D. A., Agapite, J., Steller, H., and Meier, P. (2002). The DIAP1 RING finger mediates ubiquitination of Dronc and is indispensable for regulating apoptosis. Nat Cell Biol *4*, 445-450.

Woo, M., Hakem, R., Soengas, M. S., Duncan, G. S., Shahinian, A., Kagi, D., Hakem, A., McCurrach, M., Khoo, W., Kaufman, S. A.*, et al.* (1998). Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. Genes Dev *12*, 806-819.

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, J. W., Cocina, A. E., Chai, J., Hay, B. A., and Shi, Y. (2001). Structural analysis of a functional DIAP1 fragment bound to grim and hid peptides. Mol Cell *8*, 95-104.

Wyllie, A. H. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature *284*, 555-556.

Wyllie, A. H., Kerr, J. F., and Currie, A. R. (1980). Cell death: the significance of apoptosis. Int Rev Cytol *68*, 251-306.

Xu, L., Zhu, J., Hu, X., Zhu, H., Kim, H. T., LaBaer, J., Goldberg, A., and Yuan, J. (2007). c-IAP1 cooperates with Myc by acting as a ubiquitin ligase for Mad1. Mol Cell *28*, 914-922.

Xue, D., Shaham, S., and Horvitz, H. R. (1996). The Caenorhabditis elegans cell-death protein CED-3 is a cysteine protease with substrate specificities similar to those of the human CPP32 protease. Genes Dev *10*, 1073-1083.

Yamaguchi, K., Nagai, S., Ninomiya-Tsuji, J., Nishita, M., Tamai, K., Irie, K., Ueno, N., Nishida, E., Shibuya, H., and Matsumoto, K. (1999). XIAP, a cellular member of the inhibitor of apoptosis protein family, links the receptors to TAB1-TAK1 in the BMP signaling pathway. Embo J *18*, 179-187.

Yan, N., Chai, J., Lee, E. S., Gu, L., Liu, Q., He, J., Wu, J. W., Kokel, D., Li, H., Hao, Q., *et al.* (2005). Structure of the CED-4-CED-9 complex provides insights into programmed cell death in Caenorhabditis elegans. Nature *437*, 831-837.

Yang, L., Cao, Z., Yan, H., and Wood, W. C. (2003). Coexistence of high levels of apoptotic signaling and inhibitor of apoptosis proteins in human tumor cells: implication for cancer specific therapy. Cancer Res *63*, 6815-6824.

Yang, X., Chang, H. Y., and Baltimore, D. (1998). Essential role of CED-4 oligomerization in CED-3 activation and apoptosis. Science *281*, 1355-1357.

Yang, Y., and Ashwell, J. D. (1999). Thymocyte apoptosis. J Clin Immunol *19*, 337-349.

Yang, Y., Fang, S., Jensen, J. P., Weissman, A. M., and Ashwell, J. D. (2000). Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. Science *288*, 874-877.

Yang, Y., Ludwig, R. L., Jensen, J. P., Pierre, S. A., Medaglia, M. V., Davydov, I. V., Safiran, Y. J., Oberoi, P., Kenten, J. H., Phillips, A. C.*, et al.* (2005). Small molecule inhibitors of HDM2 ubiquitin ligase activity stabilize and activate p53 in cells. Cancer Cell *7*, 547-559.

Yang, Y. L., and Li, X. M. (2000). The IAP family: endogenous caspase inhibitors with multiple biological activities. Cell Res *10*, 169-177.

Yeh, W. C., Shahinian, A., Speiser, D., Kraunus, J., Billia, F., Wakeham, A., de la Pompa, J. L., Ferrick, D., Hum, B., Iscove, N.*, et al.* (1997). Early lethality, functional NF-kappaB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. Immunity *7*, 715-725.

Yin, X. M., Wang, K., Gross, A., Zhao, Y., Zinkel, S., Klocke, B., Roth, K. A., and Korsmeyer, S. J. (1999). Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. Nature *400*, 886-891.

Yoshida, H., Kong, Y. Y., Yoshida, R., Elia, A. J., Hakem, A., Hakem, R., Penninger, J. M., and Mak, T. W. (1998). Apaf1 is required for mitochondrial pathways of apoptosis and brain development. Cell *94*, 739-750.

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.

Zacharchuk, C. M., Mercep, M., Chakraborti, P. K., Simons, S. S., Jr., and Ashwell, J. D. (1990). Programmed T lymphocyte death. Cell activation- and steroid-induced pathways are mutually antagonistic. J Immunol *145*, 4037-4045.

Zender, L., Spector, M. S., Xue, W., Flemming, P., Cordon-Cardo, C., Silke, J., Fan, S. T., Luk, J. M., Wigler, M., Hannon, G. J.*, et al.* (2006). Identification and validation of oncogenes in liver cancer using an integrative oncogenomic approach. Cell *125*, 1253-1267.

Zhao, Y., Conze, D. B., Hanover, J. A., and Ashwell, J. D. (2007). Tumor necrosis factor receptor 2 signaling induces selective c-IAP1-dependent ASK1 ubiquitination and terminates mitogen-activated protein kinase signaling. J Biol Chem *282*, 7777-7782.

Zheng, L., Fisher, G., Miller, R. E., Peschon, J., Lynch, D. H., and Lenardo, M. J. (1995). Induction of apoptosis in mature T cells by tumour necrosis factor. Nature *377*, 348-351.

Zhou, H., Du, M. Q., and Dixit, V. M. (2005). Constitutive NF-kappaB activation by the t(11;18)(q21;q21) product in MALT lymphoma is linked to deregulated ubiquitin ligase activity. Cancer Cell *7*, 425-431.

Zhou, L., Song, Z., Tittel, J., and Steller, H. (1999). HAC-1, a Drosophila homolog of APAF-1 and CED-4 functions in developmental and radiation-induced apoptosis. Mol Cell *4*, 745-755.

Zornig, M., Grzeschiczek, A., Kowalski, M. B., Hartmann, K. U., and Moroy, T. (1995). Loss of Fas/Apo-1 receptor accelerates lymphomagenesis in E mu L-MYC transgenic mice but not in animals infected with MoMuLV. Oncogene *10*, 2397-2401.

Zou, H., Henzel, W. J., Liu, X., Lutschg, A., and Wang, X. (1997). Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell *90*, 405-413.