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# THE XAF1 TUMOR SUPPRESSOR NEGATIVELY REGULATES SURVIVIN IN A p53-DEPENDENT AND –INDEPENDENT MANNER

By

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A thesis submitted to the Faculty of Graduate and Post-doctoral Studies,
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# ABSTRACT

Apoptosis is characterized by an intracellular proteolytic cascade consisting of a family of proteases called Caspases, and activation of this cascade by extracellular or intracellular stress signals results in the characteristic morphological and biochemical features of apoptotic cell death. The Inhibitors of Apoptosis Proteins (IAPs) are a family of proteins that bind and inhibit Caspases and have been demonstrated to be the only known endogenous inhibitors of the terminal caspase cascade. Defects in apoptosis allow cancer cells to survive under conditions of enormous stress and increased expression of several members of the IAP family has been observed in tumor biopsy samples and cancer cell lines. XIAP Associated Factor 1 (XAF1) was identified as a nuclear protein that binds to the X-linked Inhibitor of Apoptosis protein (XIAP) and antagonizes the anti-caspase activity of XIAP, thereby reversing the protective effect of XIAP overexpression in cancer cell lines. The expression of XAF1 is significantly reduced in cancer cell lines and several primary malignancies as a result of promoter hypermethylation, and re-activation of *xaf1* by DNA methylation inhibitors sensitizes cancer cells to apoptosis-inducing agents. *Xaf1* has recently been identified as an interferon (IFN)-stimulated gene that sensitizes tumor cells to apoptosis by TRAIL (TNF-related Apoptosis Inducing Ligand) following treatment with IFN-β.

This thesis documents the role of XAF1 in cell cycle and its potential as a negative regulator of Survivin, an unusual IAP that possesses poor anti-Caspase activity, and is known to be involved in the cell cycle as a member of the chromosomal passenger complex. In HEL 299 and wild-type mouse embryonic fibroblast (MEF) cells, FACS profiling of DNA content suggests that XAF1 overexpression triggers a $G_1$ cell cycle arrest by upregulating p21. This XAF1-mediated upregulation of p21 is not seen in p53-null MEFs and is attenuated by a

dominant-negative p53-mutant in HEL 299 cells, suggesting that XAF1 functions primarily upstream of p53 in the cell cycle arrest pathway and results in activation or repression of p53-target genes. Survivin is negatively regulated by p53 and these results suggest that XAF1 may regulate Survivin expression in a p53-dependent manner. Furthermore, overexpression of XAF1 in p53-deficient cancer cells results in significant downregulation of Survivin expression, indicating the existence of a p53-independent mechanism for the observed effect. Immunoprecipitation experiments indicate that XAF1 binds to all the IAPs tested except Survivin. Interestingly, it was observed that XAF1 complexes with Survivin only in the presence of XIAP. Furthermore, such an association was associated with a decrease in expression levels of Survivin. XIAP is a RING-bearing protein which functions as an E3 ligase and is involved in the ubiquitin-proteasome pathway. Treatment of transfected cells with proteasomal inhibitor or the use of a XIAP RING-mutant lacking E3 ligase activity was shown to restore Survivin protein expression indicating that XAF1 mediates Survivin downregulation by promoting the E3 ligase activity of XIAP. Taken together, these results suggest that XAF1 negatively regulates Survivin expression by both p53-dependent and –independent mechanisms.

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## DEDICATION

To my parents for their unending support and love.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| **5-AZA-dC** | 5-AZA-2′-Deoxycytidine |
| **ALL** | Acute Lymphoblastic Leukemia |
| **AML** | Acute Myelocytic Leukemia |
| **APAF-1** | Apoptosis Protease Activating Factor 1 |
| **ASON** | Antisense Oligonucleotide |
| **Bcl** | B Cell Lymphoma |
| **BIR** | Baculoviral IAP Repeat |
| **BIRP** | BIR-containing Protein |
| **bp** | Base Pair |
| **BRUCE** | BIR-containing Ubiquitin Conjugating Enzyme |
| **BSA** | Bovine Serum Albumin |
| **CAD** | Caspase Activated Deoxyribonuclease |
| **CARD** | Caspase Recruitment Domain |
| **CDE** | Cell-cycle Dependent Element |
| **cDNA** | Copy DNA |
| **c-FLIP** | Caspase-8 (FLICE) Like Inhibitory Protein |
| **CHR** | Cell-cycle Homology Region |
| **c-IAP** | Cellular IAP |
| **CPC** | Chromosomal Passenger Complex |
| **CLL** | Chronic Lymphocytic Leukemia |
| **dATP** | Deoxyadenosine Triphosphate |
| **DD** | Death Domain |
| **DED** | Death Effector Domain |
| **DFF40** | DNA Fragmentation Factor 40 |
| **DIABLO** | Direct IAP Binding Protein with Low PI |
| **DISC** | Death Inducing Signaling Complex |
| **DNA** | Deoxyribonucleic Acid |
| **E1** | Ubiquitin-Activating Enzyme |
| **E2** | Ubiquitin-Conjugating Enzyme |
| **E3** | Ubiquitin Protein Ligase |
| **ER** | Endoplasmic Reticulum |
| **FACS** | Fluorescence Activated Cell Sorting |
| **FADD** | Fas-Associated Protein with Death Domain |
| **FBS** | Fetal Bovine Serum |
| **FISH** | Fluorescence *in situ* hybridization |
| **GST** | Glutathione S Transferase |
| **h** | Hour |
| **HtrA** | High Temperature Requirement A |
| **IAP** | Inhibitor of Apoptosis Protein |
| **IBM** | IAP Binding Motif |
| **IFN** | Interferon |
| **IGF-BP3** | Insulin-like Growth Factor 1 Binding Protein 3 |
| **INCENP** | Inner Centromeric Protein |

| | |
|---|---|
| **IRES** | Internal Ribosome Entry Sequence |
| **JNK1** | c-Jun N-terminal Kinase 1 |
| **LRR** | Leucine Rich Repeat |
| **MAPKKK** | Mitogen Activated Protein Kinase Kinase Kinase |
| **MDM2** | Mouse Double Minute 2 |
| **MEF** | Mouse Embryonic Fibroblast |
| **MOI** | Multiplicity Of Infection |
| **MOMP** | Mitochondrial Outer Membrane Permeabilization |
| **mRNA** | messenger Ribonucleic Acid |
| **MS** | Multiple Sclerosis |
| **MxA** | Myxovirus Resistance Protein A |
| **NAb** | Neutralizing Antibody |
| **NAIP** | Neuronal Apoptosis Inhibitory Protein |
| **NCI** | National Cancer Institute |
| **NEAA** | Non-Essential Amino Acids |
| **NF-κB** | Nuclear Factor κB |
| **OMM** | Outer Mitochondrial Membrane |
| **PBS** | Phosphate Buffered Saline |
| **PFU** | Plaque Forming Unit |
| **PMSF** | Phenylmethylsulfonyl Fluoride |
| **RB** | Retinoblastoma |
| **RING** | Really Interesting New Gene |
| **RNA** | Ribonucleic Acid |
| **RNAi** | RNA Interference |
| **RPM** | Revolutions Per Minute |
| **siRNA** | Small Interfering RNA |
| **SMA** | Spinal Muscular Atrophy |
| **Smac** | Second Mitochondrial Activator of Caspases |
| **SMN** | Survival Motor Neuron |
| **TAB1** | TAK1 Binding Protein 1 |
| **TAK1** | Transforming growth factor (TGF)-beta-Activating Kinase 1 |
| **TNFα** | Tumor Necrosis Factor α |
| **TNFR** | Tumor Necrosis Factor Receptor |
| **TRADD** | TNF Associated Death Domain |
| **TRAF** | TNF Receptor-Associated Factor |
| **TRAIL** | TNF-Related Apoptosis-Inducing Ligand |
| **Ts-IAP** | Testis-specific IAP |
| **UTR** | Untranslated Region |
| **UV** | Ultraviolet |
| **XIAP** | X-linked Inhibitor of Apoptosis |
| **XAF1** | Xiap-Associated Factor 1 |
| **Zn** | Zinc |

*Chapter 1*

# LITERATURE REVIEW

## Apoptosis

In the 1960s, Lockshin and Williams introduced the term "programmed cell death" to refer to a gene-directed form of cell death (Lockshin and Williams, 1964). The term "apoptosis" was coined by Kerr, Wyllie, and Currie to describe a form of ischemia-induced hepatic cell death. The term comes from the Greek (apo + ptosis) for "falling off" and depicts a distinct morphology of dying cells characterized by cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation (Kerr JF, 1972). The realization that apoptosis is a genetically invoked form of cell death has impacted our understanding of proliferative and degenerative diseases because of the implication that tissue homeostasis can be controlled by factors that regulate cell survival and death, as well as those that affect proliferation and differentiation.

Apoptosis is a gene-directed mechanism in which unnecessary or dangerous cells are triggered to undergo self-destruction without injuring neighboring cells or eliciting any associated inflammatory response (Soini et al., 1998). The core apoptotic pathway was first described through genetic analysis in the nematode *Caenorhabditis elegans* and subsequently found in species as diverse as *Drosophila melanogaster* and humans (Tittel and Steller, 2000). In these multicellular organisms, the apoptotic process is crucial for normal development, differentiation, tissue physiology and defense against pathogens. The dysregulation of apoptosis is intricately involved in the etiology and pathogenesis of many diseases, including AIDS, autoimmune disorders, neurodegenerative diseases and cancer.

In general, apoptosis can be divided into the initiation phase, the effector phase, and the degradation phase (Susin et al., 1997). In the initiation phase, a stimulus, either extrinsic or intrinsic to the cell, triggers the apoptotic process. This stimulus may arise from a variety of sources and some general inducers include radiation, UV light, growth factor withdrawal and cytotoxic agents such as chemotherapeutic drugs. The potency of each of these stimuli to induce apoptosis, however, is cell-type dependent. Despite the differences in the initiation of apoptosis, the effector phase in which the apoptotic machinery is activated shares common biochemical features (discussed in The Apoptotic Machinery). Once cells have committed to apoptosis, the degradation phase begins and the process becomes irreversible. At this late stage, double-stranded breakdown of DNA into nucleosomal segments is manifested as DNA laddering in gel electrophoresis (Soini et al., 1998). This DNA laddering is a defining feature of apoptotic cell death that contributes to the unique morphology of apoptotic cells.

Apoptosis is characterized by a series of well-documented morphological changes that can be detected by light and electron microscopy (Clarke, 1990; Cohen, 1991; Cummings et al., 1997; Kerr JF, 1972; Walker et al., 1988; Wyllie et al., 1980). The most characteristic morphological change is seen within the nucleus as compaction of nuclear chromatin leads to sharply delineated, uniformly granular masses marginated against the nuclear envelope followed by nuclear fragmentation. As the outline convolutes, the cytoplasm also condenses and blunt blebs or protrusions appear on the plasma membrane. While the cytoplasm continues to condense, the cell disintegrates into the characteristic membrane-bound apoptotic bodies enclosing fragments of the nucleus. The integrity of the membrane encasing the apoptotic fragments is retained during the course of apoptosis until

they are engulfed by phagocytes in a "contained" manner without eliciting an inflammatory response that might be harmful to the surrounding tissues (Kerr JF, 1972; Wyllie, 1980).

In this introductory chapter, I will elaborate on our current understanding of the apoptotic processes and various protagonists and antagonists that influence cell death. The introduction concludes with a summary of the rationale and an outline for the research performed.

## The Apoptotic Machinery

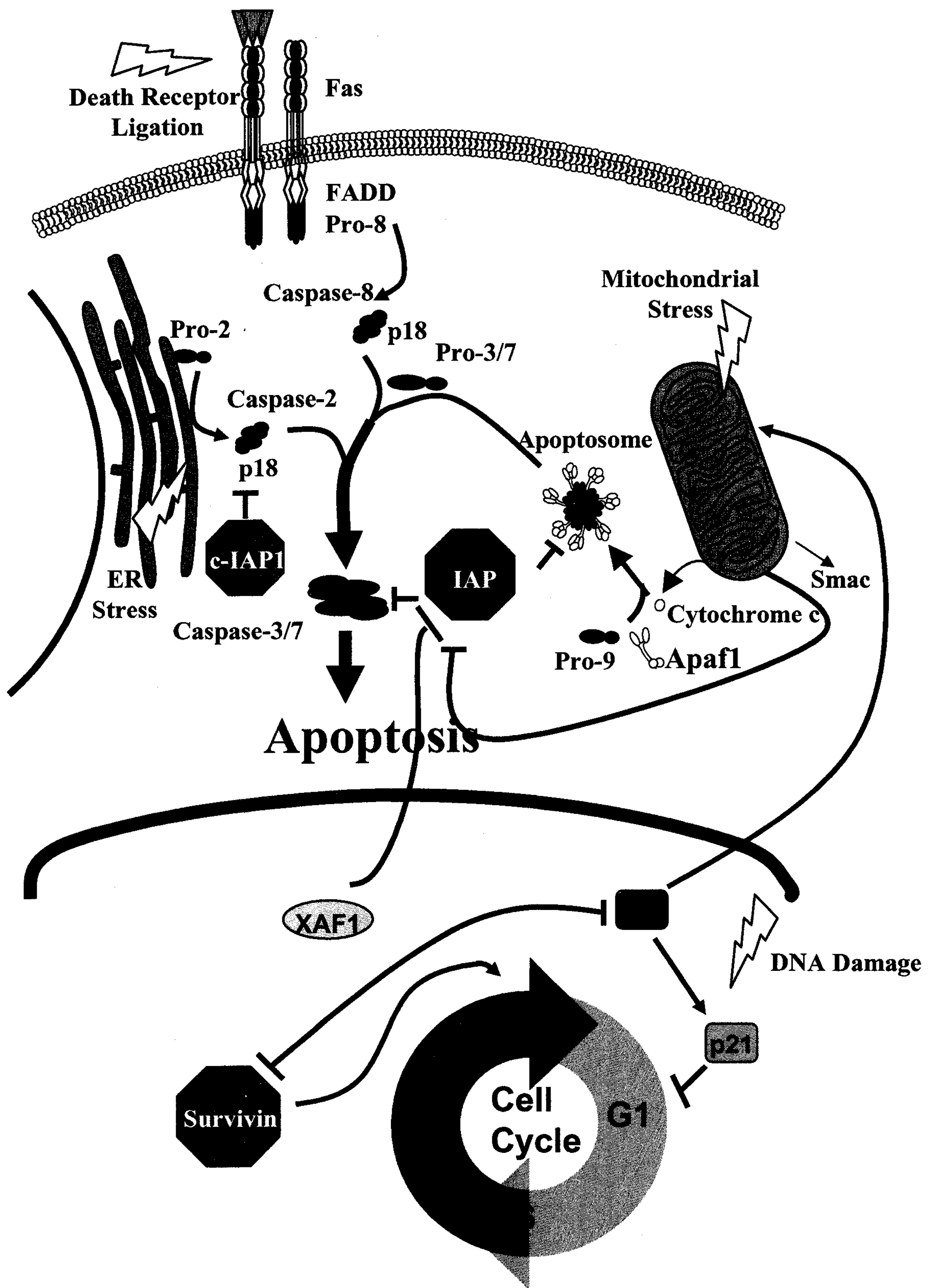
Apoptosis is first and foremost defined by its morphological features. Apoptotic cells confer a distinctive constellation of biochemical changes that underlie the structural changes. Given that diverse cell types across species exhibit morphological similarity when subjected to various death inducing stimuli, an intuitive suggestion would be that there exists a common apoptotic mechanism operating in most cells of an organism (Wyllie et al., 1980). The core of the apoptotic machinery is in fact composed of a set of conserved molecules operating within metazoan cells (Huettenbrenner et al., 2003) and is induced by a cascade of molecular events that may be initiated in a distinct manner culminating in the activation of Caspases (see Figure 1-1 for a schematic overview)

### *Caspases and Apoptotic Pathways*

The central component of the apoptotic machinery is a proteolytic system involving a family of aspartate-specific cysteine proteases, termed Caspases, which cleave many vital cellular proteins and proteolytically activate enzymes that contribute to the disassembly of a cell, such as the DNAase DFF40/CAD (Thornberry and Lazebnik, 1998). Caspases exist as

**Figure 1-1. The Convergent Apoptotic Pathways**

The extrinsic apoptotic pathway can be initiated by the ligation of death receptors Fas or TNFα by their respective ligands. Recruitment of FADD or TRADD results in proximity-induced cleavage and activation of pro-Caspase-8. Active Caspase-8 subsequently cleaves and activates effector Caspases -3 and -7. The intrinsic pathway is activated in response to intra-cellular stress, such as DNA damage, and results in the release of cytochrome *c* from the mitochondrial inner membrane space and the formation of the apoptosome consisting of Apaf-1 and Caspase-9. The apoptosome then activates Caspase-3 and -7 which leads directly to the cleavage of both nuclear and cytoplasmic substrates. ER stress results in activation of Caspase-2, -4, -9 and/or -12, which in turn activates the effector Caspases. The IAP family of proteins interfere with Caspase function at various points in the pathways. Smac/DIABLO and Omi/HtrA2 are released from the mitochondria coincidentally with cytochrome *c* and along with nuclear XAF1 are known inhibitors of XIAP function.



Death Receptor
Ligation
Fas
FADD
Pro-8
Caspase-8
p18
Mitochondrial
Stress
Pro-2
Pro-3/7
Caspase-2
Apoptosome
p18
ER
Stress
c-IAP1
IAP
Smac
Cytochrome c
Caspase-3/7
Pro-9
Apaf1
Apoptosis
XAF1
DNA Damage
p21
Survivin
Cell
Cycle
G1

zymogens in cells, but can become activated in response to apoptotic stimuli. They are organized in a cascade and can be divided functionally into two groups: initiator and effector Caspases, with upstream initiator Caspases being responsible for the activation of downstream effector Caspases (Brady, 2003). Although Caspases share distinct similarities in amino acid sequence and structure, they are highly specific in their substrate preference (Talanian et al., 1997; Thornberry, 1997). The specificity of Caspases allows them to function in an orchestrated fashion that guides the apoptotic cell to sever contacts with surrounding cells, reorganize the cytoskeleton, shut down DNA replication and repair, destroy DNA, disrupt the nuclear structure and eventually induce the cell to display signals that mark it for phagocytosis (Thornberry and Lazebnik, 1998).

Initiation of apoptosis occurs by signals from two distinct but convergent pathways: the extrinsic and intrinsic pathway. These two pathways make use of largely distinct molecular interactions and utilize different Caspases, but are also interconnected at numerous steps that ultimately converge at the level of effector Caspases activation (Strasser et al., 2000) (see Figure 1-1).

***Intrinsic Apoptotic Pathway***

The intrinsic pathway is activated in response to intra-cellular stress, such as DNA damage, hypoxia, growth factor deprivation and some chemotherapeutic drugs (Nachmias et al., 2004). This pathway, sometimes referred to as mitochondrion-mediated cell death, results in increased mitochondrial permeability, and is defined by mitochondrial outer membrane permeabilization (MOMP) that is executed by proteins from the Bcl-2 family (Green and Kroemer, 2004). The Bcl-2 family of proteins encompasses anti-apoptotic members, including Bcl-2 and Bcl-$X_L$, which preserve mitochondrial membrane integrity, and pro-

apoptotic members, such as Bax, Bad and Bid, which, through oligomerization, increase mitochondrial permeability by the formation of a transmembrane pore. The increase in permeability leads to the release of proteins normally found in the space between the inner and outer mitochondrial membranes (Goldstein et al., 2000). A pivotal protein released into the cytosol is cytochrome *c*, well known for its role in mitochondrial respiration and recognized as an essential component of a high molecular weight Caspases-activating complex known as the apoptosome (Cain et al., 2000). Apoptosome formation is caused by cytochrome *c* binding to Apaf-1, which in the presence of dATP multimerizes and facilitates the association and activation of the initiator Caspase-9 (Saelens et al., 2004). Subsequently, effector pro-caspase-3 is recruited to the apoptosome, where it is activated by Caspase-9, leading to the degradation phase of apoptosis. It should be noted, however, that recent research has also pointed to the endoplasmic reticulum (ER) as an important modulator of both mitochondrion-mediated apoptosis (Scorrano et al., 2003), as well as an ER-specific, unique pathway for Caspase activation and apoptosis (Cheung et al., 2006; Morishima et al., 2002; Nakagawa and Yuan, 2000; Rao et al., 2002; Rao et al., 2001; Zong et al., 2003).

***Extrinsic Apoptotic Pathway***

The extrinsic pathway, also know as the death receptor-induced pathway, is initiated by the ligation of death receptors belonging to the tumor necrosis factor receptor (TNF-R) superfamily, such as Fas/APO-1/CD95 and TNF-R1 (Strasser et al., 2000). Members of the TNF-R family are characterized by a cytoplasmic death domain (DD) involved in protein-protein interactions that is essential for delivering apoptotic signals (Itoh and Nagata, 1993; Tartaglia et al., 1993). Binding of ligands promotes oligomerization of the death receptors, and their cytoplasmic domains then recruit DD-containing adaptor proteins FADD and

TRADD via DD-DD interactions, leading to the formation of a death-inducing signaling complex (DISC) (Boldin et al., 1995; Chinnaiyan et al., 1995; Kischkel et al., 1995). FADD then causes the conscription of the proenzyme form of Caspase-8 and -10 through homotypic interaction of their respective death effector domains (DED) to the DISC (Boldin et al., 1996; Muzio et al., 1996). The proximity-induced activation of multiple Caspase-8 molecules by DISC (Muzio et al., 1998) in turn activates effector pro-Caspase-3 (Stennicke et al., 1998), at which point the intrinsic and the extrinsic pathways converge (Marsden and Strasser, 2003).

Evidently, Caspases occupy a central role in the regulation of apoptosis. Although the extrinsic and intrinsic apoptotic pathways, for the sake of simplicity, are generally treated as mutually exclusive, numerous opportunities for cross-talk exist and members of the Bcl-2 family play a significant and vital role in bridging the two together.

**Bcl-2 family**

The Bcl-2 family proteins may regulate apoptosis by altering the integrity of the mitochondria and by controlling calcium homeostasis (Distelhorst and Shore, 2004; Droin and Green, 2004; Kirkin et al., 2004). Members of the Bcl-2 family can be divided into three classes: 1) Anti-apoptotic (Bcl-2, Bcl-$X_L$, Bcl-w and Mcl-1); 2) Pro-apoptotic Bax-like (Bax, Bak, Bok/Mtd and Bcl-$X_S$); 3) Pro-apoptotic BH3-only (Bad, Bid, Bik/Nbk, $Bim_L$/Bod, Hrk/DP5, PUMA/Bbc3, BNIP3, Noxa and Bmf) (Droin and Green, 2004) (see Figure 1-2). Through interactions between various pro- and anti-apoptotic Bcl-2 family

**Figure 1-2. Classification of the Bcl-2 Family.**

Examples of anti-apoptotic, pro-apoptotic multi-domain or Bax-like, and pro-apoptotic BH3-only proteins are shown. Bcl-2 homology (BH) and Transmembrane (TM) domains are indicated. The BH3 domain in the pro-apoptotic members is a ligand for the hydrophobic groove formed by the BH1-BH3 domains of the anti-apoptotic members. The hydrophobic C terminus consists of a 17-23 amino-acid α-helix transmembrane domain that anchors the proteins in intracellular membranes.

Anti-apoptotic

Bcl-2
Bcl-$X_L$
Bcl-w
Mcl-1

Pro-apoptotic Bax-like

Bax
Bak
Bok
Bcl-$X_s$

Pro-apoptotic BH3-only

Bik
$Bim_L$
Hrk
BNIP3

Bad
Bid
Bmf
PUMA
Noxa

members, calcium and mitochondrial protein release, including that of cytochrome *c*, is regulated.

As already mentioned, members of the Bcl-2 family play a significant role in linking the intrinsic and extrinsic apoptotic pathways at several points. For example, in response to Fas signals, these two death pathways might cross-talk via the function of cytosolic Bid. The full-length p22 Bid is inactive and is a substrate of Caspase-8. Cleavage of p22 Bid gives rise to truncated p7/p15 Bid exposing a glycine that is *N*-myristoylated, which enables the targeting of a complex of p7 and myristoylated p15 fragments of Bid to the mitochondria (Zha et al., 2000). Upon activation, Bid induces intramembranous oligomerization of mitochondrion resident Bak (Hanahan and Weinberg, 2000; Wei et al., 2000), as well as oligomerization and integration of cytosolic Bax in the outer mitochondrial membrane (Eskes et al., 2000). Multimers of Bak or Bax form a proposed pore on the mitochondria for cytochrome *c* efflux, thereby inducing Caspase activation through the formation of apoptosomes (Kuwana et al., 1998; Li et al., 1998b; Luo et al., 1998; Wei et al., 2000). It is thus possible for an apoptotic stimulus acting through the extrinsic pathway to induce activation of the intrinsic pathway as well. By contrast, Bcl-2 inhibits apoptosis by preserving mitochondrial membrane integrity. Bcl-2 that is inserted into the outer mitochondrial membrane may, by a mechanism that has yet to be elucidated, prevent Bax/Bak oligomerization and subsequent release of apoptogenic molecules from the mitochondria (Borner, 2003; Lucken-Ardjomande and Martinou, 2005).

In addition to controlling the mitochondrial apoptotic process, Bcl-2 family proteins also regulate apoptosis by affecting calcium homeostasis. Unfortunately, a detailed discussion of this topic is beyond the scope of this thesis and the reader is referred to a

number of recent reviews dealing with this area (Armstrong, 2006; Breckenridge et al., 2003; Oakes et al., 2003; Rizzuto et al., 2003; Thomenius and Distelhorst, 2003; Walter and Hajnoczky, 2005)

**Inhibitors of Apoptosis**

The apoptotic process is tightly controlled by regulators of Caspases. An important family of endogenous Caspase inhibitors, termed the Inhibitors of Apoptosis Proteins (IAPs), was identified as a central regulatory factor that blocks the execution of apoptosis. Although other proteins have been identified that inhibit initiator Caspases, only the IAPs, and in particular the X-linked Inhibitor of Apoptosis protein(XIAP/hILP), have been demonstrated to be endogenous direct repressors of the terminal caspase cascades (Holcik et al., 2001; Liston et al., 2003). The anti-Caspase activity of the IAPs may be attributed to their characteristic 70-80 amino acid baculoviral IAP repeat (BIR) domains. However, not all BIR containing proteins function as Caspase inhibitors. Several BIR-containing proteins which do not possess a cell death program have been identified in yeast and other unicellular organisms. This has necessitated a reclassification of the IAP family as a subfamily within the larger family of BIR-containing proteins (BIRPs). Neuronal apoptosis inhibitory protein (NAIP) was the first mammalian IAP to be identified, and subsequently several other BIR-containing proteins with anti-Caspase activity have been added to the IAP family. In humans, members of this family include NAIP, XIAP/hILP, cellular IAP1 (c-IAP1/HIAP2), cellular IAP2 (c-IAP2/HIAP1), Survivin, Livin, testis-specific IAP (Ts-IAP) and Apollon/BRUCE (see Figure 1-3). In IAPs containing multiple BIRs, the third BIR domain generally interacts with and inhibits Caspase-9, while the second BIR domain is responsible

**Figure 1-3. Domain Structure of the IAP Family.**

The human BIR-containing proteins (BIRPs) are listed and amino acid sizes are noted. Proteins were not drawn to scale. The abbreviations are as follows: BIR: baculoviral IAP repeat; CARD: caspase recruitment domain; RING: RING zinc-finger; NOD: nucleotide-binding oligomerization domain; LRR: leucine-rich repeats.

BRUCE 4803

NAIP 1403

c-IAP2 618

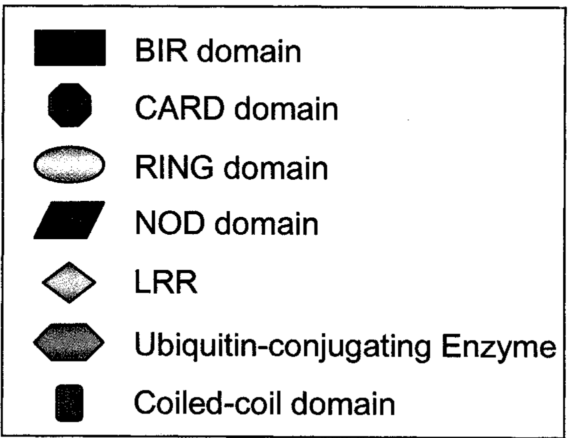
c-IAP1 604

XIAP 497

Livin 298

Ts-IAP 237

Survivin 142



for the inhibition of Caspases -3 and -7 (Roy et al., 1997; Takahashi et al., 1998). Notably, the BIR1 domains of XIAP, c-IAP1, c-IAP2, and NAIP do not display any Caspase inhibiting activity (Liston et al., 2003) but are involved in protein stabilization (Dan et al., 2004) and TRAF2 interaction (Samuel et al., 2006). Other interactions that have been mapped to the BIR domains include XIAP interaction with the TAB1 protein (Liston et al., 2003) and Smac/DIABLO and Omi/HtrA2 binding (see 'IAP Antagonists').

The current literature indicates that the anti-apoptotic activity of IAPs is not restricted to their ability to inhibit Caspase -3, -7, and -9. For example, Livin promotes the degradation of pro-apoptotic Smac/DIABLO by the ubiquitin-proteasome pathway (Ma et al., 2006). Ts-IAP, expressed exclusively in the testes, is an autosomal, retrotransposed, intronless copy of XIAP (Lagace et al., 2001). Ts-IAP is, however, an unstable protein that is unlikely to inhibit Caspase 9 in a physiological way by itself (Shin et al., 2005) and therefore might require assistance from unidentified cellular factors to be an effective inhibitor of apoptosis. Further characterization of Ts-IAP and its distribution within the testis would help in determining its role. c-IAP1 and c-IAP2 act as components of a protein complex that forms on the cytoplasmic tail of TNFα receptor 2 in association with TRAF1 and TRAF2 (Rothe et al., 1995). Although c-IAP1 displays greater activity than XIAP when exposed to TNFα-mediated cell death stimuli c-IAP1 and c-IAP2 are not as potent as XIAP with regard to Caspase inhibition (Roy et al., 1997). Interestingly, a recent study demonstrated that c-IAP1 and c-IAP2 bind but do not inhibit caspases-7 and -9 (Eckelman and Salvesen, 2006) suggesting that caspase binding, but not inhibition may be important for their physiological function., A recent study also indicates that c-IAP1 is involved in the inhibition of apoptosis in response to ER stress by binding and inhibiting Caspase-2 (Cheung et al., 2006). Some

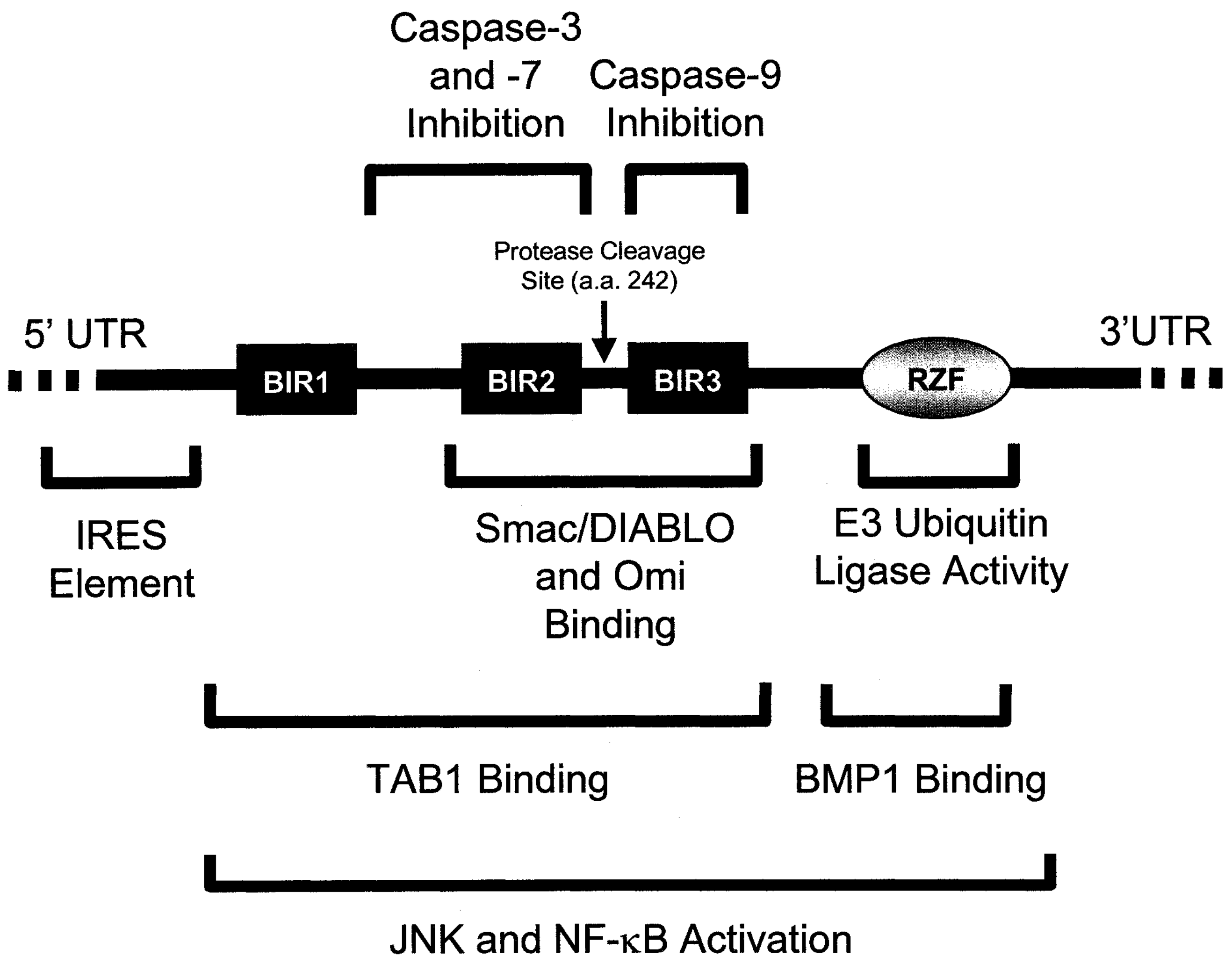
IAPs also contain a RING domain that functions as an E3 ubiquitin ligase, capable of recruiting target proteins to a complex containing an E2 enzyme for ubiquitin conjugation and proteasomal degradation (Pickart, 2001). In particular, c-IAP2 and XIAP can trigger the ubiquitination of Caspase-3 and -7 (Huang et al., 2000; Suzuki et al., 2001c), suggesting that targeting of Caspases to the proteasome may be another anti-apoptotic mechanism of the IAPs and is discussed in detail later in this section.

***XIAP***

XIAP is arguably the most potent anti-apoptotic IAP and it has been studied extensively. Xiap mRNA is expressed ubiquitously in all tissues at a fairly constant level. However, a unique mechanism has been shown to control protein synthesis of XIAP protein. The coding region accounts for only 1.5 kb of the ~9kb xiap transcript. The transcript contains a long 5′ untranslated region (UTR) which is at least 1.5kb and a 3′ UTR of 6kb (see Figure 1-4). It is believed that long UTRs interfere with efficient ribosomal translation and are rarely found in eukaryotic mRNA (Kozak, 1989). Initiation of cap-independent translation of uncapped viral mRNAs through a specific sequence termed an Internal Ribosome Entry Sequence (IRES) (Holcik et al., 2000a; Jang et al., 1988). Interestingly, an IRES element exists in the 5′ UTR of xiap mRNA, facilitating its efficient translation (Holcik et al., 1999). Several other cellular mRNAs containing IRES elements have been identified and are generally translated under stress conditions that block cap-dependent translation. IRES-mediated translation of XIAP offers improved protection against apoptosis in response to the stress condition of serum-withdrawal compared to a XIAP

**Figure 1-4. Functional Map of XIAP Protein.**

Published interactions of XIAP are indicated relative to known domains (see text for details). Some interactions have not been mapped to individual domains and have been indicated as requiring the entire protein. IRES: internal ribosome entry sequence, UTR: untranslated region, BIR: baculoviral IAP repeat, RZF: RING Zn finger.


Caspase-3
and -7
Inhibition
Caspase-9
Inhibition
Protease Cleavage
Site (a.a. 242)
5' UTR
3'UTR
BIR1
BIR2
BIR3
RZF
IRES
Element
Smac/DIABLO
and Omi
Binding
E3 Ubiquitin
Ligase Activity
TAB1 Binding
BMP1 Binding
JNK and NF-κB Activation

construct missing the IRES element (Holcik et al., 1999). This result, along with the finding that IRES-mediated translation of XIAP in response to low dose $\gamma$-radiation is upregulated (Holcik et al., 2000b), suggests that IRES-mediated translation of XIAP is critical for XIAP function by allowing for greater levels of XIAP protein relative to other cellular proteins under the same stress conditions. It is possible that the upregulation of XIAP expression in this manner under stress conditions confers greater resistance to tumor cells to death inducing stimuli.

XIAP possesses three BIR domains and has been reported to inhibit apoptosis in response to a variety of apoptotic stimuli, both intrinsic and extrinsic. The structure of the XIAP BIR2 and BIR3 domains has been determined by crystallography (Huang et al., 2001; Riedl et al., 2001). Although both BIR domains are structurally similar, different amino acid residues have been found to be critical for the inhibition of distinct Caspases. The linker region between BIR1 and BIR2 domains appears far more important than the BIR2 domain itself for interaction with Caspases -3 and -7. The linker region accounts entirely for the inhibition of Caspase-3, without the involvement of BIR2, as evidenced by the replacement of this domain by an irrelevant protein such as GST (Huang et al., 2001). However, the BIR2 domain does play a role in the inhibition of Caspase-7 by making contact with the amino terminus of the small subunit of the protease (Suzuki et al., 2001b). The isolated BIR3 domain without contribution from the upstream linker region is sufficient to inhibit Caspase-9 (Riedl et al., 2001) by bringing about a conformational shift that inactivates the enzyme (Shiozaki et al., 2003) in a manner distinct from the binding to Caspase -3 and -7.

In addition to its suppression of apoptosis by inhibiting Caspases and E3 ubiquitination of cellular proteins, XIAP has also been shown to be involved in signal

transduction pathways via TAK1, a mitogen-activated protein kinase kinase kinase (MAPKKK) leading to the activation of pro-survival NF-κB and JNK1 signaling pathways (Liston et al., 2003).

***Survivin***

Survivin is the smallest member of the IAP family with a predicted mass of 16.5 kDa. It contains only a single BIR domain and a coiled-coil domain in the carboxy-terminal sequence. Survivin lacks a RING finger domain as well as the caspase-recruitment domain (CARD) found in other members of the family (see Figure 1-3). Survivin exhibits fascinating functional properties relevant to the other IAPs in that, in addition to preventing apoptosis, it also acts as a component of the chromosomal passenger complex (CPC) and thus promotes cell proliferation as evidenced by several studies involving immunofluorescence and molecular tagging techniques (Skoufias et al., 2000; Temme et al., 2003; Uren et al., 2000; Wheatley et al., 2005). This CPC, composed of Aurora-B, Borealin, inner centromeric protein (INCENP), and Survivin, is essential for accurate spindle check point function, mitotic chromosomal movements, and proper execution of cytokinesis (Vagnarelli and Earnshaw, 2004). Protein expression of Survivin is cell cycle dependent. It is not detected in $G_1$ and S phases, and its expression peaks during $G_2$/M phase (Li et al., 1998a). Nuclear localization of Survivin however, occurs only during a very short period in $G_2$ when Survivin, along with other members of the CPC, assist each other in localizing to the centromeres (Klein et al., 2006). *Survivin* is an essential gene and cells from mouse embryos in which the gene has been knocked out display an increase in ploidy, cytokinesis failure, and large microtubule bundles (Wheatley et al., 2005), usually resulting in death of the embryos by day 5 post-coitum. Similarly, ablation of Survivin in tissue culture cells with

survivin antisense (Li et al., 1999) results in an analogous phenotype with defective chromosome movements, failure in cytokinesis, and an increase in ploidy. RNAi-based studies in tissue culture cells corroborated that antagonism of Survivin function leads to polyploidy and defects in chromosome segregation (Carvalho et al., 2003; Lens et al., 2003). Depletion of Survivin, however, did not contribute to an overall increase in apoptosis in all systems. In fact, the ability of Survivin to directly inhibit apoptosis has been a matter of much heated debate amongst researchers over the years and the possible mechanism of its anti-apoptotic function is discussed later in this section.

Since Survivin contains only a single BIR domain, it has been postulated that it may function in a manner similar to single BIR domain containing proteins found in *Caenorhabditis elegans*: BIR-1 and BIR-2, which do not contribute significantly to the inhibition of apoptosis. A study characterizing the function of BIR-1 indicated that although BIR-1 is increasingly expressed during embryogenesis, overexpression of BIR-1 does not arrest apoptosis in developing embryos, nor does the depletion of BIR-1 result in increased cell death (Fraser et al., 1999). The cells of embryos lacking BIR-1 showed a failure in cytokinesis, which can be restored, in part, by the transgenic expression of Survivin (Fraser et al., 1999). It is thus possible that Survivin likely evolved as a protein with dual roles in mitosis and apoptosis.

As mentioned earlier, the likely role of Survivin as a direct inhibitor of Caspases remains a controversial area of scientific research. Early studies suggested that Survivin directly binds to Caspases -3 and -7, thereby inhibiting apoptosis in cells exposed to various death-inducing stimuli (Tamm et al., 1998). Also, interference with Survivin expression or binding to microtubules resulted in mitotic abnormalities, and increased caspase-3 activity

with subsequent apoptosis in HeLa cells (Li et al., 1999). However, crystal structure determinations show that Survivin exists as a dimer (Verdecia et al., 2000) in functional form, raising several questions regarding the mechanism by which, if at all, Survivin binds directly to Caspases. The BIR domain of Survivin is closer in structural homology to XIAP BIR2 rather than BIR1 or BIR3. Since Survivin lacks the structural equivalent of the linker region between XIAP BIR1 and BIR2, it has been hypothesized that Survivin should not be able to directly bind to activated Caspase-3 (Verdecia et al., 2000; Wheatley et al., 2005). Still, the description of the crystal structure of Survivin has not precluded the appearance of reports in the literature indicating that Survivin binds directly to Caspase-3 and -7 (Shin et al., 2001) and is a continued cause for controversy owing to results obtained in other studies refuting these findings (Banks et al., 2000). Identification of three distinct isoforms of Survivin with differential tissue expression patterns as well as differing anti-Caspase functions has only served to complicate this issue further (Conway et al., 2000). A mitochondrial-specific Survivin has been identified and shown to be released in response to apoptotic stimuli from the intermembrane space of the mitochondrion into the cytosol where it prevents Caspase activation, yet promotes tumorigenesis (Dohi et al., 2004a). It has also been suggested that only the released mitochondrial Survivin is capable of blocking apoptosis, and not the Survivin already present in the cytosol prior to the apoptotic stimulus (Dohi et al., 2004a) although the mechanism by which this occurs has not been elucidated. How cytosolic Survivin is unable to inhibit Caspase-9, in contrast to mitochondrial Survivin raises several questions from the structural point of view. Some light has been thrown on these questions by a recent study indicating that the different functions of Survivin may be ascribed to its heterodimerization with Survivin splice variants (Caldas et al., 2005). Caldas

*et al.* demonstrated that the Survivin isoforms Survivin-2B and Survivin-ΔEx3 are expressed endogenously in Daoy and HeLa tumor cells and translated into stable proteins. Furthermore, it was shown that Survivin and Survivin-ΔEx3 interact within the mitochondria and inhibit mitochondrial-dependent apoptosis (Caldas et al., 2005). The finding that human c-IAP1 – Caspase-7 direct interaction occurs in a manner independent of the catalytically active-site pocket, in sharp contrast to XIAP – Caspase-7 interaction (Tenev et al., 2005) does raise the possibility that Survivin may be able to bind and regulate the function of Caspases through a distinct mechanism. Besides the various hypotheses in the literature regarding the nature and possible existence of direct Survivin-Caspase binding, researchers have also suggested that the regulation of Caspase function by Survivin may involve an indirect interaction. Dohi and coworkers have suggested that Survivin associates with XIAP, forming a complex in response to an apoptotic stimulus, suppresses Caspase-9 activity *in vitro* and exerts a synergistic cytoprotective effect *in vivo* (Dohi et al., 2004b). This study however, relied on co-immunoprecipitation experiments involving GST-fusion proteins to determine direct binding, and did not look at the possibility of the participation of other proteins in this complex. Furthermore, it has recently been articulated that GST tags endow BIR domains with artifactual properties and is related to the propensity of the GST tag to oligomerize which increases binding (Eckelman and Salvesen, 2006).

Whatever the case may be regarding the mechanism of action of Survivin, its close association with cancers is universally accepted. Survivin is barely detectable in terminally differentiated normal tissues or cells, but its expression is markedly increased in most tumor cells, making it a potentially useful tool for cancer diagnosis and possibly, therapy. It is an IAP consistently shown to express at elevated levels in tumor cells, and its role in mitotic

progression in dividing tumor cells may be a function of that. The functional loss of the tumor suppressor p53 in many tumor cells is associated with upregulation of Survivin. Survivin levels, in part, are regulated by transcriptional parameters. The *survivin* promoter consists of three cell cycle-dependent elements (CDEs) and a cell cycle homology region (CHR), similar to other genes active during mitosis (Wheatley et al., 2005). In addition, the promoter region contains binding sites for p53 (Mirza et al., 2002) and the RB tumor suppressor protein (Jiang et al., 2004) which repress the promoter activity, and a binding site for the transcription factor E2F1 (Jiang et al., 2004) for its activation. p53 may also regulate Survivin levels through $p21^{Waf1/Cip1}$ via the cell cycle $G_1$/S checkpoint (Lohr et al., 2003). The relationship between Survivin and p53 however, is not limited just to the repression of Survivin by p53. A circular relationship has been established between the two proteins, wherein, overexpression of Survivin leads to repression of p53 transcription and promotes degradation of p53 protein by ubiquitin-mediated proteolysis (Wang et al., 2004). Post-translational regulation of Survivin is achieved by its ubiquitination and subsequent degradation by the proteasome during the $G_1$ phase in a cell cycle dependent manner (Zhao et al., 2000). The IAP antagonist Smac/DIABLO has been shown to co-immunoprecipitate with Survivin and promotes the degradation of Survivin by the ubiquitin-proteasome pathway (McNeish et al., 2005).

***RING E3 Ligases and Ubiquitination***

Ubiquitination has emerged as a key regulatory mechanism in eukaryotic cells for regulating the half-life of proteins by targeting them for proteasomal degradation. This is brought about by the conjugation of the 76-amino acid ubiquitin peptide to protein substrates involving a cascade of three to four enzymes known as E1 (ubiquitin-activating enzyme), E2

(ubiquitin-conjugating enzyme), E3 (ubiquitin protein ligase) and E4 respectively. Ubiquitin is activated by E1 and conjugated by E2 to a lysine residue identified by the ubiquitin protein ligase E3 in the target protein. E3 ligases thus provide specificity for ubiquitin-conjugating activity, and hence proteasomal degradation (Pickart, 2001). Several members of the IAP family possess a carboxy-terminus RING Zn finger motif characterized by seven cysteines and one histidine that chelate two zinc ions (Borden, 2000). Interestingly, RING finger proteins have been recognized as the largest class of E3 ubiquitin ligases (Joazeiro and Weissman, 2000) and thus presents another manner by which IAPs may regulate apoptosis. However, as mentioned earlier, not all IAPs are created equal, and appear to function in a distinct manner with regard to ubiquitination. XIAP and c-IAP2 have been reported to trigger the ubiquitination of Caspase -3 and -7 *in vitro* (Huang et al., 2000; Suzuki et al., 2001c) . Some studies have shown that the IAP antagonist Smac/DIABLO is a substrate for XIAP-mediated ubiquitination *in vitro* but does not undergo degradation (MacFarlane et al., 2002). Other studies indicate that although XIAP and Livin can promote Smac/DIABLO ubiquitination, only c-IAP1 and c-IAP2-mediated ubiquitination results in proteasomal degradation of Smac/DIABLO (Yang and Du, 2004). Interestingly, it seems, the mammalian IAP antagonist Smac/DIABLO can also promote auto-ubiquitination and degradation of the IAPs themselves. Proteasomal loss of Smac/DIABLO induced by c-IAP1 and c-IAP2 is associated with loss of the IAP itself (Yang and Du, 2004). In another study, Smac3, a Smac/DIABLO splicing variant that retains the IAP-binding motif (IBM), promoted the auto-ubiquitination and degradation of XIAP (Fu et al., 2003). It is possible that one mechanism of Smac antagonism of IAPs, and vice-versa, involves a self-sacrificial co-degradation process.

Thus, in contrast to the known properties of the BIR domains of IAPs in inhibiting Caspases, the RING domains have been shown to perform the dual function of not only targeting Caspases and other pro-apoptotic binding partners of the IAPs for ubiquitination and proteasomal degradation, but also the targeting of the IAPs themselves for proteasomal degradation in response to apoptotic triggers. Whether this added function of auto-ubiquitination of the IAPs by the RING domains accentuates their anti-apoptotic activity or antagonizes it, remains unclear owing to the mixed results obtained in some studies. In an early study, treatment of cells with etoposide or glucocorticoids led to the degradation of XIAP and c-IAP1, which was blocked by the use of proteasomal inhibitors (Yang et al., 2000). This study further demonstrated that the over-expression of RING-deleted XIAP and c-IAP1 mutants in cell culture attenuated the auto-ubiquitination and proteasomal degradation of these IAPs and was associated with improved protection against apoptotic triggers compared to over-expression of wild-type XIAP and c-IAP1. Another study however, indicated that XIAP RING-deletion mutants were less efficacious in protecting cells from apoptosis induced by the over-expression of Caspase-3 or Fas than wild-type XIAP (Suzuki et al., 2001c). A possible explanation for this supposedly contradictory behavior may lie in the abundance of IAP binding partners, and the diverse structural mechanisms by which this interaction may occur. Furthermore, the IAPs may function in non-apoptotic healthy living cells as E3 ligases designed to promote the degradation of accidentally activated Caspases or accidental release of small amounts of mitochondrial IAP antagonists such as Smac/DIABLO. Under such conditions, the co-degradation of IAPs would not result in significant decreases in IAP levels, and as a consequence, no increase in

apoptosis. However, under conditions of severe apoptotic stress, the significant loss of IAPs through auto-ubiquitination would be sufficient to trigger cell death.

**IAP Antagonists**

The anti-apoptotic activity of the IAPs must be strictly regulated in order to maintain tissue homeostasis. In addition to the mechanisms outlined earlier, three proteins, XIAP-Associated Factor1 (XAF1), second mitochondrial activator of caspases protein a.k.a Direct IAP Binding protein with Low PI (Smac/DIABLO) and Omi/HtrA2, have been identified that directly bind IAPs and suppress their activity.

***Smac/DIABLO***

The Smac/DIABLO protein resides in the inner mitochondrial membrane space of healthy cells and is released into the cytosol along with cytochrome *c* upon receiving an apoptotic signal via the intrinsic apoptotic pathway. During release, the amino-terminal 55 amino-acid mitochondrial localization signal peptide of Smac/DIABLO is proteolytically cleaved (Du et al., 2000; Verhagen et al., 2000) and the active, mature Smac/DIABLO protein binds to and neutralizes IAPs. Smac has been demonstrated to bind to all IAPs tested (Du et al., 2000; Vucic et al., 2002) and can bind to either BIR2 or BIR3 of XIAP, thereby interfering with either Caspase -3, -7, or Caspase-9 inhibition. However, Smac/DIABLO - XIAP interaction is much stronger on BIR3 and the newly generated amino terminus is critical for this interaction (Srinivasula et al., 2000). A co-crystallization examination of Smac/DIABLO and XIAP BIR3 demonstrated that the amino-terminal tetrapeptide sequence of mature Smac/DIABLO (Ala-Val-Pro-Ile) functions as an IAP Binding Motif (IBM) and binds to the surface groove of the BIR3 domain (Liu et al., 2000). Further elaboration of this

structural interaction established that the surface contacts between Smac/DIABLO and XIAP BIR3 overlap completely with those of Caspase-9/XIAP BIR3 contacts (Sun et al., 2000). The tetrapeptide IBM sequence of Smac/DIABLO is similar to the cleavage site in the linker region of Caspase-9 ($Ala_{316}$-Thr-Pro-Phe) and it was shown that $Ala_{316}$ cleavage site mutants of Caspase-9 were fully active and not inhibited by XIAP (Ekert et al., 2001; Srinivasula et al., 2001). Taken together, these findings suggested that Smac promotes apoptosis by relieving partially processed Caspase-9 from binding to XIAP BIR3 in a competitive manner. In addition to the direct binding and inhibition of anti-caspase activity of IAPs by Smac/DIABLO, the identification of Smac splice variants that promote auto-ubiquitination of IAPs (see above), yields another mechanism by which this protein and its isoforms negatively regulate IAP function and allow the apoptosis program to continue.

***Omi/HtrA2***

A second mitochondrial IAP-binding protein called Omi or HtrA2 was identified (Hegde et al., 2002; Suzuki et al., 2001a; Verhagen et al., 2002) subsequent to the identification of Smac/DIABLO. It is a ubiquitously expressed mitochondrial serine protease that shares sequence and structural homology to the *Escherichia coli* serine protease high temperature requirement A (HtrA). At normal temperatures, HtrA functions as a chaperone. However, at higher temperatures, it acts as an endoprotease that targets damaged or misfolded proteins for degradation (Spiess et al., 1999). Considering the extensive homology between the mammalian and bacterial genes, it was hypothesized that the mammalian Omi/HtrA2 may function in a similar manner to *Escherichia coli* HtrA. This was supported by the finding that both the expression and proteolytic activity of Omi/HtrA2 were upregulated in response to heat shock (Gray et al., 2000). The nuclear-encoded precursor

Omi/HtrA2 protein possesses an amino-terminal mitochondrial localization sequence which is cleaved upon import of the protein into the mitochondria. This is followed by the processing and removal of a second leader sequence resulting in the generation of mature Omi/HtrA2 possessing an exposed amino-terminal IBM (Hegde et al., 2002; Suzuki et al., 2001a; Verhagen et al., 2002). Once released into the cytosol by an apoptotic trigger that causes permeabilization of the outer mitochondrial membrane (OMM), it contributes to apoptosis by both Caspase-dependent and Caspase-independent ways. Similar to Smac/DIABLO, Omi/HtrA2 binds and inhibits XIAP, c-IAP1, and c-IAP2, thereby promoting the activity of Caspases -3, -7, and -9 (Hegde et al., 2002; Suzuki et al., 2001a; Verhagen et al., 2002). However, it was found that Omi/HtrA2 binds preferentially to XIAP BIR2, in contrast to Smac/DIABLO (Verhagen et al., 2002). Omi/HtrA2 further contributes to Caspase-dependent apoptosis through proteolytic cleavage of its IAP binding partners (Srinivasula et al., 2003; Suzuki et al., 2004; Yang et al., 2003). In their report, Suzuki *et al.* also indicate that extramitochondrial expression of an ubiquitin-fusion Omi/HtrA2 construct also induces permeabilization of the OMM with subsequent cytochrome *c* release and activation of Caspases, thereby suggesting another possible mechanism by which Omi/HtrA2 promotes Caspase-dependent apoptosis.

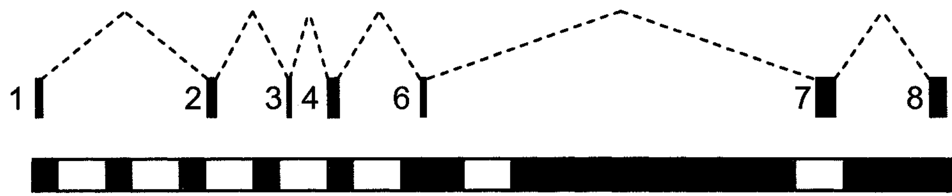
Additionally, it is assumed that Omi/HtrA2 may contribute to apoptosis in a Caspase-independent manner through its serine protease activity. This function is independent of its IAP-binding activity and involves the proteolysis of yet to be identified target proteins (Hegde et al., 2002; Suzuki et al., 2001a; Verhagen et al., 2002).

### *XAF1*

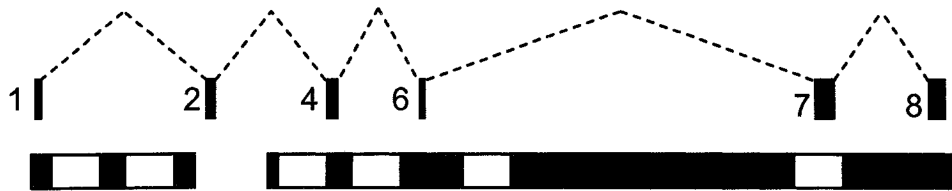
The 34 kDa zinc-finger rich nuclear protein XAF1, was isolated by yeast two-hybrid screening with XIAP (Liston et al., 2001). In addition to the original xaf1 cDNA subsequently, two other alternatively spliced xaf1 cDNAs, xaf1B and xaf1C, were identified (Fong and Korneluk, unpublished results) (Yin et al., 2006) (see Figure 1-5). XAF1 contains seven zinc fingers, while splicing of the 57 bp exon 3 results in an in-frame 19 amino-acid deletion and a complete loss of the third zinc finger in XAF1B (see Figure 1-5). The XAF1C isoform exhibits a truncation of the protein distal to the fifth zinc finger along with the addition of 24 novel amino-acids to the carboxy-terminus (see Figure 1-5). Interaction between XIAP and XAF1 was confirmed *in vitro* using purified recombinant proteins and it was further demonstrated that this interaction interferes with XIAP-mediated Caspase-3 inhibition (Liston et al., 2001). The XAF1B isoform also binds XIAP, however, XAF1C does not (Fong and Korneluk, unpublished results). XAF1 overexpression using recombinant adenoviruses resulted in reversal of XIAP-mediated protection to apoptosis induced by etoposide. Subcellular distribution analysis showed that expression of XAF1 triggers a redistribution of most of the endogenous XIAP protein to the nucleus. This suggested that possible sequestration of XIAP in the nuclear inclusions by XAF1, in addition to its biochemical neutralization, aids in the reversal of Caspase inhibition (Liston et al., 2001). A recent report has indicated a similar mechanism for the suppression of XIAP activity by XAF1 in rat neurons following transient focal ischemia of the rat brain, as evidenced by the redistribution of the two proteins, resulting in enhanced neuronal susceptibility to degeneration upon reperfusion (Siegelin et al., 2005).

**Figure 1-5. XAF1 Splice Isoforms.**

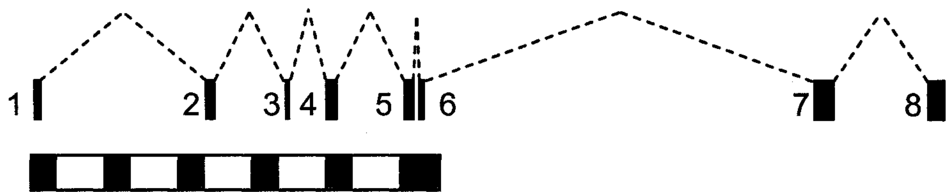
Three cDNAs are formed by alternative splicing of the *xaf1* gene which contains 8 exons. XAF1 represents the originally isolated xaf1 cDNA and contains 7 zinc fingers. Splicing of exon3 results in an in-frame deletion of the third zinc finger in XAF1B. The addition of exon5 into XAF1C causes the truncation of the protein after the fifth zinc finger. Black boxes represent exons and are numbered. White blocks represent zinc fingers.




XAF1




XAF1B




XAF1C

Xaf1 mRNA is ubiquitously expressed in normal tissues, but is expressed at very low levels or not at all in majority of the cancer cells of the 60-cell-line panel of the National Cancer Institute (NCI) (Fong et al., 2000; Liston et al., 2001) indicating that XAF1 downregulation may be important in the development of malignancy. Other studies have also documented the low levels of XAF1 expression in primary malignancies such as human gastric adenocarcinomas (Byun et al., 2003), melanomas (Ng et al., 2004), colorectal cancers (Ma et al., 2005), prostate cancer (Fang et al., 2006), and urogenital malignancies (Lee et al., 2006). The *xaf1* gene was localized by fluorescence *in situ* hybridization (FISH) analysis to the locus 17p13.2, distal to the *p53* gene (Fong et al., 2000). This region is associated with a loss of heterozygosity of polymorphic markers in cancer cells and a decrease in heterozygosity within the *xaf1* region was observed in the NCI 60-cell-line panel. However, this allelic loss failed to explain the significant reduction in XAF1 expression since many of the cell lines tested did not show a homozygous deletion of the *xaf1* gene (Fong et al., 2000). Characterization of a possible transcriptional regulatory mechanism responsible for the low levels of expression has indicated a strong association between hypermethylation of CpG sites in the promoter region of *xaf1* gene and its silencing in cancers (Byun et al., 2003; Fang et al., 2006; Lee et al., 2006). Aberrant DNA methylation has been implicated not only in the downregulation of the xaf1 transcript, but also for splicing alterations resulting in deletions in the zinc finger domains that interfere with the functional interaction between XAF1 and XIAP (Fang et al., 2006). Inhibition of DNA methylation results in expression of full length xaf1 mRNA.

Gene array studies have identified *xaf1* as a novel interferon (IFN)-stimulated gene associated with IFN-β-induced apoptosis (Leaman et al., 2002). Although xaf1 mRNA is

upregulated by IFN-α and IFN-β, high levels of XAF1 are predominantly seen in cell lines sensitive to the pro-apoptotic effects of IFN-β. Conversely, tested melanoma cell lines resistant to apoptosis displayed little or no upregulation of XAF1 protein expression. A correlation was also found between XAF1 and a critical mediator of IFN-β-induced apoptosis, namely, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Leaman et al., 2002). Melanoma cell lines resistant to IFN-β-induced apoptosis were resistant to recombinant TRAIL protein as well. Pre-treatment of these cell lines with IFN-β to induce XAF1, or overexpression of XAF1 in these cells increased sensitivity of these cells to TRAIL-induced apoptosis (Leaman et al., 2002). A recent study has demonstrated that pre-treatment of ACHN renal cell carcinoma and A375 melanoma cells, both of which are resistant to IFN-β-induced apoptosis, with the DNA demethylating nucleoside analog 5-AZA-2′-deoxycytidine (5-AZA-dC) augmented the expression of IFN-stimulated genes, particularly *xaf1* by up to 10 fold and sensitized the cells to apoptosis (Reu et al., 2006). Overexpression of XAF1 was able to sensitize these cells to IFN-induced apoptosis. Conversely, siRNA to xaf1 following pre-treatment with 5-AZA-dC was shown to inhibit IFN-β-induced apoptosis (Reu et al., 2006).

XAF1 has also been implicated to play a role in the regulation of the cell cycle. Our laboratory has previously observed (Fong and Korneluk, unpublished results) that overexpression of XAF1 by adenoviral vectors in human fibroblast cells HEL 299 and WI-38 induced a $G_1$/S cell cycle arrest and is associated with an upregulation of the p21 $^{Waf1/Cip1}$ cell cycle protein. Recently, it was reported that XAF1 overexpression in cell cultures results in a post-translational activation of p53 with the resultant activation of p53-target genes (Lee et al., 2006).

**p53**

p53 is a multi-faceted tumor suppressor gene that is capable of inducing temporary growth arrest and DNA repair, irreversible growth arrest, terminal differentiation, or apoptosis in response to potentially oncogenic cellular stress such as DNA damage (Sigal and Rotter, 2000). Therefore, it is imperative that functional p53 be present *in vivo* for tumor growth suppression (Jimenez et al., 2000). The function of the p53 gene is lost by mutation in over 50% of human cancer and a loss of heterozygosity often accompanies tumor progression (Haupt and Haupt, 2004; Vogelstein and Kinzler, 1992), (Haupt and Haupt, 2004). Unlike many other tumor suppressor genes, more than 85% of p53 mutations result in single amino-acid substitutions rather than deletions or frame-shifts (Slee et al., 2004). Most of the missense mutations occur in the DNA binding core domain (amino acids 102-292) region of p53 that is evolutionarily conserved between p53 and its homologues from *Drosophila* and *C. elegans*. In human tumors, amino-acid residues that are essential for contact with DNA target sequence (two repeats of PuPuPuC(A/T)(A/T)GpyPyPy; in which Pu is a purine and Py is a pyrmidine) are frequently found to be mutated (Haupt and Haupt, 2004). In addition, mutations of residues that do not contact DNA directly but are required for structural maintenance also cause disruption of the p53-DNA interaction. Frequently, mutations in one allele are sufficient to interfere with p53-dependent apoptosis by a dominant negative mechanism since in most cases mutant p53 negates wild-type p53 function through heteromerization.

Under normal conditions, p53 has a short half-life and is maintained at very low levels by Mdm2-mediated degradation (Haupt et al., 1997). However, in response to stress by DNA damage, hypoxia, oxidative stress or oncogene activation, p53 is stabilized and

activated by post-translational modification (Haupt and Haupt, 2004). In tumor cells, transcriptionally inactive mutant p53 is unable to induce the expression of the Mdm2 protein which would normally provide a feedback mechanism that down-regulates p53 protein levels (Midgley and Lane, 1997). Moreover, some p53 mutants exhibit lower affinity for association with Mdm2 (Buschmann et al., 2000). Hence, mutant p53 proteins which are impervious to these negative regulations accumulate to high levels in cancer cells and negate the functions of the wild-type protein.

Pathways through which p53 induces apoptosis may involve both transcriptional transactivation and transrepression of multiple p53-target genes, as well as transcription-independent mechanisms that engage the mitochondrial-apoptotic pathways (Slee et al., 2004). In general, apoptotic target genes of p53 may be divided into two major categories: 1) proteins acting at the level of receptor signaling for apoptosis and 2) proteins acting downstream by activating apoptotic effector proteins (Sionov and Haupt, 1999). The former includes the insulin-like growth factor-1-binding protein 3 (IGF-BP3), which induces apoptosis by blocking the IGF-1 survival signal (Buckbinder et al., 1995) and Fas/APO-1/CD95, that functions in the T-cell killing triggered by anti-cancer drugs (Friesen et al., 1996). Essential downstream p53-targeted apoptotic effector proteins are primarily associated with mitochondrial changes, including Caspase-9 and its cofactor Apaf-1 in Myc oncogene-induced apoptosis (Soengas et al., 1999), and Bax, necessary for p53-mediated cell death in brain tumors (Yin et al., 1997). In addition to acting as a regulatory gene coordinating the expression of many proteins involved in apoptosis, recent research also suggests that p53 is involved in mediating apoptosis at the mitochondrial level by directly

and physically interacting with the Bcl-2 member Bak, resulting in the release of cytochrome *c* from the mitochondria (Erster et al., 2004; Leu et al., 2004).

**Rationale and Outline for the Thesis Project**

XIAP-Associated Factor 1 (XAF1) was identified as a novel XIAP antagonist which interestingly resides in the nucleus. The expression of XAF1 is significantly reduced in cancer cell lines and several primary malignancies as a result of silencing by promoter hypermethylation. Subsequent characterization of the *XAF1* gene localized it to chromosome 17, telomeric to the tumor suppressor *p53* gene. Our laboratory had previously observed that XAF1 overexpression in normal cell lines results in cell cycle arrest in the $G_1$ phase raising the possibility that XAF1 may function as a potential tumor suppressor protein with an ability to impede cell growth and reverse the protective effect conferred by XIAP to apoptosis in cancer cells. Survivin is a unique IAP with roles in cell cycle progression and cell death. It is negatively regulated by p53 in a reciprocal manner and is markedly upregulated in most cancers. Furthermore, it was suggested that survivin recruits XIAP, forming an anti-Caspase complex in response to apoptotic stimuli (Dohi et al., 2004b).

XAF1 appears to play an opposing role to that of Survivin, both in terms of function as well as reciprocal expression in normal tissues vs. cancers. This suggests that XAF1 may play a critical role in cancer progression by acting as a negative regulator of Survivin function. This thesis documents the possible nature of this interaction between the two proteins. Keeping in view the negative relationship between p53 and Survivin, I began by further elaborating the role of XAF1 in the cell cycle with regard to the p53 status of the cell by FACS and Western Blot analysis. I was able to determine that XAF1 induces cell cycle

arrest in normal cells in a p53-dependent manner. Subsequently, I examined Survivin protein expression levels in cancer cells in response to XAF1 overexpression. Interestingly, XAF1 overexpression was able to downregulate Survivin protein expression irrespective of the p53 status of the cell, suggesting a p53-independent mechanism for this negative regulation. *Xaf1* has been shown to be an IFN-stimulated gene (Leaman et al., 2002). In order to demonstrate that endogenous XAF1 expression results in the downregulation of Survivin, I treated several cell lines with IFN-β and examined protein expression profiles in these cells by Western Blot analysis. Following this, I attempted to determine whether XAF1 binds directly to Survivin and other IAPs besides XIAP by co-immunoprecipitation experiments. Furthermore, I also investigated the ability of XAF1 to interfere with the formation of the proposed survivin-XIAP complex (Dohi et al., 2004b). I found that XAF1 does not bind directly to Survivin, and was unable to show an interaction between Survivin and XIAP in the absence of XAF1 by co-immunoprecipitation experiments. Surprisingly, and interestingly, I found that co-transfection of cells with expression plasmids encoding XAF1, XIAP and Survivin resulted in significantly lower levels of Survivin protein in cells compared to those transfected with Survivin alone, or Survivin and XIAP only, as determined by Western Blot analysis. Also, co-immunoprecipitation analysis demonstrated a definite interaction between the three proteins. These findings suggested that XAF1 may regulate Survivin levels by promoting the E3 ubiquitin ligase activity of XIAP, and experiments with proteasomal inhibitors and a XIAP mutant construct lacking in E3 ligase activity confirmed this assumption. Finally, I pre-treated cancer cells with RNAi against XIAP, followed by adenoviral overexpression of XAF1 to further characterize the XIAP-mediated downregulation of Survivin by XAF1.

*Chapter 2*

# MATERIALS AND METHODS

## Cell Culture and Harvesting

All media were supplemented with heat-inactivated 10% fetal bovine serum (FBS) (Invitrogen), 1 U/ml penicillin and 1 $\mu$g/ml streptomycin (Fisher) and all cell cultures were maintained at 37°C in 5% $CO_2$ in 100 mm tissue-culture grade dishes (Corning) and passaged every 48-72 hours. Human embryonic lung fibroblast cells: HEL 299 and WI-38, human embryonic kidney (HEK) 293T cells, and human malignant melanoma A375 cells (ATCC), were maintained in Dulbecco's minimal essential medium (DMEM) (Invitrogen). Human malignant melanoma cell line SK-MEL-5 (ATCC) and renal cell carcinoma cell line ACHN (ATCC) were maintained in minimum essential medium (MEM) (Invitrogen), supplemented with 1% non-essential amino acids (NEAA) (Gibco). Human neuroblastoma cell line SK-N-AS was maintained in DMEM supplemented with 1% NEAA and 1% glutamine (Gibco). Human neuroblastoma cell line SK-N-BE(2) was maintained in Dulbecco's modified eagle's medium/nutrient mixture F-12 Ham's (DME/F-12 1:1) (Invitrogen), supplemented with 1%NEAA and 1% glutamine. Primary wild-type mouse embryonic fibroblast (wt MEF) cells and p53 -/- mouse embryonic fibroblast (p53-/- MEF) cells were maintained in minimum essential medium - alpha modification (MEM Alpha) (Invitrogen), supplemented with 1% NEAA and 1% glutamine.

Cells were harvested from dishes by scraping and re-suspended in 1ml PBS. The cell suspensions were collected in eppendorf tubes, incubated on ice and centrifuged at 4°C at 200 x g for 5 minutes. The supernatant was discarded and the cell pellets lysed in 50 mM

Tris-HCl, pH 8.0, containing 150 mM NaCl, 1% Triton-X 100, 1mM NaF and a cocktail of protease inhibitors consisting of 0.1mM phenylmethylsulfonyl fluoride (PMSF), 10 $\mu$g/ml aprotonin, 5 $\mu$g/ml pepstatin A, and 10 $\mu$g/ml leupeptin (lysis buffer-1). The cell lysates were incubated on ice for 20 minutes followed by centrifugation at 12000 x g for 15 minutes to pellet the insoluble cell debris. Alternatively, in order to obtain whole cell extracts, cells were harvested and lysed in 1% SDS, 62.5mM Tris-HCl, pH 6.8, containing protease inhibitors (lysis buffer-2). The cell lysates were incubated at room temperature for 15 minutes, sonicated in a water bath for 10 minutes, and subsequently boiled for 5 minutes. The samples were allowed to cool, and centrifuged at room temperature at 12000 x g for 15 minutes. The supernatant from each lysate sample was transferred to a new eppendorf tube and the pellet discarded. Protein content was determined by the Bio-Rad protein assay using bovine serum albumin (BSA) as a standard.

**Plasmid DNA Constructs and Transient Transfections**

The primers 5′-CGGATCCATGGGACCTAAAGACAGT and 5′-CTCGAGCTAGGACAGGAAGGTGCGCACGCGGCTGC were used to amplify a full-length livin cDNA clone (Open Biosystems) using Platinum® *Pfx and Taq* DNA polymerase (Invitrogen) as per the manufacturer's protocol. The amplified PCR product was cloned into the PCR2.1 vector using the TOPO TA cloning kit (Invitrogen) and sequenced. The coding region was then subcloned using BamH1/Xho1 ligation sites into a 6myc-pcDNA3 expression vector. Mini-prep plasmid DNA was isolated by the standard alkaline lysis method (Current Protocols in Molecular Biology, Ch. 1.6). Maxi-prep plasmid DNA was

prepared using the Genelute HP Plasmid Maxiprep Kit (Sigma) as per the manufacturer's protocol.

The following plasmid DNA constructs were obtained courtesy of various members of Dr. Korneluk's and Dr. Liston's research groups: pCI-XAF1, 6-myc-pcDNA3-XAF1, 6-myc-pcDNA3-XIAP, 6-myc-pcDNA3-c-IAP1, 6-myc-pcDNA3-c-IAP2, 6-myc-pcDNA3-NAIP, 6-myc-pcDNA3-TsIAP, 6-myc-pcDNA3-Survivin, pcDNA3-Survivin, and the XIAP RING Finger mutant 6-myc-pcDNA3-$XIAP_{H467A}$ in which replacement of $His_{467}$ residue with Ala results in loss of E3 ubiquitin ligase activity (Yang et al., 2000).

Cells were seeded at low to medium density in 6-well plates and transfections were performed in triplicate using Lipofectamine 2000$^{TM}$ (Invitrogen) as described in the manufacturer's protocol.

**Recombinant Adenoviral Vectors and Cell Infection**

The adenoviral vectors adeno-lacZ, adeno-xaf1, adeno-p53, and adeno-xiap were generated as reported previously (Liston et al., 2001). GFP-RNAi-luciferase and GFP-RNAi-xiap were generated by Dr. Dan McManus and Charles Lefebvre (Aegera Therapeutics Inc.). The dominant-negative p53 mutant adenoviral construct adeno-p53 22/23*ΔPro lacking the N-terminal Transactivation domain 1 and Polyprolene domain was provided courtesy of Dr. Ruth Slack (University of Ottawa). Cells were seeded at low density in 60-100 mm dishes and transduced at a multiplicity of infection (MOI) of 50 plaque forming units (pfu)/cell. Transduced cells were collected 48 h post-infection or exposed to further treatment with various agents before harvesting.

**Preparation of Cells for Fluorescence Activated Cell Sorting (FACS) Analyses**

Cells were harvested and centrifuged for 5 minutes at 200 x g. The supernatant was discarded and the pellet re-suspended in 1 ml of PBS, pH 7.0, by vortexing at approximately ¾ maximum speed. While continuing to vortex, 4 ml of -20°C absolute ethanol was added. The sample was fixed at -20°C for at least 15 minutes. The ethanol fixed cells were subsequently centrifuged for 5 minutes at 200 x g. The ethanol was carefully aspirated and the cells re-suspended in 1 ml of PBS, pH 7.0, by vortexing. 100 $\mu$l of DNase free 0.1 mg/ml stock RNase solution (Roche Applied Science) was added and the samples incubated at 37°C for 30 minutes. 20 $\mu$l of 1mg/ml propidium iodide solution (BD Biosciences Pharmingen) was added to each sample and allowed to incubate for at least 10 minutes at room temperature in the dark. Subsequently, FACS analyses were performed using a Coulter XL cytometer (Coulter Canada).

**Treatment of cells with Proteasomal Inhibitors**

Untransfected or transfected 293T cells were treated with 10$\mu$M proteasomal inhibitor MG-132 (Calbiochem) at 20 h post-transfection for 4 h. The cells were harvested at 24 h post-transfection.

**Interferon β treatment**

Untransduced and adenoviral vector transduced cells were treated with recombinant interferon-β (IFN-β) (Sigma) 500U/ml 48 h post-infection for a period of 24 h and harvested for subsequent analysis.

**Immunoprecipitation**

100 mm dishes of transfected and untransfected cells were rinsed in 1X PBS. The cells were harvested by scraping, re-suspendeded in an eppendorf tube with 500 $\mu$l of 1X PBS, and centrifuged for 5 minutes at 4°C at 700 x g. The supernatant was discarded and the cell pellet re-suspended in lysis buffer (50 mM Tris-HCl, pH 6.8, 150 mM NaCl, 1% Triton-X 100) containing protease inhibitors. Lysates were incubated on ice for 20 minutes and then centrifuged at 12000 x g for 20 minutes. Protein quantification was done by Bio-Rad protein assay using BSA as a standard as above. 250 $\mu$g protein extract per lysate sample was used to perform the immunoprecipitation using the anti-c-Myc Immunoprecipitation kit (Sigma) as instructed by the manufacturer's protocol.

**Western Blot Analysis**

Protein extracts (10 $\mu$g/lane) in 1X sample buffer (62.5 mM Tris-HCl, pH 6.8 containing 2% SDS, 5% glycerol, 1% βME, 0.1% bromphenol blue) and dual color protein markers (BIO RAD) were electrophoresed on discontinuous polyacrylamide gels with an upper stacking gel (4.8% acrylamide mix, 125 mM Tris-HCl, pH 6.8, 0.1% SDS, 0.1% ammonium persulfate) and a lower separating gel (12% – 15% acrylamide mix, 380mM Tris-HCl,, pH 8.8, 0.1% SDS, 0.1% ammonium persulfate). Proteins were transferred onto Protran® BA85 0.45 $\mu$m Nitrocellulose membrane (Whatmann) at 15V for 30 minutes using a semi-dry transfer apparatus (Hoefer Semiphor). Blots were placed in Licor blocking buffer (LI-COR):PBS 1:1 and incubated for 1 hour. Subsequently, blots were probed with primary antibody diluted in Licor:PBS 1:1, 0.1% Tween-20 and incubated overnight. Blots were washed in 1X TBS-T (20mM Tris-HCl, pH 7.5, 250mM NaCl, 0.1% Tween-20) 4 x 6

minutes and then incubated with AlexaFluor 680 fluorescent-conjugated secondary antibodies (Molecular Probes) diluted in Licor:PBS 1:1, 0.1% Tween-20 and incubated for 1 hour. The blots were then washed in 1X TBS-T 6 x 5 minutes, followed by 2 x 5 minute washes in PBS. The blots were allowed to dry and antibody binding was detected by imaging of the blots using the Odyssey Infrared Imaging System and software (LI-COR) at the requisite wavelength emissions.

**Antibodies**

Polyclonal anti-XAF1 and anti-XIAP antibodies were generated by immunizing rabbits with GST-XAF1A or GST-XIAP fusion protein in RIBI adjuvant (Sigma) as previously described (Liston et al., 2001). The following primary antibodies with the stated dilutions were used to detect individual proteins for Western immunoblot analysis: polyclonal anti-XAF1 at 1:500, polyclonal anti-p53 (Cell Signaling) at 1:1000, monoclonal anti-p21$^{Waf1/Cip1}$ at 1:2000, monoclonal anti- mouse p53 (1C12) mAb (Cell Signaling) at 1:2000, monoclonal anti- mouse p21$^{Waf1/Cip1}$ mAb (Biosource) at 1:250, polyclonal anti-XIAP at 1:1000, monoclonal anti-Survivin (Cell Signaling) at 1:1000, monoclonal anti-Myc tag (Stressgen) at 1:1000, and monoclonal anti-β Actin (Sigma) at 1:5000. Bound primary antibodies were conjugated with Alexa Fluor® 680 goat anti-mouse IgG or Alexa Fluor® goat anti-rabbit IgG at 1:10000.

*Chapter 3*

# Results

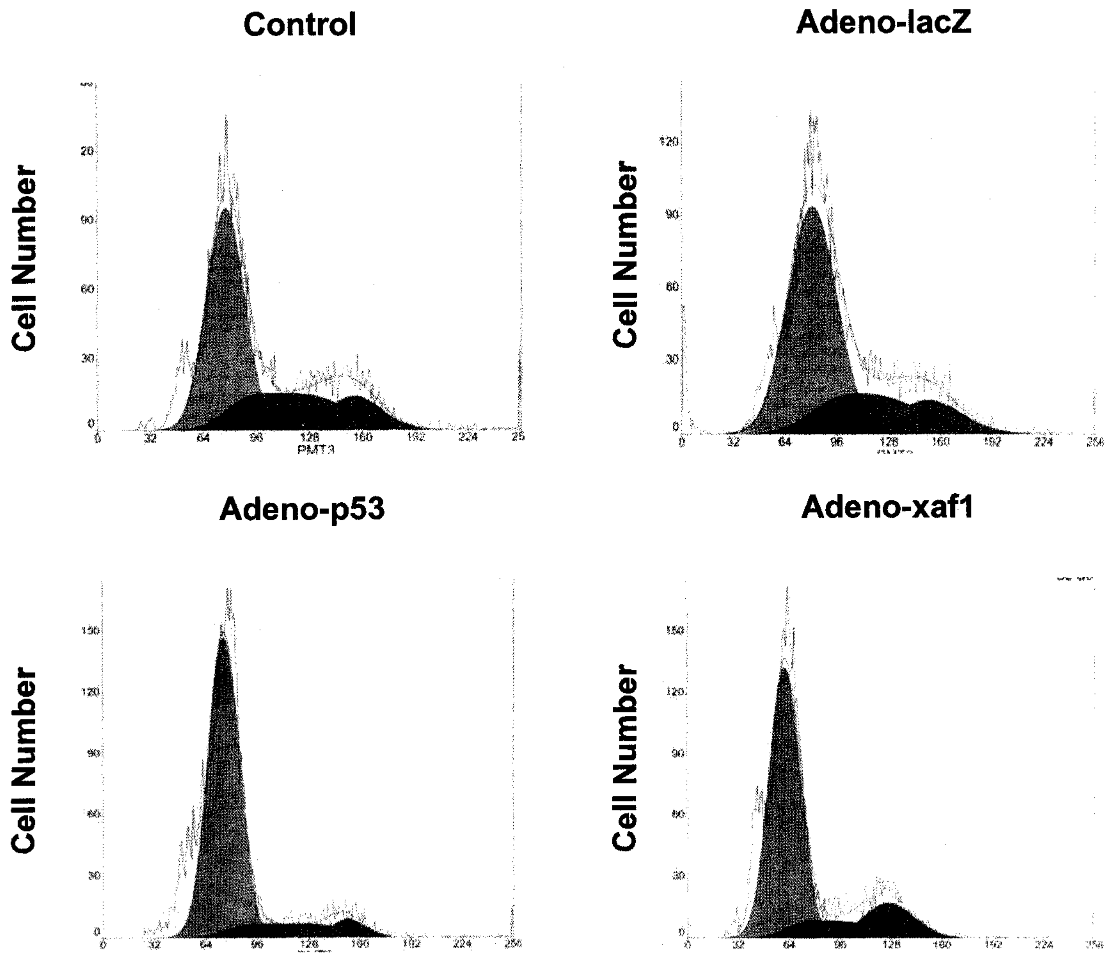
## XAF1 induced cell cycle arrest in normal cells is p53-dependent

Tumor suppressor genes often interfere with the development of cancer cells by signaling through pro-apoptotic pathways or by inhibiting the cell cycle. To assess the role of XAF1 as a potential tumor suppressor I examined the effect of XAF1 overexpression on the proliferation of human embryonic lung fibroblasts HEL 299 and WI-38. Both HEL 299 and WI-38 are relatively normal cell lines with a limited replicative lifespan. HEL 299 cells were plated at a low density and transduced at a multiplicity of infection (MOI) of 50 plaque forming units (pfu)/cell. Infected cells were collected 48 h post-infection, fixed with ethanol and stained with the DNA dye propidium iodide. Fluorescence activated cell sorting (FACS) analysis (see Figure 3-1A) indicated that cells infected with control adeno-p53 vector inhibited cell cycle progression at G1, as measured by the significant loss of S phase cells (11.9%) when compared to adeno-lacZ infected cells (22.5%). Adeno-xaf1 infected cells also significantly suppressed cell cycle progression through $G_1$ phase resulting in 12.5% cells in S phase (see Figure 3-1B). Comparable results were obtained for WI-38 cells with the percentage of S phase cells being 18.7%, 4.6%, and 10.2% for adeno-lacZ, adeno-p53, and adeno-xaf1 transduced cells respectively.

To elucidate the possible mechanism for the observed G1 arrest, protein extracts from adeno-p53 and adeno-xaf1 transduced HEL 299 cells were examined for

**Figure 3-1. XAF1 Overexpression Induces $G_1$ Cell Cycle Arrest.**

(A) HEL 299 cells were transduced with adeno-lacZ, adeno-p53 or adeno-xaf1 at a MOI of 50 pfu/cell. Cells were collected 48 h post-infection and analyzed by fluorescence activated cell sorting (FACS). Untransduced cells were used as a control. (B) The percentage of G1 and S-phase cells were determined for HEL 299 cells transduced with adeno-lacZ, adeno-p53 or adeno-xaf1. Mean values of three replicates were analyzed for siginificant differences between exposure groups with one-way ANOVA. (Bonferroni post hoc, $p < 0.01$).




**B**

| | Uninfected | Ad-lacZ | Ad-p53 | Ad-xaf1 |
|---|---|---|---|---|
| G1 | 61.3+/-3.16 | 62.4+/-3.29 | 79.4+/-5.83 | 72.4+/-1.31 |
| S | 26.6+/-1.29 | 23.7+/-1.57 | 9.37+/-1.99 | 13.3+/-2.25 |

**DNA Content (G1 and S Phase)**

expression of the cell cycle regulating protein p21$^{waf1/cip1}$, the upregulation of which is associated with $G_1$/S cell cycle arrest. As expected, the overexpression of p53 upregulated p21$^{waf1/cip1}$ protein expression. Similarly, XAF1 overexpression was also found to the upregulate p21$^{waf1/cip1}$ (see Figure 3-2). To determine whether XAF1-induced cell cycle arrest was dependent or independent of the p53 pathway, I examined the effect of XAF1 overexpression on proliferation of wild type MEFs and p53-/- MEFs. Cells were plated at low density and infected with adeno-lacZ, adeno-p53 or adeno-xaf1 at a MOI of 50 pfu/cell. FACS analysis of transduced wt MEFs demonstrated a decrease in the percentage of S-phase cells from 7.3% in the control adeno-lacZ infected sample to 5.5% in cells overexpressing p53 (see Figure 3-3 A and B). XAF1 overexpression resulted in a comparable loss of S-phase cells to 4.5%. Although an analogous drop in percentage of S-phase cells was seen between p53 and XAF1 respectively, whether this is truly a $G_1$ arrest cannot be conclusively stated owing to the already low percentage of proliferating cells in the control population. Interestingly, overexpression of p53 or XAF1 in p53-/- MEFs induced cell death in 36.6% and 31.9% of the cell population respectively, as determined by FACS analysis (see Figure 3-4 A and B).

I next determined whether or not XAF1-mediated p21$^{waf1/cip1}$ upregulation is a p53-dependent event. Examination of p21$^{waf1/cip1}$ protein expression in wt MEFs demonstrated an upregulation of expression in response to XAF1 overexpression similar in response to p53 (see Figure 3-5A). In contrast, an increase in p21$^{waf1/cip1}$ levels was not seen in p53 -/- MEFs overexpressing XAF1 (see Figure 3-5B). These findings suggest that XAF1 induces cell cycle arrest through a p53-dependent pathway. However, in the absence of p53, XAF1 is able to trigger cell death through an as yet unknown mechanism. To further confirm that

**Figure 3-2. XAF1 Overexpression Triggers p21$^{waf1/cip1}$ Protein Expression in HEL 299 Cells.**

HEL 299 cells were transduced with adeno-lacZ, adeno-p53 or adeno-xaf1 at an MOI of 50 pfu/cell. Cells were collected 48 h post-infection and the proteins analyzed by Western blot for XAF1, p53, and p21$^{waf1/cip1}$. An anti-β actin antibody was used as a loading control. XAF1 induces an increase in p21$^{waf1/cip1}$ protein expression similar to that seen in adeno-p53 transduced cells.

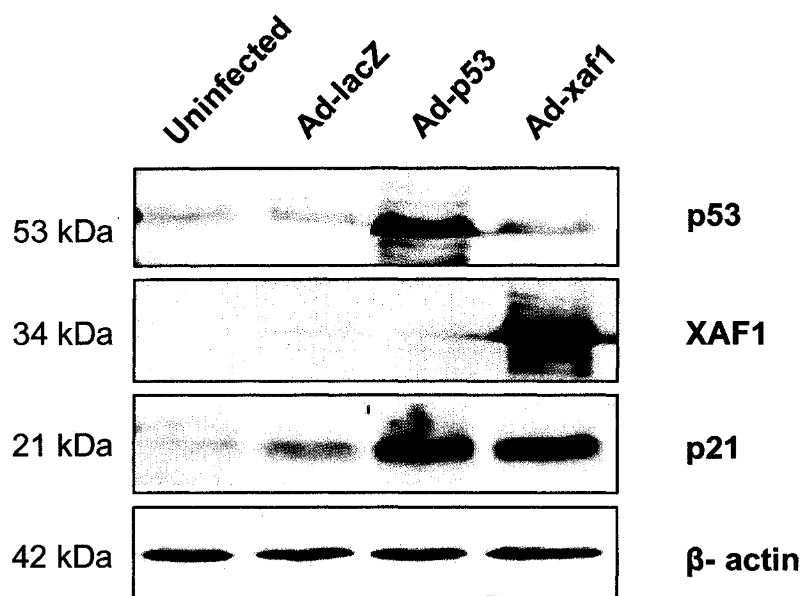
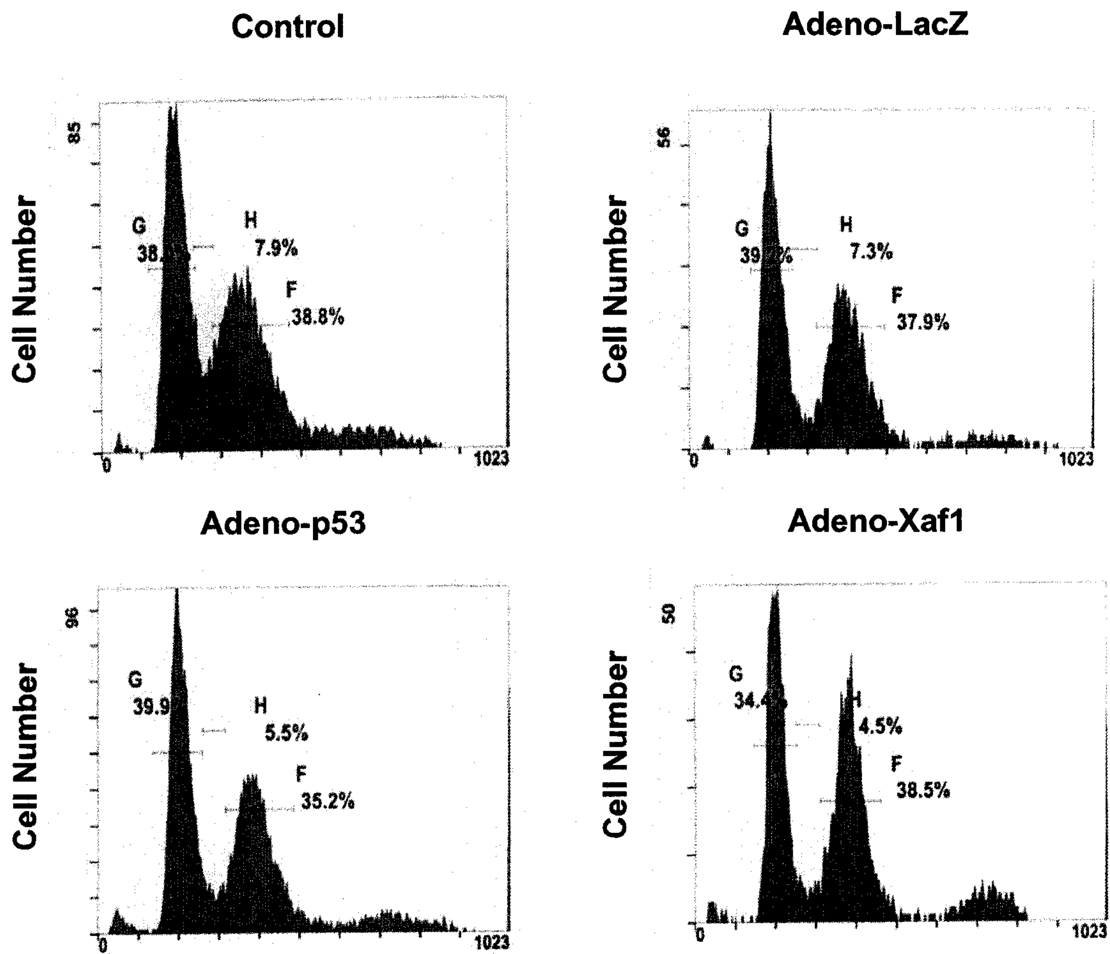

Uninfected
Ad-lacZ
Ad-p53
Ad-xaf1
53 kDa
p53
34 kDa
XAF1
21 kDa
p21
42 kDa
β- actin

**Figure 3-3. XAF1 Induces $G_1$ Cell Cycle Arrest In wt MEFs.**

(A) Wt MEF cells were transduced with adeno-lacZ, adeno-p53 or adeno-xaf1 at a MOI of 50 pfu/cell. Cells were collected 48 h post-infection and analyzed by FACS. Untransduced cells were used as a control. (G: cells in G1 phase, H: cells in S phase, F: cells in G2 phase). (B) The percentage of S-phase cells was determined for wt MEFs transduced with adeno-lacZ, adeno-p53 or adeno-xaf1. Mean values of three replicates were analyzed for siginificant differences between exposure groups with one-way ANOVA. (Bonferroni post hoc, $p < 0.05$). XAF1 induces a reduction in S-phase content, similar to p53 overexpression.

A



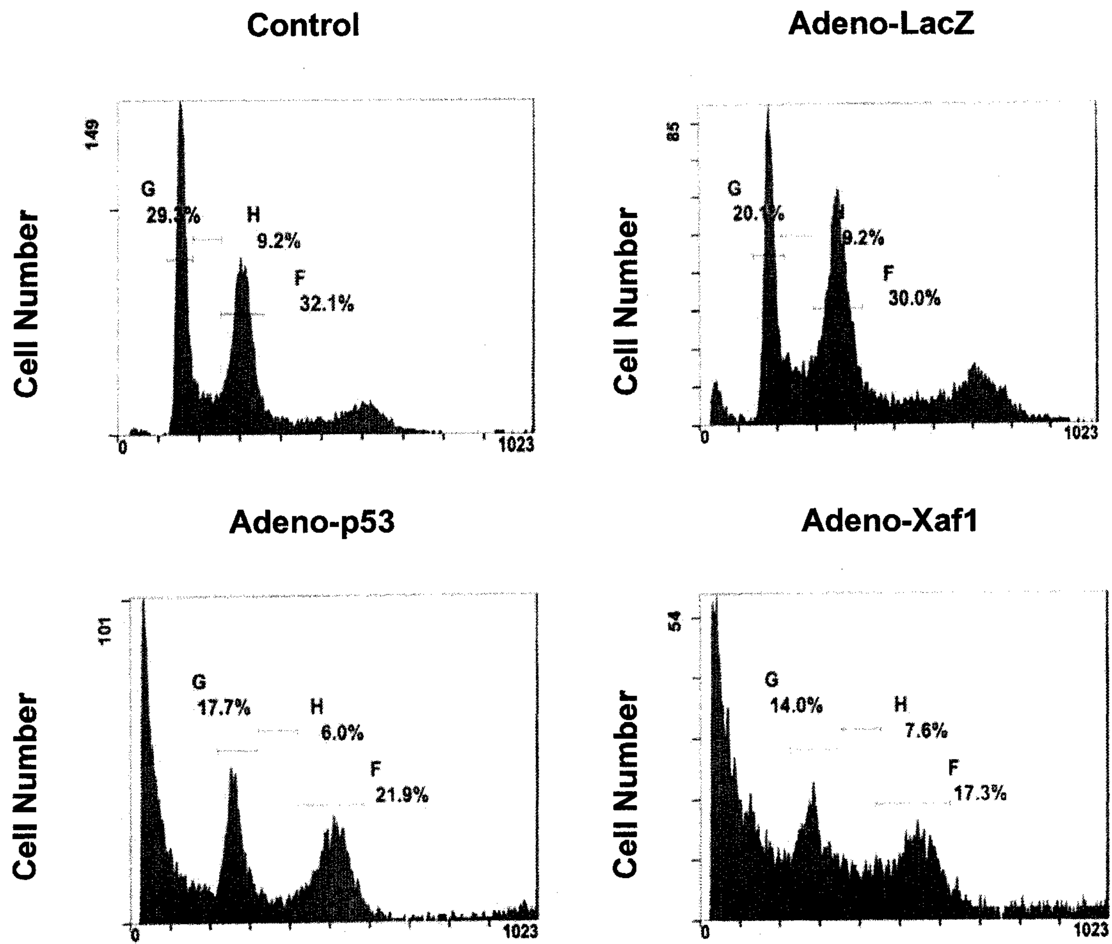

B

| | Uninfected | Ad-lacZ | Ad-p53 | Ad-xaf1 |
|---|---|---|---|---|
| Wt MEFs | 7.33+/-0.737 | 7.17+/-0.153 | 5.23+/-0.252 | 5.7+/-1.66 |

DNA Content (S Phase)

**Figure 3-4. XAF1 Induces Cell Death In p53 -/- MEFs.**

(A) p53-/- MEF cells were transduced with adeno-lacZ, adeno-p53 or adeno-xaf1 at a MOI of 50 pfu/cell. Cells were collected 48 h post-infection and analyzed by FACS. Untransduced cells were used as a control. XAF1 overexpression induced massive cell death, similar to that seen in adeno-p53 transduced cells. (B) Percentage of non-viable cells (sub-G1 cells) was determined for p53-/- MEFs transduced with adeno-lacZ, adeno-p53, and adeno-xaf1. Overexpression of XAF1 results in comparable levels of cell death to p53 overexpression.



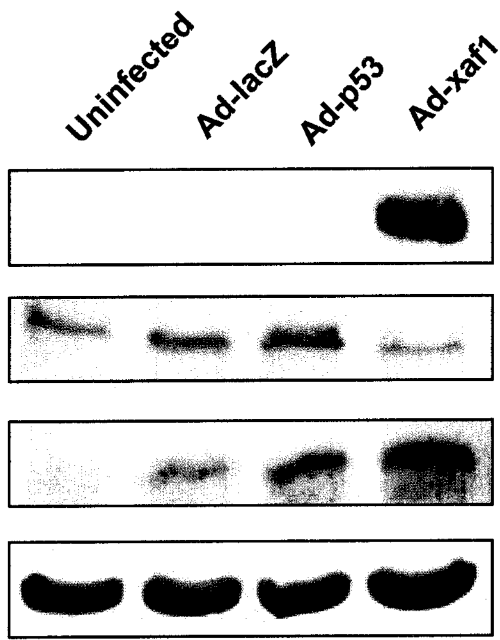

**B**

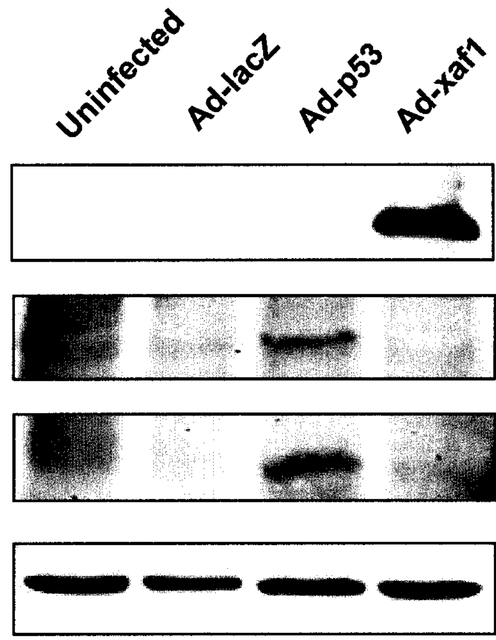
| | Uninfected | Ad-lacZ | Ad-p53 | Ad-xaf1 |
|---|---|---|---|---|
| p53-/- MEFs | 5.9 | 13.9 | 36.6 | 31.9 |

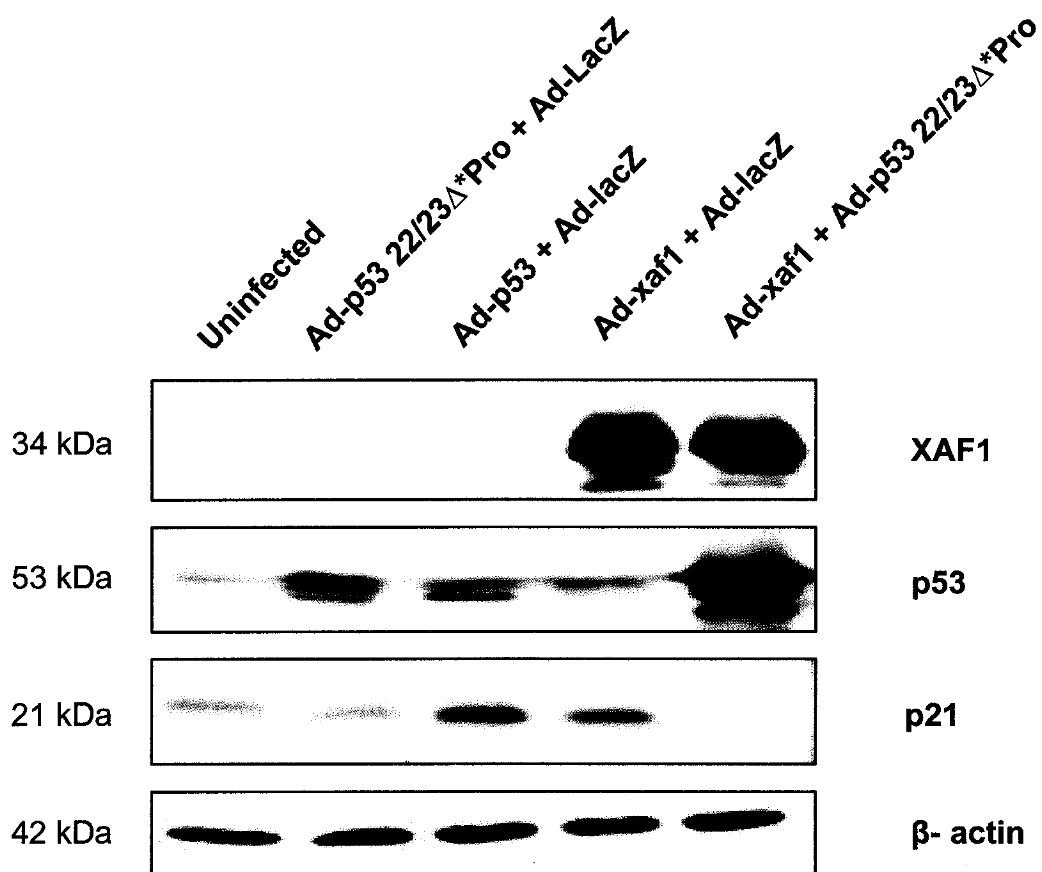
**Percentage of sub-G1 Cells**

**Figure 3-5. XAF1-Mediated Upregulation Of p21 $^{waf1/cip1}$ Protein Expression Is p53-Dependent.**

(A) Wt MEF cells were tranduced with adeno-lacZ, adeno-p53 or adeno-XAF1 at a MOI of 50 pfu/cell. Cells were collected 48 h post-infection and the proteins analyzed by Western blot for XAF1, p53, and p21$^{waf1/cip1}$. An anti-β-actin antibody was used as a loading control. XAF1 induces an increase in p21$^{waf1/cip1}$ protein expression similar to that seen in adeno-p53 transduced cells. (B) p53-/- MEF cells were tranduced with adeno-lacZ, adeno-p53 or adeno-xaf1 at a MOI of 50 pfu/cell. Cells were collected 48 h post-infection and the proteins analyzed by Western blot for XAF1, p53, and p21$^{waf1/cip1}$. An anti-β actin antibody was used as a loading control. XAF1 did not induce p21$^{waf1/cip1}$ protein expression, indicating it acts through the p53 pathway. (C) HEL 299 cells were transduced with adeno-lacZ, adeno-xaf1, adeno-p53, or p53 dominant-negative mutant adenoviral construct adeno-p53 22/23Δ*Pro at a MOI of 50 pfu/cell. Cells were harvested 48 h after infection and analyzed by Western blot for protein expression levels of XAF1, p53 and p21$^{waf1/cip1}$. Anti-β actin antibody was used as a loading control. p21$^{waf1/cip1}$ expression induced by XAF1 (lane 4), is attenuated by the dominant-negative p53 mutant (lane 5).


A
Uninfected
Ad-lacZ
Ad-p53
Ad-xaf1
XAF1
p53
p21
β- actin


B
Uninfected
Ad-lacZ
Ad-p53
Ad-xaf1


C
Uninfected
Ad-p53 22/23Δ*Pro + Ad-LacZ
Ad-p53 + Ad-lacZ
Ad-xaf1 + Ad-lacZ
Ad-xaf1 + Ad-p53 22/23Δ*Pro
34 kDa
53 kDa
21 kDa
42 kDa
XAF1
p53
p21
β- actin

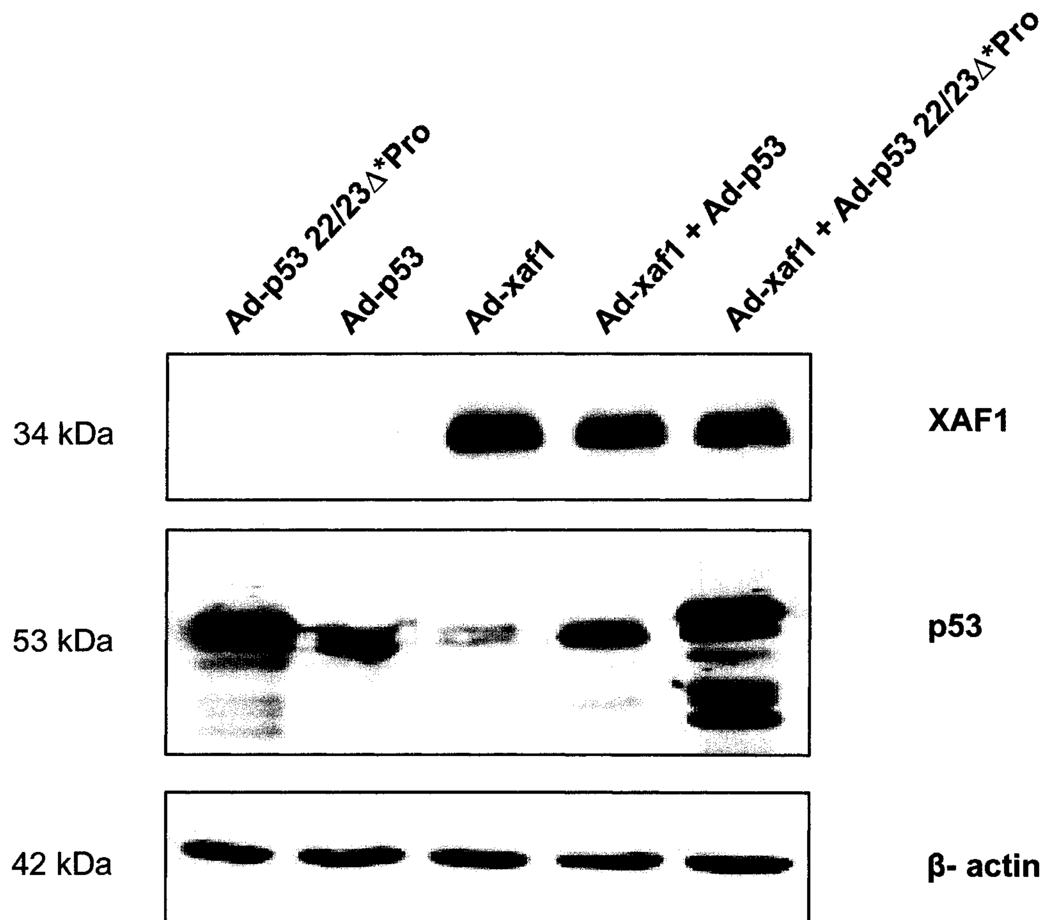
XAF1 induces p21$^{waf1/cip1}$ via the p53 pathway, I transduced HEL 299 cells with adeno-xaf1 with or without an adenoviral vector expressing a dominant-negative p53 mutant construct (see Figure 3-5C). Cells infected only with adeno-p53 or the dominant-negative mutant construct served as control. As expected, XAF1 was able to induce p21$^{waf1/cip1}$ protein upregulation similar to the induction achieved by p53. Moreover, this phenomenon was found to be completely abrogated in protein extracts from cells co-infected with the dominant negative p53 mutant (see Figure 3-5C). Furthermore, cells co-infected with adeno-xaf1 and adeno-p53 22/23*ΔPro displayed markedly increased levels of p53 mutant protein compared to those infected with mutant construct alone (see Figure 3-5C). Increase in p53 protein levels was not seen when HEL 299 cells were co-infected with adenoviral vectors expressing wt p53 and XAF1 compared to those infected with adeno-p53 alone (see Figure 3-6). It is possible that XAF1 promotes stability of p53 protein expression, either by increasing p53 transcription or by stabilizing p53 at the post-translational level. A recent study using the protein synthesis inhibitor cyclohexamide indicated that XAF1 stabilizes p53 at the post-translational level, resulting in the accumulation and prolonged activation of p53 in the human bladder cancer cell line 253J (Lee et al., 2006). However, I did not see accumulation of wt p53 protein in HEL 299 cells, and future experiments assessing the effect of XAF1 expression on *p53* promoter activity would be beneficial to delineate the underlying mechanism.

**XAF1 downregulates Survivin independently of p53**

Survivin is an IAP that plays a critical role in cell cycle progression, acting as a component of the CPC. Survivin protein levels are regulated in a cell cycle dependent

**Figure 3-6. XAF1 Increases Protein Expression of Dominant-Negative Mutant p53.**

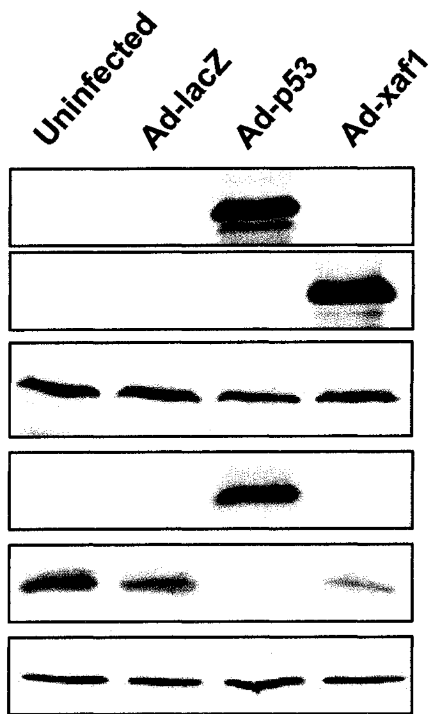
HEL 299 cells were transduced with adeno-p53 22/23Δ*Pro, adeno-p53, and adeno-xaf1 at a MOI of 50 pfu/cell. Cells were harvested 48 h after infection and analyzed by Western blot for protein expression levels of p53 and XAF1. Anti-β actin antibody was used as a loading control. XAF1 induces upregulation of the dominant-negative p53 mutant but not wt p53.

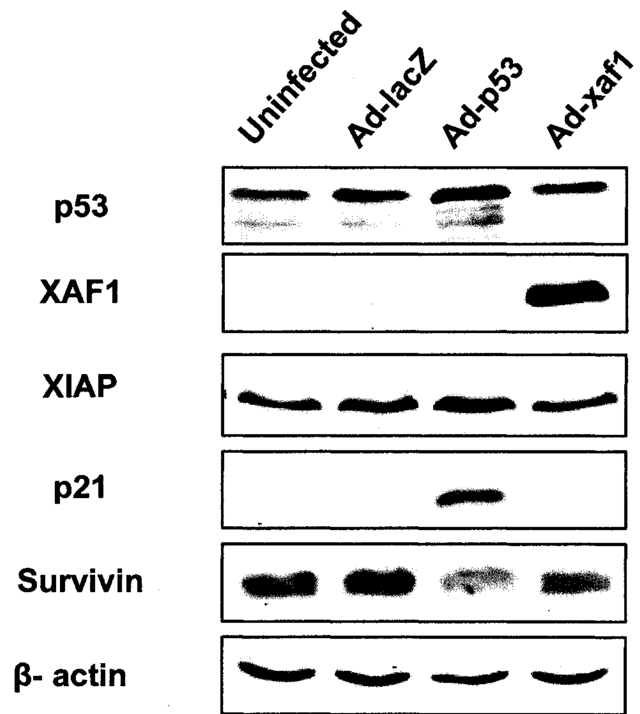

Ad-p53 22/23Δ*Pro
Ad-p53
Ad-xaf1
Ad-xaf1 + Ad-p53
Ad-xaf1 + Ad-p53 22/23Δ*Pro
34 kDa
XAF1
53 kDa
p53
42 kDa
β- actin

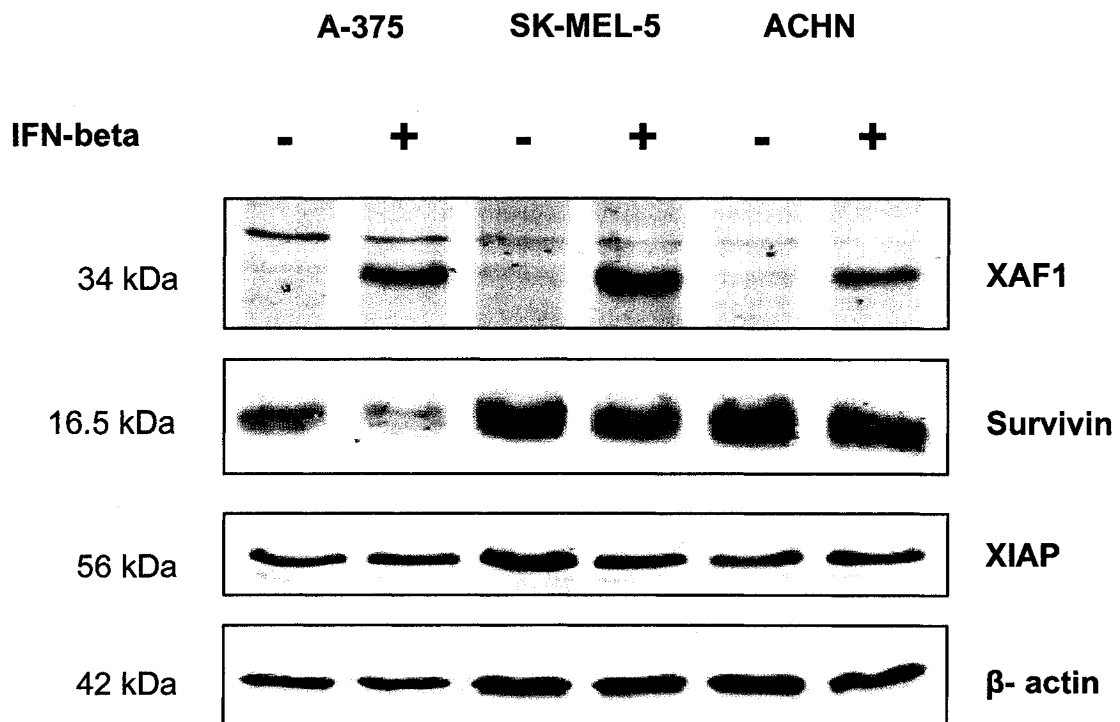
manner by both transcriptional and post-translational mechanisms. It is barely detectable in terminally differentiated cells, but its expression is markedly increased in the majority of tumor cells. Conversely, tumor cells express little or no XAF1. p53 has been shown to directly repress the promoter activity of the *survivin* gene (Mirza et al., 2002) and may also regulate Survivin levels via $p21^{waf1/cip1}$ activation of the $G_1/S$ cell cycle checkpoint (Lohr et al., 2003). The functional interactions of XAF1 with IAPs other than XIAP have not been documented thus far. Being a predominantly nuclear protein, I hypothesized that XAF1 may negatively regulate Survivin function. The above results suggest that XAF1, acting through the p53 pathway, may regulate Survivin levels by activating the $G_1/S$ checkpoint. However, the increased levels of Survivin protein seen in tumor cells are frequently associated with a functional loss of p53. It would be interesting, from a therapeutic point of view, if XAF1 was able to negatively regulate Survivin in tumor cells lacking functional p53 protein. I therefore selected two neuroblastoma cell lines: SK-N-AS which are p53 deficient in every aspect, that is, undetectable p53 protein, no p53-specific transactivation functions and absence of $G_1$ arrest in response to irradiation (Birgit Geoerger, 2005) and SK-N-BE(2) which express a non-functional mutant p53 protein (Torkin et al., 2005), and assessed the effects of XAF1 overexpression on Survivin protein levels. SK-N-AS and SK-N-BE(2) cells were plated at low density and infected with adeno-lacZ, adeno-p53 and adeno-xaf1 at a MOI of 50 pfu/cell. Cells were collected 48 h post-infection and whole cell lysates were generated and examined for expression of Survivin, $p21^{waf1/cip1}$, p53, XIAP, and XAF1 proteins by Western blot analysis. Extracts from cells infected with adeno-lacZ were used as control. As expected, XAF1 overexpression did not induce any change in $p21^{waf1/cip1}$ protein expression in both SK-N-AS cells (see Figure 3-7A) and SK-N-BE(2) cells (see Figure 3-7B) compared to those

**Figure 3-7. XAF1 Downregulates Survivin Protein Expression**

Neuroblastoma cell lines SK-N-AS and SK-N-BE(2) were infected with adeno-lacZ, adeno-p53 and adeno-xaf1 at a MOI of 50 pfu/cell. Cells were collected 48 h post-infection and analyzed by Western Blot for protein expression of p53, XAF1, XIAP, $p21^{waf1/cip1}$, and survivin. Adeno-lacZ infected cell lines were used as control. Survivin expression is decreased in adeno-xaf1 infected cells (A) SK-N-AS cells and (B) SK-N-BE(2) cells. (C) A-375, SK-MEL-5, and ACHN cell lines were treated with IFN-β (500U/ml) for 24 hrs to induce endogenous XAF1 expression. The cells were harvested and analyzed by Western blot for protein expression levels of XAF1, Survivin and XIAP. XAF1 induction in A-375 and SK-MEL-5 cells was found to downregulate Survivin.


A
Uninfected
Ad-lacZ
Ad-p53
Ad-xaf1
p53
XAF1
XIAP
p21
Survivin
β- actin


B
Uninfected
Ad-lacZ
Ad-p53
Ad-xaf1


C
A-375
SK-MEL-5
ACHN
IFN-beta
-
+
-
+
-
+
34 kDa
XAF1
16.5 kDa
Survivin
56 kDa
XIAP
42 kDa
β- actin

expressing the wt p53 construct. Wt p53 completely repressed Survivin protein expression in SK-N-AS cells and to a lesser extent in SK-N-BE(2) cells. Interestingly, XAF1 overexpression resulted in downregulation of Survivin in both cell lines, albeit to a lesser degree compared to p53. XIAP levels were not affected in either of the cell line tested. Although the effects of XAF1 overexpression on Survivin levels appear not to be as potent as those seen with p53, these results strongly suggest the existence of a distinct p53-independent mechanism by which XAF1 may negatively regulate Survivin protein expression.

*Xaf1* has been shown to be an IFN-stimulated gene (Leaman et al., 2002). To confirm that endogenous XAF1 expression downregulates Survivin, three cell lines resistant to IFN-β induced apoptosis were studied. A-375 (melanoma), SK-MEL-5 (melanoma), and ACHN (renal cell carcinoma) display a marked increase in XAF1 expression upon IFN-β treatment (500u/ml,24 h) as has been previously reported (Leaman et al., 2002). Whole cell lysates were generated and analyzed by Western blot for the expression of XAF1, Survivin and XIAP proteins. Untreated cells were used as control. IFN-β induction of XAF1 downregulated Survivin in A-375 and SK-MEL-5 cells, but not in ACHN cells (see Figure 3-7C).

**XAF1 does not bind Survivin**

XAF1 has been shown to bind and antagonize the anti-Caspase activity of XIAP and the above results indicate that XAF1 negatively regulates Survivin protein expression. However, its direct interaction with other members of the IAP family, including Survivin, has not been documented so far. To determine whether XAF1 directly binds Survivin and other IAPs, I co-transfected 293T cells with pCI-XAF1 and a panel of myc-tagged IAP

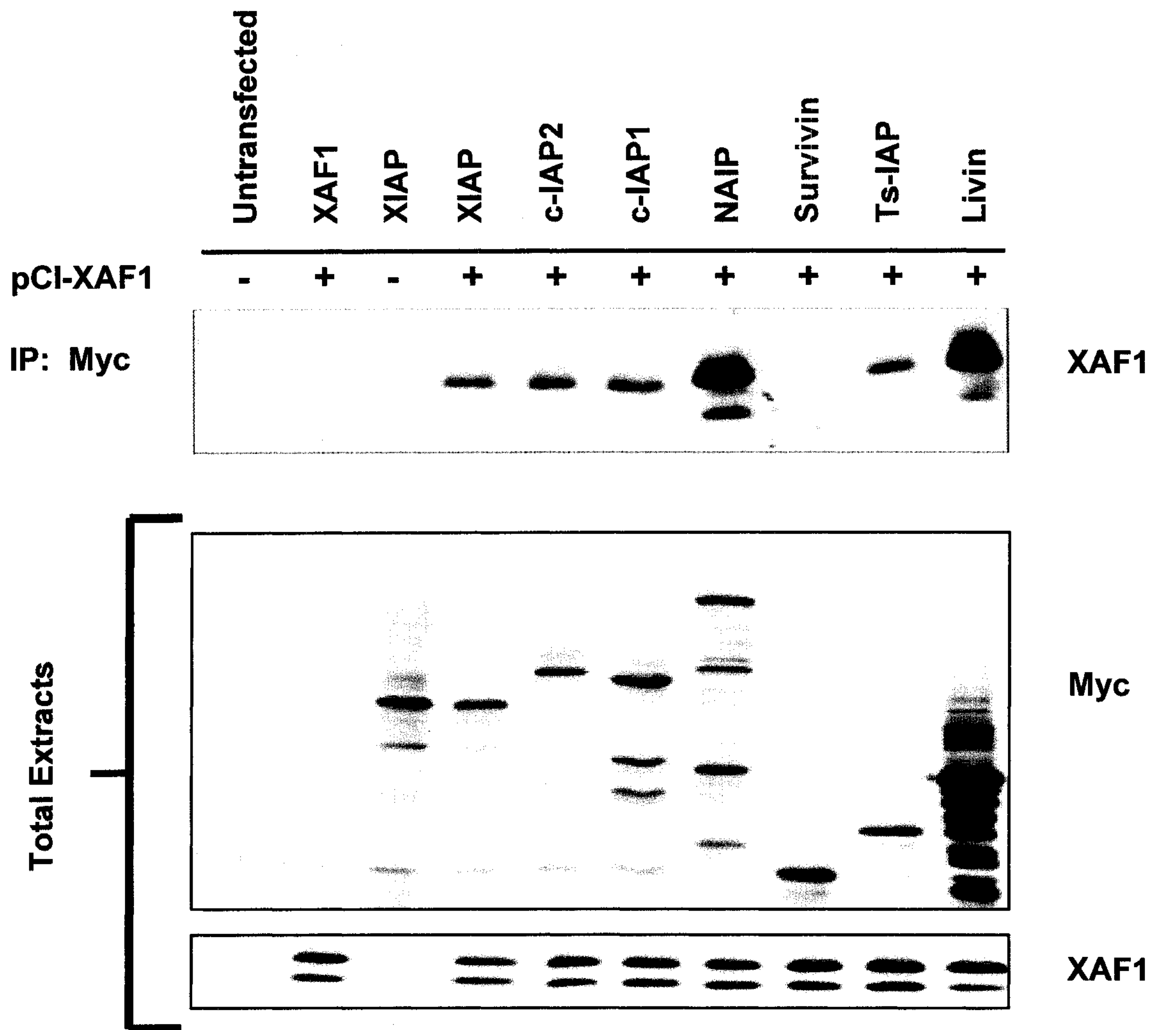
expression plasmids, namely, XIAP, c-IAP1, c-IAP2, NAIP, Survivin, Ts-IAP, and Livin. 48 h post- transfection, the cells were collected and protein extracts were examined by Western blot for the expression of myc-tagged IAPs and XAF1 (see Figure 3-8). Immunoprecipitation with anti-myc antibody was performed in order to isolate IAP complexes and subsequently analyzed by Western blot for the presence of XAF1. Extracts from cells transfected with pCI-XAF1 alone were used as control. To my surprise, XAF1 was found to bind all IAPs tested except Survivin (see Figure 3-8). This suggests that XAF1-mediated downregulation of Survivin requires additional factors. This result may also provide the explanation for why XAF1 induces apoptosis in absence of XIAP (Xia et al., 2006).

**XAF1-mediated downregulation of Survivin is XIAP-dependent**

The ability of Survivin to bind and directly inhibit Caspases remains controversial, but the emerging consensus is that direct Survivin-Caspase inhibition does not occur in physiological contexts. It has been suggested that Survivin associates with XIAP and suppresses Caspase-9 activity *in vitro* in a synergistic manner (Dohi et al., 2004b). I questioned whether the ability of XAF1 to bind and antagonize XIAP would possibly interfere in the formation of such an IAP-IAP complex. I therefore co-transfected 293T cells with pcDNA3-Survivin, in combination with myc-tagged XIAP, or myc-tagged XAF1. Furthermore, 293T cells were also co-transfected with pcDNA3-Survivin, pCI-XAF1 and myc-tagged XIAP. 48 h post-transfection, cells were collected and protein extracts were analyzed by Western blot for expression of Myc-tag, XAF1 and Survivin proteins. Cells transfected with pcDNA3-Survivin alone served as control. Interestingly, Survivin protein expression was significantly reduced in cells co-expressing myc-tagged XIAP and XAF1 compared to those expressing Survivin alone (see Figure 3-9). Survivin protein levels in

**Figure 3-8. XAF1 Physically Interacts With All IAPs Tested Except Survivin.**

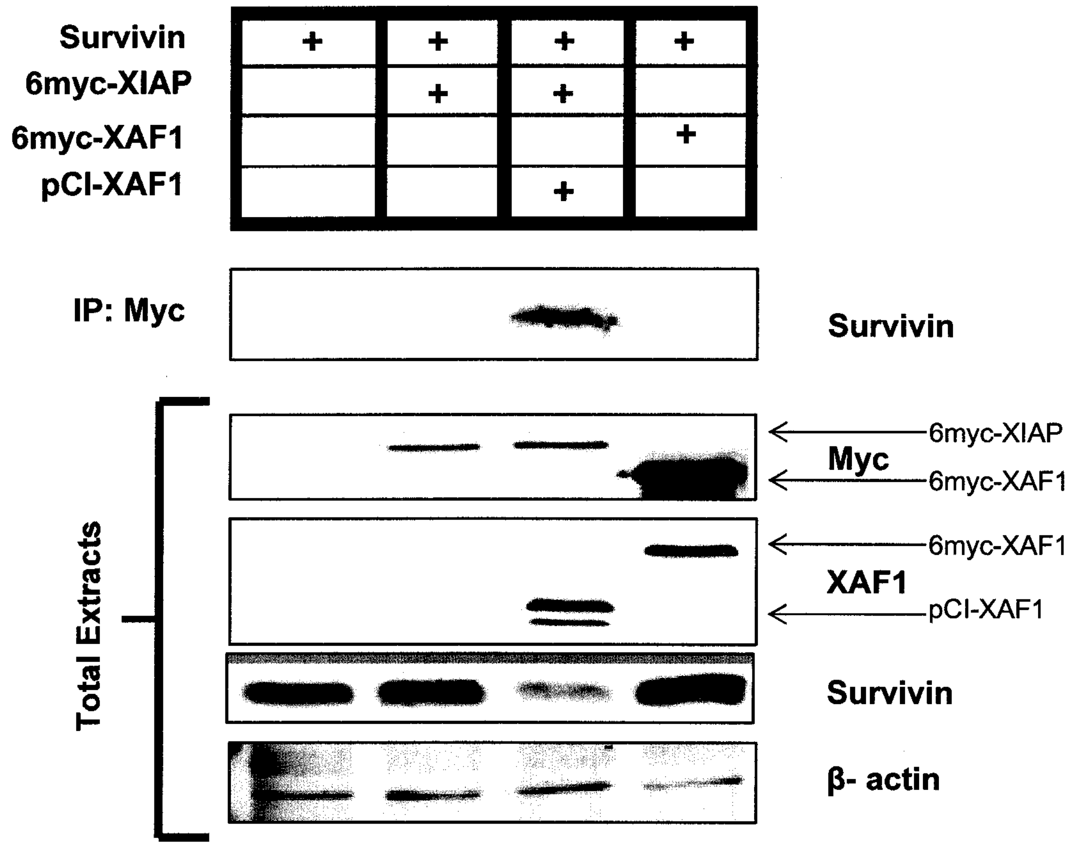
293T cells were transfected with various 6-myc tagged IAP expressing plasmids as indicated and co-transfected with pCI-XAF1 (lanes 4 to 10) respectively. The cells were harvested and analyzed by Western blot for expression of myc tagged IAPs and XAF1. Immunoprecipitation of cell lysates (250 μg) with anti-c-Myc agarose suspension (20 μl) was performed and subsequently analyzed by Western blot for XAF1. Cells transfected with pCI-XAF1 alone were used as control. XAF1 was shown to bind all IAPs tested except survivin (lane 8).



Untransfected
XAF1
XIAP
XIAP
c-IAP2
c-IAP1
NAIP
Survivin
Ts-IAP
Livin
pCI-XAF1
- + - + + + + + + +
IP: Myc
XAF1
Total Extracts
Myc
XAF1

**Figure 3-9. XAF1 Promotes Survivin-XIAP Physical Interaction.**

293T cells were co-transfected in triplicate with either pcDNA3-6myc-XIAP, pcDNA3-6myc-Xaf1, or pcDNA3-6myc-XIAP and pCI-Xaf1 in the presence of pcDNA3-Survivin. The cells were harvested and analyzed by Western blot for expression of myc, XAF1 and survivin. Representative blot is shown. Survivin levels were uniformly decreased in cells co-expressing XAF1 and 6myc-XIAP (lane 3). Immunoprecipitation of cell lysates (250 μg) with anti-c-Myc agarose suspension (20 μl) was performed and subsequently analyzed by Western blot for survivin. Co-transfection of cells with XAF1 promoted binding of Survivin to XIAP (lane 3).



Survivin
6myc-XIAP
6myc-XAF1
pCI-XAF1
+
+
+
+
+
+
+
+
IP: Myc
Survivin
Total Extracts
6myc-XIAP
Myc
6myc-XAF1
6myc-XAF1
XAF1
pCI-XAF1
Survivin
β- actin

cells co-expressing myc-tagged XIAP or myc-tagged XAF1 were similar to those seen in control cells. I next immunoprecipitated the extracts from transfected cells and probed the immune complexes by immunoblotting for Survivin. Immunoprecipitated Survivin appeared to co-associate with myc-XIAP only in cells co-expressing XAF1 (see Figure 3-9). I was unable to see an interaction between survivin and XIAP in the absence of XAF1. Dohi et. al. have suggested that Survivin-XIAP interaction requires an apoptotic signal, and it is possible that such a signal is critical for association. XAF1, a pro-apoptotic protein, appears to promote an association between Survivin and XIAP. Furthermore, these results indicate that XAF1-mediated association of these IAPs significantly decreases Survivin protein levels in cells.

Survivin is regulated, in part, by the ubiquitin-proteasome pathway (Zhao et al., 2000). I questioned whether XAF1 negatively regulates Survivin levels by promoting interaction of Survivin with XIAP, subsequently activating the E3 ubiquitin ligase activity of the XIAP RING Zn finger resulting in the proteasomal degradation of Survivin. I propose a model in which XAF1 acts as a 'switch', converting XIAP from an anti-apoptotic protein to a pro-apoptotic protein. In this scenario, XAF1 activates the latent E3 activity of XIAP and triggers the destruction of Survivin. Loss of Survivin then results in mitotic catastrophe, as has been documented with RNAi studies (Carvalho et al., 2003; Lens et al., 2003). Similarly, XAF1 may be able to convert other IAP family members, particularly those that possess RING fingers (c-IAP1 and 2, Livin, Ts-IAP). Whether XIAP-XAF1 complexes ubiquitinate Survivin, cells were transfected and treated with the proteasomal inhibitor MG-132. The effect on Survivin levels was assessed and compared to untreated transfected cells. 293T cells were transfected with Survivin, 6myc-XIAP, and XAF1 expression plasmids. 20 h post-

transfection, cells were treated with MG-132 (10$\mu$M) for 4 h and harvested for analysis by Western blot for expression of Myc-tag, XAF1, and Survivin proteins. MG-132 treated cells showed markedly greater expression levels of Survivin protein compared to untreated cells (see Figure 3-10A). Furthermore, increased expression of XAF1 protein was seen in cells treated with the proteasomal inhibitor, suggesting that XAF1 is also degraded by the ubiquitin-proteasome pathway.

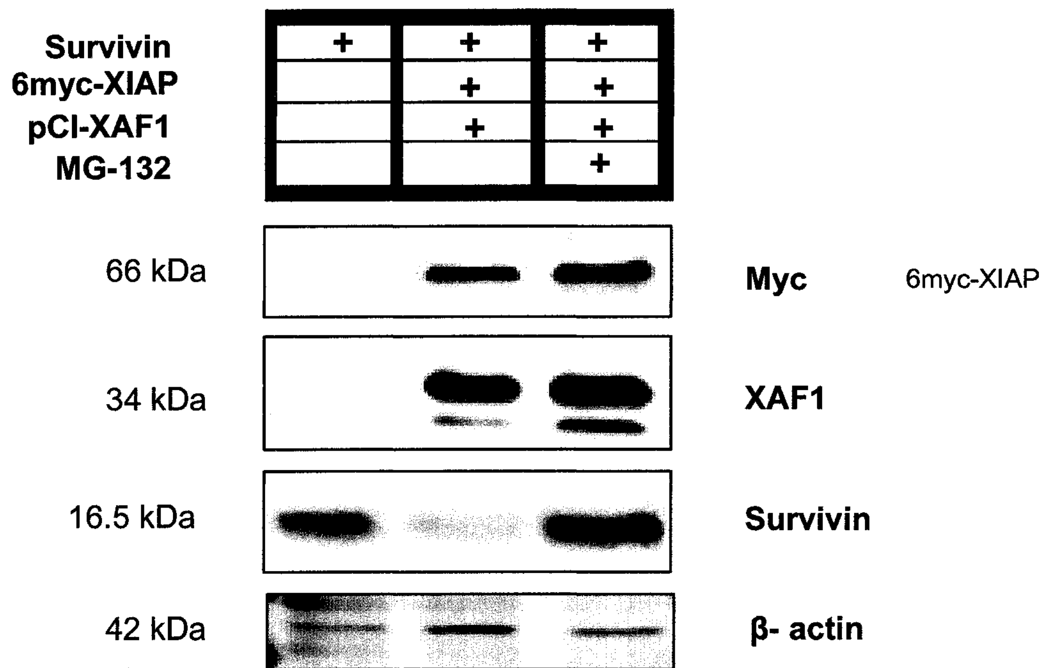
To determine whether the proteasomal degradation of XAF1 and Survivin is mediated by XIAP upon association of these proteins, I co-transfected 293T cells with Survivin and XAF1 expression plasmids in combination with plasmid constructs expressing either wt XIAP, or a XIAP-RING mutant. The 6myc-$XIAP_{H467A}$ expression plasmid encodes a mutant in which $His_{467}$ residue is replaced by an Alanine, resulting in loss of E3 ligase activity (Yang et al., 2000). 24 h post-transfection cells were collected, and the protein extracts generated were analyzed by Western blot for expression of XIAP, XAF1, and Survivin. Cells expressing the XIAP-RING mutant displayed increased Survivin expression compared to cells expressing wt XIAP. These expression levels were comparable to survivin expression levels seen in cells expressing survivin alone (see Figure 3-10B). A Similar increase in XAF1 protein expression was seen in cells expressing the RING mutant compared to wt XIAP. My results thus suggest that XAF1-mediated downregulation of survivin is dependent on the RING E3–ubiquitin ligase activity of XIAP.

To further verify the XIAP-dependent and p53-independent nature of this mechanism, SK-N-BE(2) neuroblastoma cells, expressing non-functional p53 mutant, were infected with recombinant adenoviral vectors expressing RNAi to luciferase and XIAP at a

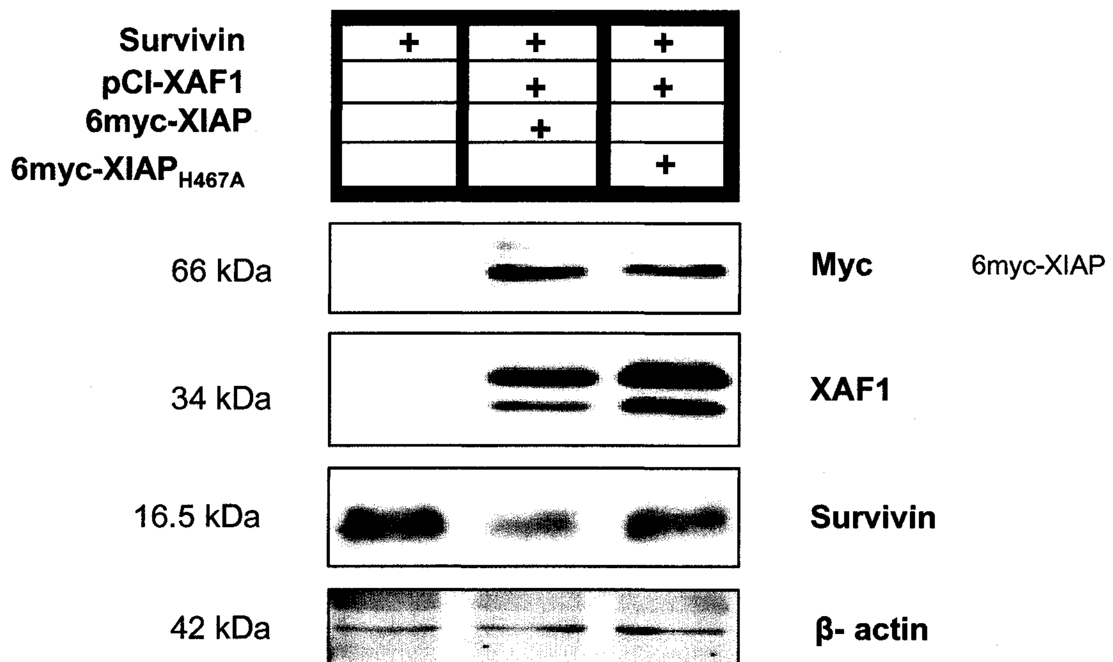
**Figure 3-10. XAF1 Promotes RING E3 Ligase Activity of XIAP and Downregulates Survivin in a Proteasomal Dependent Manner.**

(A) 293T cells were transfected in triplicate with pcDNA3-Survivin, pcDNA3-6myc-XIAP, and pCI-XAF1. Transfected cells were treated with 10 μM proteasomal inhibitor MG-132 for 4 hrs and harvested at 24hrs for analysis by Western Blot. Representative blot is shown. MG-132 treated cells showed greater Survivin expression levels compared to untreated cells. (B) 293T cells were transfected in triplicate with pcDNA3-Survivin, pCI-XAF1, and pcDNA3-6myc-XIAP or the mutant construct pcDNA3-6myc-$XIAP_{H467A}$ lacking E3 ligase activity. Use of XIAP-RING-mutant showed greater expression levels of Survivin compared to wild type XIAP.

A



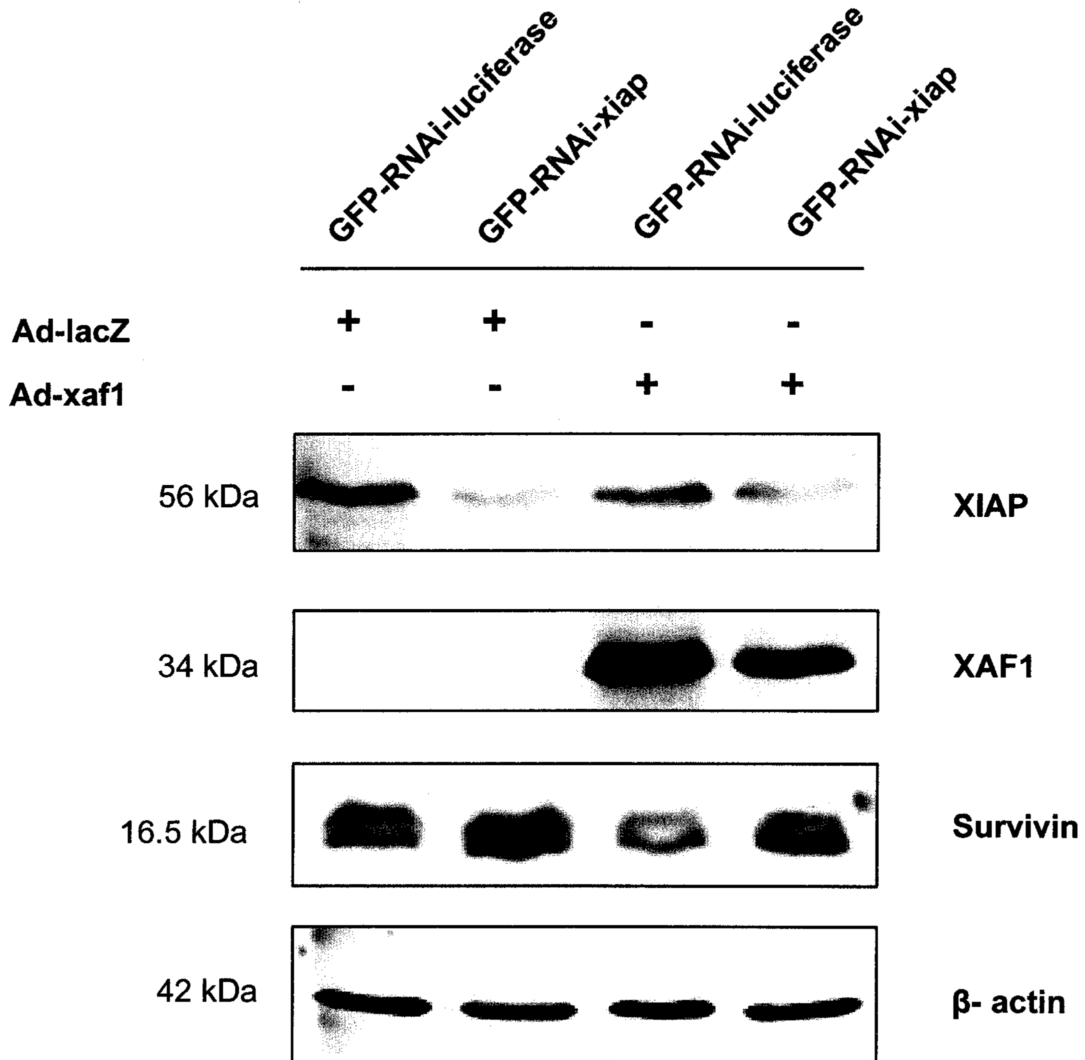

B



MOI of 50 pfu/cell. After allowing for sufficient time for downregulation of XIAP to occur (48 h post-infection) cells were re-infected with adeno-xaf1 or adeno-lacZ (to maintain equal viral load in control population) at a MOI of 50 pfu/cell. The cells were collected 24 h post-re-infection and XIAP, XAF1, and Survivin protein expression was analyzed by Western blot. The control cells expressing RNAi-luciferase and RNAi-xiap in the absence of XAF1 expression did not exhibit any change in Survivin protein expression (see Figure 3-11). Overexpression of XAF1 resulted in downregulation of Survivin compared to control as expected. However, this decrease in Survivin was not seen in cells in which XIAP levels were downregulated by pre-treatment with RNAi to XIAP (see Figure 3-11). Together, these results demonstrated that XAF1-mediated downregulation of Survivin is XIAP-dependent and does not require functional p53. Thus XAF1 affects cell cycle and apoptosis by regulating survivin in a novel p53-independent manner.

**Figure 3-11. XIAP-Dependent XAF1-Mediated Downregulation of Survivin.**

SK-N-BE(2) cells line were transduced with GFP-RNAi-luciferase and GFP-RNAi-xiap for 48 h and subsequently transduced with adeno-xaf1 (lanes 3 and 4) for another 24 h at a MOI of 50 pfu/cell. The cells were harvested and analyzed by Western blot for XIAP, XAF1, and Survivin protein expression. An anti-β actin antibody was used as loading control. XAF1 overexpression was unable to downregulate Survivin when XIAP levels were decreased (lane 4).



GFP-RNAi-luciferase
GFP-RNAi-xiap
GFP-RNAi-luciferase
GFP-RNAi-xiap
Ad-lacZ
+
+
-
-
Ad-xaf1
-
-
+
+
56 kDa
XIAP
34 kDa
XAF1
16.5 kDa
Survivin
42 kDa
β- actin

*Chapter 4*

# DISCUSSION

The balance between proliferation and cell death is lost in malignant tumors, and defects in apoptosis mechanisms allow neoplastic cells to survive beyond normal levels of stress. Under normal circumstances, defects in DNA repair and chromosome segregation would lead to apoptosis as a defense mechanism for the removal of unstable cells. Clearly, defects in apoptosis would allow these unstable cell populations a survival advantage, providing opportunities for selection of progressively aggressive clones (Ionov et al., 2000) with additional genetic alterations that further deregulate cell proliferation, interfere with differentiation, accelerate angiogenesis, and increase cell motility and invasiveness during tumor progression (Reed, 2003). Anti-cancer treatments usually utilize cytotoxic agents and radiation to kill cancer cells causing irreparable cellular damage that, in turn, triggers apoptosis (Sjostrom and Bergh, 2001). A major hurdle in cancer therapies is therefore quite apparent: inherent defects in apoptotic pathways render incipient cancer cells resistant to drugs and radiation, thereby requiring higher, more toxic doses for tumor killing, and ultimately contributing to the undesirable side effects of cancer therapy. In recent years, strategies aiming to overcome the aberrant control of apoptosis in cancer cells have become the focus of well-designed, rational anti-cancer regimens in an effort to increasing the sensitivity of these cells to conventional cytotoxic agents, thereby lowering the toxicity and burden on normal cells. Delineating the underlying mechanisms that cause cancer cells to escape from the apoptotic machinery has therefore, not surprisingly, been the subject of intense research.

Induction of cell cycle arrest or apoptosis is an essential function of p53 as a tumor suppressor and the accumulated evidence for the important role this protein plays in suppressing tumorigenesis is convincing. Conversely, Survivin, a member of the IAP family, promotes cell proliferation by acting as a member of the chromosomal passenger complex which is essential for accurate spindle check point formation, mitotic chromosomal movements and proper execution of cytokinesis (Vagnarelli and Earnshaw, 2004). p53 negatively regulates Survivin by transcriptional repression (Mirza et al., 2002) and through $p21^{waf1/cip1}$ via the cell cycle $G_1/S$ checkpoint (Lohr et al., 2003). XAF1 was originally identified as a nuclear protein that binds and inactivates the anti-caspase activity of XIAP (Liston et al., 2001). Given that little or no XAF1 is expressed in tumor cells, it is possible that XAF1 plays a role in the suppression of malignancy. Here we demonstrate novel and distinct interactions of the XAF1 protein with the cell cycle-associated p53 and Survivin proteins. Overexpression of XAF1 causes a $G_1$ cell cycle arrest in human fibroblast cells which is correlated with an increase in $p21^{waf1/cip1}$ protein expression. Furthermore, concurrent with a recently published report (Lee et al., 2006), we provide evidence that XAF1 acts upstream of p53 and induces $p21^{waf1/cip1}$ and this triggers a $G_1$ cell cycle arrest. Future experiments to determine whether XAF1 activates *p53* promoter activity or prolongs half-life of p53 protein will help to understand the nature of XAF1-mediated stabilization. Our results indicate that in addition to a p53-dependent mechanism, XAF1 may also suppress tumorigenesis in a p53-independent manner. Downregulation of Survivin in p53-deficient tumor cells by XAF1 demonstrates that p53 is dispensable for XAF1 to exert its effects on apoptosis and cell cycle. Upon further examination of this p53-independent mechanism, we found that XAF1 promotes association of Survivin with XIAP which results in activation of

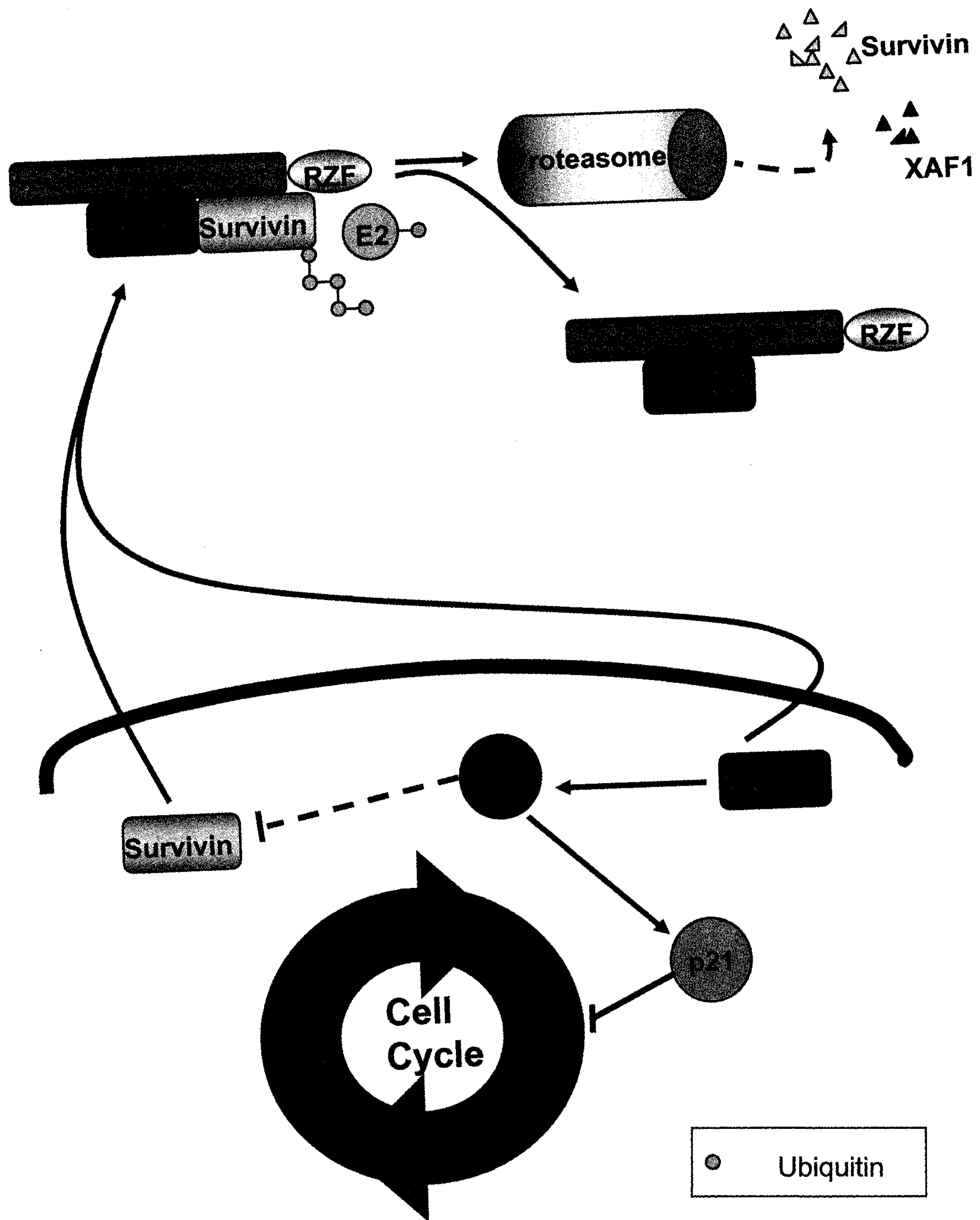
the RING E3 ligase activity of XIAP and the subsequent proteasomal degradation of Survivin. Taken together, our results show that the downregulation of Survivin by XAF1 can occur through two distinct mechanisms: 1) p53-mediated, cell-cycle dependent and 2) XIAP-mediated p53-independent (see Figure 4-1 for a schematic overview).

We were unable to see a direct interaction between XIAP and Survivin in the absence of XAF1. These results are in contrast to those observed in another recent report (Dohi et al., 2004b). That study relied on co-immunoprecipitation experiments involving GST-fusion proteins to assess direct binding between the two IAPs and it has recently been shown that GST tags endow BIR domains with artifactual properties and is related to the propensity of the GST tag to oligomerize which increases binding (Eckelman and Salvesen, 2006). Furthermore, the possibility of other proteins being involved in such an interaction was not assessed. However, Dohi et. al. have suggested that direct Survivin-XIAP interaction requires an apoptotic signal, and demonstrated that XIAP and Survivin synergistically suppressed cell death induced by Bax or Fas in transfected cells. It is possible that such an apoptotic signal is critical for association and furthermore, XAF1 expression may potentially serve as an apoptotic signal.

Our knowledge of the function of RINGs in IAPs is still in its rudimentary stages. RING domains act as E3 ubiquitin-ligases which bind E2 ubiquitin-conjugating enzymes and catalyze the transfer of ubiquitin from the E2 to a substrate (Lorick et al., 1999). This has led to the suggestion that IAPs may polyubiquitinate proteins such as caspases that interact with them directly and target them for the degradation by the 26S Proteasome. However, multi-functional IAPs such as XIAP also bind to a host of other proteins besides caspases. The

**Figure 4-1. XAF1 Negatively Regulates Survivin By p53-Dependent and p53-Independent pathways.**

The abbreviations are: RZF – RING Zinc Finger; E2 – Ubiquitin-Conjugating Ligase; (See text for details).


Survivin
XAF1
RZF
Survivin
E2
Proteasome
RZF
Survivin
p21
Cell
Cycle
Ubiquitin

number of identified targets of IAP-mediated ubiquitination is thus increasing and the results can be fairly complex. The complexity arises from the fact that many ubiquitin modifications alter the activity of the modified protein and do not necessarily result in degradation (Vaux and Silke, 2005). As a general rule, monoubiquitination (one ubiquitin on one lysine residue) or multi-monoubiquitination (one ubiquitin on several lysine residues within the substrate) does not lead to proteasomal degradation of the substrate. Ubiquitin itself contains several lysine residues that may be ubiquitinated in turn giving rise to polyubiquitin chains. Polyubiquitin chains that contain more than four ubiquitins linked together through the lysine residue K48 (K48-linkage) acts as a strong signal for the recruitment to the proteasome for degradation (Vaux and Silke, 2005). On the other hand, non-K48 linkage (predominantly K63 linkage) results in modified activity of the substrate. Questions such as the decision whether to monoubiquitinate or polyubiquitinate, however, remain unanswered.

The ubiquitin functions of the non-IAP RING-bearing protein MDM2 have been characterized and the diversity of interactions and outcomes may serve to exemplify the complexity we might expect to see with the IAPs. The MDM2 RING domain displays a similar organization to that seen in the RING domain of IAPs. It was hypothesized that MDM2 was responsible for the polyubiquitination and subsequent targeting of p53 for proteasomal degradation. Interestingly, results indicate that MDM2 multi-monubiquitinates p53 and leads to the sequestration of p53 in the cytoplasm (Lai et al., 2001; Li et al., 2003) and not its degradation. It has been suggested that MDM2-mediated K48-linked polyubiquitination of p53 may require the transcriptional co-activator p300 (Grossman et al., 2003). Furthermore, modification of p53 by MDM2 with a ubiquitin-like molecule called NEDD8 in some situations has been shown to destabilize p53 *in vitro* and inhibit its

transcriptional activity (Xirodimas et al., 2004). Therefore, it is likely that the outcome of ubiquitination of substrate proteins by IAPs may similarly vary, depending on the association of other proteins interacting directly or indirectly with IAPs as a part of multi-subunit complexes.

Mammalian IAPs have been shown to dimerize through their RING domains (Silke et al., 2002) and it has thus been suggested that they may trans-ubiquitinate each other to regulate their respective protein levels in cells. Although Survivin lacks a RING domain, our results indicate that oligomerization of different IAPs for subsequent ubiquitination need not necessarily occur through the RING domains and can possibly be mediated by the presence of a third party such as XAF1. It is possible that XAF1 thus regulates IAP levels in cells by mediating association with XIAP to maintain the apoptotic threshold. Further experiments involving other members of the IAP family would be beneficial in this regard. The significant loss of Survivin and to some extent of XAF1, seen upon interaction of these two proteins with XIAP was not associated with any change in the protein levels of XIAP itself. However, Dohi et. al. reported that formation of a Survivin-XIAP complex interferes with the auto-ubiquitinating activity of XIAP and results in the reduction in the relative amounts of XIAP-ubiquitin conjugates. Such contradictory findings should not be surprising owing to the complexity of outcomes that can result depending on various interactions of RING-bearing proteins, as mentioned above. Moreover, our findings are in contrast to those seen in studies investigating the ubiquitination activities of c-IAP1 and c-IAP2 on Smac/DIABLO where a self-sacrificial co-degradation process was identified as a means of regulating Smac/DIABLO and vice versa. It is possible, that far from acting as a strict roadblock to the induction of apoptosis mediated by caspases, commensurate with its reputation as the most

potent IAP, XIAP may actually function as a central 'gatekeeper', opening or closing the door to the apoptotic process by regulating levels of both pro- and anti-apoptotic proteins, including itself, depending on its myriad and complex interactions. For such a function to be accomplished successfully, it would be vital that XIAP be maintained at fairly constant levels. The increased apoptotic threshold of cancer cells may, in part, be due to the loss of intermediaries such as XAF1, resulting in the escape of anti-apoptotic target proteins from XIAP-mediated proteasomal degradation.

The functional interactions of XAF1 with XIAP and Survivin with XIAP have not been mapped to individual domains. Preliminary studies by members of Dr. Korneluk's laboratory suggest that XAF1 associates with XIAP-BIR2 and Dohi et. al. indicate in their report that Survivin binds to GST-XIAP-BIR1 or –BIR3, but only weakly to –BIR2 *in vitro* (Dohi et al., 2004b). However, it is too early to say whether formation of the XIAP-XAF1-Survivin complex involves the direct binding of both XAF1 and Survivin to a single XIAP monomer, or requires XIAP oligomerization with XAF1 and Survivin interactions occurring on separate XIAP molecules within the complex. Further characterization and mapping of the individual domain interactions by crystallography, mutagenesis and co-immunoprecipitation studies would help in this regard.

Xaf1 mRNA is ubiquitously expressed in normal tissues, but is expressed at very low levels or not at all in majority of the cancer cells of the NCI 60 panel of cell lines (Liston et al., 2001) and several primary malignancies (Byun et al., 2003; Fang et al., 2006; Ma et al., 2005; Ng et al., 2004). These studies further demonstrated that the silencing of the *xaf1* gene in cancers is associated with the hypermethylation of CpG sites in the promoter region of the gene. Aberrant DNA methylation which results in silencing of many tumor suppressor genes

is an epigenetic phenomenon and occurs at least as frequently as mutations or deletions in cancer cells (Baylin, 2005). Apart from silencing of *xaf1*, hypermethylation of the *xaf1* promoter has also been shown to result in the expression of a truncated splice variant that affects its functional interaction with XIAP in prostate cancers (Fang et al., 2006). The truncated splice isoform XAF1C isoform does not bind XIAP (Fong and Korneluk, unpublished results) and it has been reported that an artificially truncated 174 amino acid form that lacks the carboxy-terminus acts in a dominant-negative manner (Leaman et al., 2002). In an analogous situation, heterodimerization of Survivin splice isoforms has been shown to alter function of the protein. It is possible that XAF1 truncated splice variants may similarly associate and thus inhibit the pro-apoptotic function of XAF1. Considering that methylation of apoptotic genes results in loss of function in tumors, it has been suggested that analysis of methylation status may serve as a marker for the detection and prognosis of cancers (Gopisetty et al., 2006). Epigenetic silencing of *xaf1* has been shown to be highly prevalent in malignant urogenital tumors (Lee et al., 2006), gastric adenocarcinomas (Byun et al., 2003), and colorectal cancers (Ma et al., 2005) compared to adjacent normal tissues or benign tumors. Furthermore, unlike mutations, DNA methylation can be reversed by treatment with DNA methylation inhibitors such as azacitidine or decitabine (5-AZA-dC). Restoring the functionality of intact tumor suppressor genes, thereby provides a selective, tumor-specific therapy. Treatment of prostatic cancer cell lines with decitabine results in the expression of full length xaf1 mRNA (Fang et al., 2006) and has been shown to sensitize tumor cells to apoptosis induction by etoposide or 5-fluorouracil (5-FU) (Lee et al., 2006) suggesting that re-expression of methylated-silenced pro-apoptotic genes can sensitize cells to existing chemotherapy agents. Azacytidine and decitabine have been extensively used in

clinical trials to treat solid tumors and hematological disorders (Gopisetty et al., 2006). Although results for patients with hematological disorders have been promising, drug-induced demethylation has not been consistently seen in all patients and a better understanding of the tumor response to demethylation and re-expression of genes such as *xaf1* is necessary to effectively target tumors.

Interferons (IFNs) are a family of cytokines that elicit immunomodulatory, cell differentiative, anti-angiogenic and anti-proliferative effects. Although IFN-α has been used for several years to treat various cancers, a substantial proportion of patients do not respond to treatment (Chawla-Sarkar et al., 2003). IFNs can exert their effects on tumor cells directly through inhibition of cell proliferation, cell cycle arrest, or induction of apoptosis. Alternatively, IFNs can indirectly effect cells by stimulation of immunologic functions or anti-angiogenesis. Initially, induction of apoptosis was not considered an action of IFNs. However, subsequent studies have demonstrated that the caspase cascade is activated in cells in response to IFN treatment and induces apoptosis. Based on *in vitro* studies, induction of apoptosis has been shown to be IFN species and cell type-specific (Chawla-Sarkar et al., 2003). Gene array studies have identified more than 15 pro-apoptotic IFN-stimulated genes and include *Fas/CD95*, *TRAIL*, *caspase-4* and *-8* amongst others. *In vitro* studies in melanomas (Chawla-Sarkar et al., 2001) and multiple myeloma (Chen et al., 2001) demonstrated that IFNs induced apoptosis by activating the extrinsic apoptotic pathway as evidenced by the strong induction of TRAIL and/or Fas. However, treatment of cancer cells with IFNs does not always result in cell death. It has been suggested that silencing of one or more IFN-stimulated genes in cancer cells may confer resistance in these cells to IFN-induced apoptosis.

*Xaf1* was recently identified as a novel IFN-stimulated gene that augments TRAIL-induced apoptosis following treatment of cells with IFN-β (Leaman et al., 2002). Although xaf1 mRNA is upregulated by IFN-α2 and IFN-β, high levels of XAF1 are predominantly seen in cell lines sensitive to the pro-apoptotic effects of IFN-β. Furthermore, a recent study has also found that pre-treatment of ACHN renal cell carcinoma and A375 melanoma cells with decitabine increases the expression of IFN-stimulated genes and sensitizes the cells to apoptosis (Reu et al., 2006). Superficial bladder cancers have been successfully treated in patients receiving Mycobacterium bovis bacillus Calmette-Guerin (BCG) immunotherapy, which induces IFN-γ, TNF-α and TRAIL (Jackson et al., 1995), in combination with IFN-therapy (Luo et al., 1999). It has also been demonstrated that patients that respond to BCG therapy have significantly higher urine TRAIL levels than non-responders indicating that TRAIL plays an important role in BCG-induce tumor regression (Ludwig et al., 2004). Understanding the mechanism for TRAIL resistance in non-responsive bladder tumors would be very helpful in restoring IFN sensitivity. Interestingly, epigenetic silencing of *xaf1* is frequent in invasive, high-grade bladder tumors compared to low-grade tumors (Lee et al., 2006) and this inactivation possibly contributes to TRAIL resistance. Restoration of XAF1 expression in such tumors may sensitize them to IFN therapy. Thus, the expression and methylation status of *xaf1* may serve as a potentially useful marker to identify IFN-resistant tumors and combination therapy with DNA methylation inhibitors and IFNs may yield a better prognosis for such patients. The potential for use of XAF1 as a prognostic marker for IFN therapy is, however, not just limited to cancers. A recent study has compared the significance and specificity of the IFN-stimulated genes XAF1, TRAIL, and myxovirus resistance protein A (MxA) as biological markers for IFN-β therapy of multiple sclerosis

(MS) (Gilli et al., 2006). IFN-β is used as a treatment for MS, but circulating neutralizing antibodies (NAbs) are a significant problem. XAF1 induction was used as a marker of IFN-β function, but was less sensitive than MxA. It is possible that roles for XAF1 as an IFN-stimulated gene may be identified in other immune disorders and such an understanding might help in devising treatment regimens that overcome IFN resistance.

Tissue homeostasis is dependent on maintaining the balance between cell proliferation and cell death. Thus, cell cycle and apoptosis are intimately linked processes which can be ascertained from the fact that a number of morphological features are shared between mitosis and apoptosis, such as cell rounding, cell shrinkage and chromatin condensation. Several proteins with dual regulatory functions in cell cycle and apoptosis have been characterized and include c-Myc, p53, RB protein, NF-κB, and Bcl-2 amongst others. As mentioned earlier, induction of cell cycle arrest or apoptosis is an essential function of p53 as a tumor suppressor and most human cancers display either defects in the p53 pathway or mutations in p53. Transcriptional control by p53 is important for apoptosis induction and it has recently been shown that the development of tumors in p53-null mice is associated with the loss of apoptotic function of p53 which leads to genomic instability (Attardi, 2005). Thus, it is not surprising that considerable effort is being made to restore p53 function by gene therapy approaches (Clayman et al., 1998; Vattemi and Claudio, 2006; Vecil and Lang, 2003). Learning how p53 controls apoptosis through its targets may help us to identify markers to better predict the prognosis in p53 gene therapy designed to reactivate its function. However, unlike cancer cells that compensate for a lack of nutrients and oxygen by compromising the apoptotic pathway, normal cells that possess intact pathways would also be subject to the same p53-mediated apoptotic stresses upon such treatment, and

therefore, are more likely to undergo apoptosis leading to side-effects associated with other cancer chemotherapies. Radiotherapies that predominantly induce p53-dependent apoptosis have severe side effects and preclude their use in many patients. Thus, p53 gene therapy might be more effective in patients with localized, accessible tumors and its therapeutic effects in patients with head and neck cancers are undergoing investigation (Clayman et al., 1998; Liu et al., 1995; Vattemi and Claudio, 2006; Vecil and Lang, 2003). XAF1 appears to act upstream of p53 and exerts its effects partly by stabilizing p53 expression resulting in activation or repression of p53 target genes. Therefore, it is possible that reactivation of *XAF1* in tumor cells, either by adenoviral gene therapy, use of methylation inhibitors, or IFN induction could be particularly beneficial.

As has already been discussed, apoptosis suppression is fundamental to cancer cell survival, and so it is not surprising that components of the apoptotic pathway have emerged as important therapeutic targets. A variety of antisense oligonucleotides, traditional small molecules, biologically active peptides, peptidomimetics, monoclonal antibodies and gene therapy payloads have been incorporated into strategies that target apoptotic pathways in cancer cells (Bell et al., 2003; Denicourt and Dowdy, 2004; Reed, 2003; Shankar and Srivastava, 2004; Wetzker and Rommel, 2004; Xiong, 2004). Although factors such as unexpected toxicities, poor pharmacokinetics, stability and oral bioavailability may limit the use of these compounds in cancer treatment, these apoptosis-based anti-tumor agents might still serve as precursor molecules for the development of more effective therapies.

By virtue of their anti-caspase activity, the IAPs serve as pivotal regulators of the core apoptotic machinery, thereby representing another promising target for enhancing the re-activation of the death program. Numerous proof-of-principle studies have demonstrated

that the downregulation of XIAP leads to enhanced chemotherapy sensitivity in various types of cancer cells (Holcik and Korneluk, 2001; Liston et al., 2003; Reed, 2003). For example, in both *in vitro* and *in vivo* xenograft human lung cancer models, antisense oligonucleotides (ASONs) targeting XIAP induce apoptosis and enhance chemotherapeutic activity (Hu et al., 2003). The validation of XIAP as an important gate keeper of the apoptosis cascade has led to the launching of Phase I clinical trials for a XIAP-specific ASON in a variety of cancers types (Holcik, 2005). An alternative approach to suppress IAP function utilizes short peptides or small molecules that mimic IAP antagonists. In an intracranial malignant glioma xenograft model *in vivo*, synthetic Smac based peptides are able to induce complete regression of the tumors in combination with TRAIL without detectable toxicity to normal brain tissue (Fulda et al., 2002). Similarly, non-peptidyl small molecules XIAP antagonists screened from combinatorial chemical libraries have been shown to sensitize cancer cells to chemotherapeutic drugs and to suppress growth of established tumors in xenograft models in mice, while displaying little toxicity to normal tissues (Li et al., 2004; Schimmer et al., 2004). Clearly, the effective tumor suppression activities of these IAP antagonists warrant further studies into their applicability in anti-cancer regimens. Although our understanding of the role XAF1 plays in tumor suppression is still in its infancy, the handful of reports in the literature present a very promising picture for this novel IAP antagonist and tumor suppressor as a potential prognostic marker for the treatment of cancers and immune disorders, allowing for the design of patient and disease-specific therapeutic approaches.

***Summary and Future Directions***

XAF1 was identified as a novel binding partner of XIAP that antagonizes its anti-caspase activity and reverses the protective effect of XIAP overexpression in cancer cell lines. Research characterizing this protein has revealed that the expression of *xaf1* is significantly reduced by epigenetic silencing in cancer cell lines and several primary tumors. Re-activation of *xaf1* by DNA methylation inhibitors has been shown to sensitize cancer cell lines to killing by cytotoxic agents. Furthermore, *xaf1* has recently been identified as an IFN-stimulated gene that sensitizes cells resistant to IFN-induced apoptosis to killing by TRAIL following pre-treatment with IFN-β. The majority of tumors display either mutations or decreased expression of the tumor suppressor p53, which is usually associated with increased expression levels of the cell cycle-associated IAP Survivin which promotes cell cycle progression.

In this thesis, I have examined the mechanisms by which XAF1 may suppress tumorigenesis in cells, possibly by regulating Survivin expression levels. Since p53 and Survivin are closely associated with the cell cycle and negatively regulate one another, I began by delineating the effects of XAF1 overexpression on the cell cycle of normal cells and further examined the dependence of the effects seen on the p53 pathway. My results demonstrated that XAF1 induces a $G_1$ cell cycle arrest via upregulation of $p21^{waf1/cip1}$ in human and mouse embryonic fibroblast cells in a p53-dependent manner. It appears that XAF1 stabilizes p53 protein expression leading to repression or activation of p53 target genes such as *p21*. Further experiments need to be performed to assess the nature of this stabilization by examining the effect of XAF1 expression on the activity of the *p53* promoter as well as the half-life of p53 protein. These results suggested that XAF1 may negatively

regulate survivin expression through p53-mediated transcriptional repression and the $G_1/S$ cell cycle check point.

I next determined whether XAF1 could negatively regulate survivin expression in p53-deficient cells. XAF1 overexpression resulted in significant downregulation of survivin in both cell lines tested indicating a p53-independent mechanism for the effect observed. My immunoprecipitation studies indicated that XAF1 does not bind directly to Survivin and only associates with it in the presence of XIAP. Formation of a XIAP-XAF1-Survivin complex was found to be associated with reduction of Survivin protein levels. The XIAP RING finger domain functions as an E3 ligase involved in the ubiquitin-proteasome pathway and treatment of transfected cells with proteasomal inhibitors or transfection with a XIAP RING mutant construct lacking E3 ligase activity were able to restore Survivin expression. Furthermore, downregulation of Survivin by XAF1 was not seen in cells pre-treated with RNAi to XIAP. Taken together, these results strongly suggest that XAF1 mediates Survivin downregulation by promoting the RING E3 ligase activity of XIAP. The functional interactions between XAF1 and XIAP, as well as Survivin and XIAP, have not been mapped to individual domains and experiments using mutagenesis and co-immunoprecipitation studies need to be performed. It is possible that one function of XAF1 is to negatively regulate IAP levels by promoting XIAP-mediated proteasomal degradation. Therefore, future studies should also investigate the interaction of XAF1 and XIAP with other IAPs.

Finally, endogenous expression of XAF1, induced by IFN-β, was found to similarly decrease Survivin protein expression in cell lines tested. This indicates that XAF1 negatively regulates Survivin at the physiological level and could be particularly beneficial for the treatment of IFN-sensitive tumors expressing high levels of Survivin. Of prime relevance to

this thesis, are experiments to determine whether endogenous XAF1-mediated downregulation of Survivin occurs by the mechanisms outlined above. Furthermore, XAF1 expression induced by IFN has been shown to sensitize cancer cells to killing by TRAIL. Experiments investigating whether downregulation of XAF1, using RNAi strategies following pre-treatment with IFN, reduces sensitivity to TRAIL-induced apoptosis and how the observed effect correlates with Survivin expression would be beneficial to determine the anti-apoptotic potential of Survivin. It would also be interesting to investigate whether downregulation of XIAP, using RNAi strategies, would impact the effect of XAF1 expression on TRAIL-induced apoptosis.

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# CURRICULUM VITAE


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**Education:**

2002 – Present — **Master of Science - Microbiology and Immunology**
University of Ottawa (Ottawa, Ontario, Canada)

***Thesis Title***: The XAF1 tumor suppressor downregulates survivin in a p53-dependent and –independent manner
Supervisors: Dr. R.G.Korneluk and Dr. Peter Liston

2001 – 2001 — **E-Commerce Business Solutions Developer Diploma**
The Institute for Computer Studies (Ottawa, Ontario, Canada)

1993 – 1999 — **Bachelor of Medicine and Surgery (M.B.B.S)**
Jawharlal Institute of Post-Graduate Medical Education and Research (J.I.P.M.E.R), University of Pondicherry (Pondicherry, India)

**Research Experience:**

2002 – Present — *MSc. Candidate*: Investigated the effects of Xiap Associated Factor 1 (XAF1) on the cell cycle by FACS and Western Blot analysis of cell cycle associated protein expression levels. Studied the regulation of survivin protein levels by XAF1 overexpression in various cancer cell lines and further characterized the possible role of XAF1 in the promotion of E3 ubiquitin ligase activity of Xiap and the ubiquitin-mediated loss of survivin by the XAF1-XIAP complex.

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**Publications and Book Chapters:**

Cheung HH, **Arora V**, Korneluk RG. Abnormalities of cell structures in tumors: apoptosis in tumors. EXS. 2006;(96):201-21. Review.

**Arora V**, Cheung HH, Plenchette S, Liston P, Korneluk RG. The XAF1 tumor suppressor downregulates survivin protein levels by promoting E3-ubiquitin ligase activity of XIAP. (Manuscript in preparation).

**Presentations, Posters and Abstracts:**

Cellular Senescence and Cell Death, Keystone, Colorado, USA (2005)
The XAF1 tumor suppressor triggers cell cycle arrest through a p53 and Survivin dependent pathway.
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**Conferences attended:**

Cellular Senescence and Cell Death, Keystone, Colorado, USA (2005)

BioNorth Ottawa Life Sciences Council Conference, Ottawa, Ontario, Canada (2003). Student Delegate.

Anesthesia National Conference, Pondicherry, India (1993). Student Volunteer.

49th National Conference on Tuberculosis and Chest Diseases, Pondicherry, India (1996). Student Volunteer.

National Conference of Obstetrics and Gynecology, Pondicherry, India (1998). Student Volunteer.

**Professional Memberships:** Medical Council of India.

**Work Experience:**

1999 – 2001 *General Physician* (Private practice), New Delhi, India. Practiced as a primary health care provider.

1998 – 1999 *Resident Medical Intern*, Jawaharlal Institute of Post-graduate Medical Education and Research, Pondicherry, India.

**Other:**

Good working knowledge of software programming languages C/C++, JAVA, Perl, Microsoft Visual Basic 6.0, HTML, and JavaScript