AN INVESTIGATION OF THE FUNCTION OF IκB FAMILY MEMBER BCL-3 AND ITS ROLE IN ONCOGENESIS

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ABSTRACT

David Kashatus: An investigation of the function of IκB family member Bcl-3 and its role in oncogenesis
(Under the direction of Albert S. Baldwin Jr.)

Bcl-3 was originally identified as a protein that is highly expressed in certain B-cell lymphomas harboring a 14;19 translocation, and more recent evidence indicates that its expression is characteristic of a large number of human cancers. A member of the IκB family of NF-κB inhibitors, Bcl-3 interacts with NF-κB dimers containing p50 or p52 and promotes transcription of a subset of NF-κB dependent genes. However, neither the role of Bcl-3 in oncogenesis, nor its role in normal physiology, has been clearly defined.

My research has focused on elucidating the function of Bcl-3 in the context of DNA damage signaling in order to gain insight into its physiological and pathological roles. Experiments presented here indicate that Bcl-3 is inducible by DNA damage and is required for the induction of Hdm2 gene expression and the suppression of persistent p53 activity. Furthermore, constitutive expression of Bcl-3 suppresses DNA damage-induced p53 activation and inhibits p53-induced apoptosis through a mechanism that is at least partly dependent on the upregulation of Hdm2. The results provide insight into a mechanism whereby altered expression of Bcl-3 leads to tumorigenic potential. Since Bcl-3 is required for germinal center formation, these results further indicate that Bcl-3 may play a critical in the B-cell maturation process by suppressing p53-dependent apoptosis in response to somatic hypermutation and class switch recombination.

As aberrant expression of Bcl-3 has been found in an increasing number of human cancers, my second project set out to investigate whether Bcl-3 plays a role in hepatocellular carcinoma (HCC). The data indicate that nuclear staining of Bcl-3 is present in a high percentage of primary HCC tumors compared to adjacent normal tissue. Furthermore, western blot analysis indicates that high Bcl-3 expression correlates positively with expression of Hdm-2. These data suggest that Bcl-3 may play a causal role in HCC and indicate that it may be functioning through inhibition of p53.

Collectively, the work described here proposes a novel function for the oncoprotein Bcl-3 that provides insight into both its normal and oncogenic roles and provides further evidence for the expanding role of Bcl-3 in human cancer.

To my wife Jen, for all of her support.

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LIST OF ABBREVIATIONS

AP	activating protein
APAF	apoptosis protease-activating factor
ARD	ankyrin repeat domain
ARF	alternative reading frame
ATM	ataxia-telangiectasia mutated
ATR	ATM and Rad3 related
BAFF	B cell activating factor belonging to the TNF family
Bcl	B-cell lymphoma
B-CLL	B-cell chronic lymphocytic leukemia
BCR	B-cell receptor
BH	Bcl-2 homology
bp	Base pair
53BP	p53 binding protein
BRCA	Breast cancer associated
CDK	cyclin dependent kinase
CHK	checkpoint
CBP	CREB binding protein
CDC	cell division cycle
ChIP	chromatin immuno-precipitation
c-IAP	cellular inhibitor of apoptosis protein
CK2	casein kinase 2

ELKS	glutamate, leucine, lysine, serine
HCC	hepatocellular carcinoma
HDAC	histone deacetylase
Hdm	human double minute
HUS	hydroxyurea sensitive
IκB	inhibitor of κB
IKK	IκB kinase
IL	interleukin
IRF	interferon response factor
J/m^2	joules per meter squared
JNK	c-Jun N-terminal kinase
kD	kilodalton
LT-β	lymphotoxin-beta
ml	milliliter
μl	microliter
LPS	lipopolysaccharide
mM	millimolar
μM	micromolar
mdm	mouse double minute
MMP	matrix metallo-proteinase
MnSOD	manganese superoxide dismutase
NIK	NF-κB-inducing kinase
NF-κB	nuclear factor kappa B

NLS	nuclear localization sequence
p/CAF	p300/CBP-associated factor
PFT	pifithrin
RHD	Rel homology domain
RITA	reactivation of p53 and induction of tumor cell apoptosis
RNAi	RNA interference
siRNA	short interfering RNA
TA	transactivation domain
TCR	T-cell receptor
TLR	toll-like receptor
TNF	tumor necrosis factor
TNFR	TNF receptor
TRADD	TNFR associated death domain
TRAF	TNFR associated factor
β-TrCP	beta-transducin repeat-containing protein
UV	ultraviolet
ZAP	zeta-chain associated protein

LIST OF SYMBOLS

α	alpha
β	beta
ε	epsilon
γ	gamma
κ	kappa

INTRODUCTION

The genomes of mammalian cells are constantly exposed to a wide variety of insults from both exogenous and endogenous sources. In order to maintain cell viability and to ensure the proper formation and maintenance of complex tissues, evolution has provided cells with numerous mechanisms to repair the damage incurred, when possible, and to minimize the deleterious effects the genetic lesions may have on cellular function. Failure of these protective mechanisms can lead to the accumulation of mutations of genes encoding key components of the cellular machinery, mutations that can compromise cellular function and ultimately result in disease, most notably, cancer.

Recent years have seen major progress in our understanding of how cells respond to genomic insults. A variety of biochemical pathways have been identified that can recognize DNA damage and elicit complex responses leading to repair of damaged DNA, arrest of the cell cycle, and when warranted, elimination of damaged cells through the process of programmed cell death. Each of these pathways involves the coordinated interaction of numerous proteins and integrates multiple signals to arrive at the appropriate cellular response. While our understanding of these pathways has grown significantly in recent years, a complete understanding of these complex processes remains incomplete.

The p53 tumor suppressor protein has been identified as a critical mediator of the cell's response to DNA damage. Often referred to as the guardian of the genome, p53 is activated by a wide variety of genomic insults and its activation leads to critical cell fate decisions, including cell cycle arrest and apoptosis (Vousden and Lu 2002). A sequence

specific transcription factor, activated p53 binds to the promoter regions of numerous genes including the cell cycle inhibitor p21 and pro-apoptotic proteins Bax, Noxa and Puma to induce their transcription. In a poorly understood way, the cell then integrates the multiple signals initiated by p53 to execute the appropriate response (Oren 2003).

Another transcription factor that plays an important, though poorly understood, role in response to genomic insults is NF-κB. The role of NF-κB in mediating innate and adaptive immunity has been studied in great depth and the molecular details of its response to cytokines, growth factors and various infectious agents has been increasingly well defined in recent years (Ghosh and Karin 2002). However, the influence of NF-κB in a much wider set of cellular processes has also been emerging, providing a wealth of open questions and research opportunities for scientists interested in NF-κB.

The NF-κB family consists of five mammalian homologues (p65, RelB, C-Rel, p50 and p52) which can homo- or hetero-dimerize to form active transcriptional complexes. NF-κB is normally maintained in a latent state through its interaction with its inhibitor IκB. There are at least six members of the IκB family, including IκBα, IκBβ, IκBε, Bcl-3, p100 and p105, each of which can interact with NF-κB through its internal ankyrin repeat domain (Hayden and Ghosh 2004). Traditionally, much of the research focus has been on so-called classical NF-κB, which consists of a heterodimer of the p50 and p65 subunits, as this dimer plays a dominant role in many of the well-defined pathways of NF-κB activation. However, as the far-reaching influence of NF-κB has begun to be uncovered, so to have the roles of many of the other NF-κB and IκB family members.

Bcl-3 is one of the factors whose cellular function has only begun to emerge. Originally cloned as a breakpoint junction in certain B-cell chronic lymphocytic leukemias

(McKeithan et al. 1987), Bcl-3 is unique among IκB family members in that, unlike the other IκBs that bind to NF-κB dimers and sequester them in the cytoplasm, Bcl-3 is predominantly nuclear, and has a C-terminal transactivation domain. Shortly after it was cloned, Bcl-3 was shown to preferentially bind to p50 and p52 homodimers and was subsequently shown to promote transactivation at NF-κB dependent promoters (Ohno et al. 1990; Franzoso et al. 1992; Franzoso et al. 1993; Nolan et al. 1993).

In addition to its apparent role in B-CLL, Bcl-3 has been shown to be present at high levels in the nuclei of numerous solid tumors (Cogswell et al. 2000; Thornburg et al. 2003), suggesting an oncogenic function. Additionally, sporadic evidence has hinted at a role for Bcl-3 in the cellular response to DNA damage (Boulton et al. 2002; Rocha et al. 2003; Watanabe et al. 2003). This evidence has prompted a more thorough investigation into the specific function of Bcl-3, which is the focus of this doctoral dissertation.

As described earlier, DNA damage signals lead to the activation of the transcription factor p53. The work described here identifies Bcl-3 as a critical factor in mediating this response. In cells expressing low basal levels of Bcl-3, DNA damage signals result in a transient increase in Bcl-3 protein levels, suggesting it may have an important role in the damage response. Intriguingly, when expressed at higher levels, Bcl-3 inhibits DNA damage-mediated stabilization of p53 and leads to protection against p53-dependent apoptosis. Additionally, loss of Bcl-3 leads to an enhanced p53 response and subsequent sensitization to DNA-damage induced apoptosis. The mechanism of this inhibition of p53 is at least partly through Bcl-3 mediated induction of the inhibitor of p53, mdm2.

These findings provide insight into a novel mode of regulation of the critically important tumor suppressor p53. Importantly, they may also shed light on the still poorly

understood role that the NF-κB family plays in response to genomic damage. Finally, these results allow us to begin to better understand both the normal physiological function of Bcl-3, as well as its potential oncogenic function. This understanding will offer new tools to fight the growing number of cancers that over-express Bcl-3.

I. NF-κB

The NF-κB Family

NF-κB was originally identified in 1986 as a nuclear factor that bound to an enhancer element in the gene encoding immunoglobulin (Ig)κ light-chain and was thought to be specifically confined to B-cells (Sen and Baltimore 1986). Investigation of this factor quickly revealed however that its expression is in fact ubiquitous, though it is normally retained in the cytoplasm in a latent state through specific interactions with a family of inhibitors collectively known as IκB (Haskill et al. 1991; Beg et al. 1992). Subsequent studies of NF-κB showed that it is a member of a family of dimeric transcription factors of which there are five known mammalian homologues (p65, RelB, c-Rel, p50 and p52). These proteins share a highly conserved 300 amino acid sequence in the N-terminus known as the Rel homology domain (RHD), which is composed of two immunoglobulin-like domains (Ghosh et al. 1990; Nolan et al. 1991). The RHD is critical for dimerization and binding to the consensus κB binding sequence, GGGRNNYYCC (R = purine, Y = pyrimidine and N = any base). Additionally, the RHD mediates the interaction between NF-κB and its inhibitor IκB (Hayden and Ghosh 2004).

The use of different dimer combinations allows NF-κB to elicit a diverse array of transcriptional responses as different dimers exhibit different binding affinities to NF-κB consensus sequences (Fujita et al. 1992; Kunsch et al. 1992). Additionally, the p65, c-Rel and RelB subunits each contain two potent acid-rich transactivation domains of 30 amino acids (TA1 and TA2) in their C-terminus (Schmitz et al. 1994). These domains promote transcription through the recruitment of transcriptional co-activators, a process that seems be dependent on specific post-translational modifications such as phosphorylation and acetylation (Gerritsen et al. 1997; Zhong et al. 1998; Chen et al. 2001; Zhong et al. 2002). Notably, p50 and p52, which are initially formed as 105 and 100 kD precursors (p105, p100) respectively, lack such transactivation domains and consequently require either dimerization to one of the other family members, or an interaction with IκB family member Bcl-3, to form transcriptionally active complexes.

Regulation of NF-κB

As a potent transcriptional activator with a diverse set of target genes, NF-κB is tightly regulated through a variety of mechanisms. The best-characterized mechanism of regulation involves the interaction between NF-κB dimers and members of the IκB family of inhibitors. There are at least six mammalian members of the IκB family, IκBα, IκBβ, IκBε, Bcl-3 and the NF-κB precursor proteins p100 and p105. IκB proteins each contain an internal ankyrin repeat domain (ARD) consisting of six or seven 33 amino acid ankyrin repeats. Structural and functional studies have revealed that the ARD mediates tight binding between IκB and NF-κB dimers (Hatada et al. 1992). Importantly, many of the IκB proteins contain a potent nuclear export sequence and mask the nuclear localization sequence present

in the RHD of most NF-κB proteins (Huxford et al. 1998; Malek et al. 1998). Thus, NF-κB/IκB complexes are maintained in the cytoplasm, where NF-κB is unable to promote transcription of its target genes.

Although activation of NF-κB can occur in a number of different ways, disruption of the NF-κB/IκB complex is essential as it allows NF-κB to translocate to the nucleus and bind to target gene promoters. The most common way that this occurs is through the regulated proteosomal degradation of IκB following stimulus specific phosphorylation on two key serine residues and subsequent polyubiquitination (Chen et al. 1995; Scherer et al. 1995). For example, IκBα, which binds to a number of NF-κB dimers including the most abundant p65/p50 heterodimer, is inducibly phosphorylated on serines 32 and 36 following stimulation of cells by TNFα or other pro-inflammatory cytokines (Brown et al. 1995; DiDonato et al. 1996). This phosphorylation results in the recruitment of a ubiquitin ligase complex containing the E3 ubiquitin ligase β-TrCP which covalently attaches a poly-ubiquitin chain to lysines 21 and 22 of IκBα (Yaron et al. 1998). Poly-ubiquitination of IκBα leads to its degradation by the 26S proteosome, allowing the freed NF-κB dimers to accumulate in the nucleus and activate transcription.

Similarly, stimulation of cells with lymphotoxin-β leads to activation of RelB/p100 NF-κB dimers through phosphorylation of serines 927 and 932 in the C-terminus of p100. This phosphorylation leads to poly-ubiquitinaton and degradation of the ankyrin repeat-containing c-terminus, resulting in RelB/p52 dimers, which can then translocate to the nucleus and activate transcription (Senftleben et al. 2001; Lang et al. 2003).

The key player in mediating the disruption of the NF-κB/IκB complex, and thus a key regulator of NF-κB activity, is the IκB kinase complex (IKK). First identified in 1996 as a

700-900 kD complex, the various components of IKK were subsequently cloned and shown to consist of two kinases, IKKα and IKKβ, that share 52% sequence identity, and a regulatory subunit IKKγ/NEMO (Chen et al. 1996; Lee et al. 1997). IKKα and IKKβ are each characterized by an N-terminal kinase domain, a C-terminal helix-loop-helix domain, and a leucine zipper domain (DiDonato et al. 1997; Zandi et al. 1997). IKKγ, which is unrelated to IKKα and IKKβ, contains a C-terminal zinc finger-like domain, a leucine zipper, and N- and C-terminal coiled-coil domains (Yamaoka et al. 1998). IKK phosphorylates members of the IκB family on multiple residues and has other targets as well, including p65.

Kinase activity of IKK requires dimerization, which is mediated by the leucine zipper domains, and available data indicate that heterodimers of IKKα and IKKβ are predominant (Mercurio et al. 1997). While knockout experiments indicate some overlap in function, the two kinases exhibit different specificities for the different IκB family members. As such, IKKβ seems to be the dominant kinase for IκBα, while IKKα is dominant in the lymphotoxin-β mediated phosphorylation of p100 (Li et al. 1999a; Li et al. 1999b; Tanaka et al. 1999; Senftleben et al. 2001). IKKγ, while lacking intrinsic kinase activity, is a key structural component of IKK whose presence is required for the kinase activity of the complex (Rothwarf et al. 1998; Rudolph et al. 2000).

Ultimately, if not exclusively, the numerous signals that lead to activation of NF-κB converge on the IKK complex leading to its phosphorylation of IκB proteins. The subsequent ubiquitination and degradation of IκB and release of NF-κB is the key regulatory step allowing NF-κB to activate its target genes.

Biological Function of NF-κB

The complex regulation of NF-κB activity is critical considering the wide variety of biological processes in which it is involved. Much of what we know about the biological roles of the NF-κB and IκB family members comes from analyses of the various knock-out mice that have been generated for these factors. The data confirm a crucial role for NF-κB in immune function and also highlight the important roles for NF-κB in other cellular and physiological processes.

The p65 knockout mice exhibit embryonic lethality at day E15/16 due to massive liver apoptosis (Beg et al. 1995b). This apoptosis was shown to be dependent on TNFα signaling as this lethality could be rescued by crossing the mice with either the TNFα or TNFR knockout mice (Doi et al. 1999; Alcamo et al. 2001). These double knockout mice exhibit increased susceptibility to bacterial infection, confirming a role for p65 in innate and adaptive immunity. Further, fibroblasts generated from the p65 null embryos displayed a loss of induction of known NF-κB target genes following induction with TNFα, confirming the critical importance of the p65 subunit for NF-κB activity (Beg et al. 1995b).

Genetic analysis of the other NF-κB subunits also highlights the role of the NF-κB family in immune function. The p50 knockout mouse exhibits decreased immunoglobulin production and defective humoral immune responses (Sha et al. 1995). The p52 knockout mice fail to develop normal B-cell follicles and have defects in their splenic microarchitecture and Peyer's patch development (Caamano et al. 1998; Franzoso et al. 1998; Paxian et al. 2002). RelB deficient mice exhibit decreased NF-κB activity in the thymus and spleen and have increased inflammatory infiltration in multiple organs and deficits in adaptive immunity (Weih et al. 1995). Finally, c-Rel deficient mice fail to

generate an effective humoral immune response and their peripheral B and T cells fail to respond to mitogenic stimuli (Kontgen et al. 1995).

Knockout mice have also been generated for many of the IκB family members and suggest some overlap, but not redundancy, in function. IκBα mice exhibit increased, but not constitutive, basal NF-κB activity (Beg et al. 1995a; Klement et al. 1996). Furthermore, analysis of cells lacking IκBβ and IκBε, and double and triple knockouts of all three have revealed that while all three can effectively inhibit NF-κB function, the difference in their activity seems to be primarily due to differences in the rate of their degradation and re-synthesis (Hoffmann et al. 2002). Notably, IκBα is the only IκB known to be transcriptionally regulated by NF-κB and thus it provides critical negative feedback regulation following NF-κB activation. The knockout of IκB family member Bcl-3 also reveals an important role in immune function and will be discussed in detail later.

Genetic analysis of the NF-κB and IκB family members has revealed an important role for this family in all aspects of immune function and in addition to a potentially much broader role regulating apoptosis. The breadth of its influence has only increased since these analyses were performed. As research has progressed, myriad pathways have been found to activate NF-κB resulting in a variety of biological responses. We turn our attention to the pathways leading to NF-κB activation, emphasizing DNA damage, a poorly understood aspect of NF-κB biology and the focus of this dissertation.

Signaling Pathways to NF-κB.

The most intensely studied pathway leading to NF-κB activation has been the tumor necrosis factor (TNF) signaling pathway. The TNF superfamily consists of at least 19

soluble ligands with 29 known receptors and performs multiple roles in the innate and adaptive immune response (Aggarwal 2003). Of these family members, TNFα has been the most widely studied, and has become the canonical model for activation of NF-κB. Upon binding to the TNF receptor (TNFR), TNFα induces receptor dimerization and subsequent recruitment of a host of adaptor molecules including the TNFR associated death domain protein TRADD. Ultimately, TRADD recruitment leads to activation of numerous effector molecules including the pro-apoptotic Caspase 8 and the c-Jun N-terminal Kinase (JNK). In addition, through interactions with the TNFR associated factor (TRAF) family of proteins, the IKK signaling complex is recruited to the receptor and activated (Aggarwal 2003).

While the activation of caspase 8 intitiates a pro-apoptotic signaling cascade, activation of IKK leads to rapid phosphorylation of IκBα and subsequent release of the p65/p50 NF-κB heterodimer. This activation of NF-κB leads to the rapid induction of anti-apoptotic genes such as cIAP1/2 and Bcl-X_L and ultimately the termination of the apoptotic response (Wang et al. 1998). In addition, NF-κB activation leads to induction of pro-inflammatory cytokines such as IL-6 and TNF, which promote an inflammatory response, and manganese superoxide dismutase (MnSOD), which scavenges free radicals and potentiates the JNK response (Collart et al. 1990; Shimizu et al. 1990; Jones et al. 1997; Sakon et al. 2003).

The understanding of the TNF pathway has revealed a clear role for canonical NF-κB (ie- p65/p50) in the regulation of apoptosis. While much of this knowledge can be extended to other NF-κB inducing pathways, the complexity of the signaling urges caution. As the wealth of research on NF-κB has shown, the cellular context and use of different arms of the NF-κB signaling pathway can lead to different outcomes.

An alternative pathway to NF-κB discussed earlier is that downstream of the Lymphotoxin-βR or BAFF-R. Upon ligand activation, these receptors recruit factors that, in a still undefined way, lead to activation of the NF-κB inducing kinase (NIK), which phosphorylates and activates IKKα. IKKα then phosphorylates p100, leading to its processing into p52, allowing p52/RelB dimers to enter the nucleus and activate target genes, including certain chemokines involved in secondary lymphoid organogenesis (Senftleben et al. 2001).

Another important NF-κB pathway is downstream of the Toll-like receptors (TLR). TLRs are evolutionarily conserved pattern recognition receptors that interact with distinct pathogen-associated patterns present on various microbes and elicit a powerful innate immune response. TLRs can bind to diverse ligands such as bacterial associated Lipopolysaccharide (LPS), viral associated dsRNA and non-methylated CpG DNA. Upon activation, these receptors recruit adaptors, including MyD88, and other effector molecules, which results in the activation of transcription factors AP-1, IRF3 and, through activation of IKK, NF-κB. In the context of TLR signaling, NF-κB activation leads to the induction of a set of genes, including pro-inflammatory cytokines, important for recruiting immune cells involved in combating the infection (Janeway and Medzhitov 2002).

The anti-apoptotic effects of NF-κB have also been shown to be important in adaptive immunity. As such, NF-κB is induced in both B and T-cells following activation of the B-cell receptor (BCR) and T-cell receptor (TCR), respectively, to allow for antigen specific proliferation and maturation of lymphocytes into effector cells. The details of NF-κB activation by BCR and TCR are less well understood, but are thought to involve recruitment

of IKK to the immunological synapse and the activity of certain key tyrosine kinases such as ZAP70 (Ruland and Mak 2003).

Each of the pathways discussed thus far involves activation of NF-κB through the recruitment of IKK to a trans-membrane receptor complex followed by release of NF-κB to the nucleus. The differences in the responses to these diverse initiating signals is likely mediated by the timing of activation, the different IKK complexes that are formed at the receptor leading to different IKK activities (ie- TNF vs LT-β), and the interactions of NF-κB with the other signaling pathways downstream of these receptors. Activation of NF-κB by cell stresses, such as DNA damage, therefore presents a unique challenge in that the paradigm of receptor-mediated IKK activation that has been established does not apply. Because of this, and other factors, our knowledge of this important aspect of NF-κB signaling has lagged.

DNA damage signaling to NF-κB

Given the ability of NF-κB to induce anti-apoptotic genes, and the use of DNA damaging therapeutic agents to treat various cancers, a complete understanding of how these agents induce NF-κB and the physiological consequences of that induction are of the utmost importance. It was recognized early on that DNA damage is able to activate NF-κB (Brach et al. 1991) however, discrepancies in the systems used to study this phenomenon have resulted in inconsistent data and an unclear picture of the molecular events leading to NF-κB activation and the subsequent cellular response.

Inducers of DNA double strand breaks, including ionizing radiation and the topoisomerase inhibitor camptothecin, have been shown to induce NF-κB in a process

requiring the protein ataxia-telangiectasia mutated (ATM) (Li et al. 2001). One report showed that treatment of cells with topoisomerase inhibitors results in activation of IKK in a process dependent on the zinc finger domain of IKKγ (Huang et al. 2002). More recent studies have shown that induction of DNA double strand breaks leads to ATM mediated phosphorylation of IKKγ on serine 85, leading to its sumoylation and subsequent nuclear export. In the cytoplasm, sumoylated IKKγ then causes the activation of IKK in a manner dependent on the protein ELKS (ADD REF, WU, 2006, Science). Other studies have shown that the protein RIP is required for activation of IKK in response to DNA damage, but how this requirement relates to the role of IKKγ sumoylation is unclear (Hur et al. 2003).

NF-κB is also activated by UV-C irradtiation, which results in several types of DNA lesions, including pyrimidine dimers. NF-κB activation following UV-C treatment has been shown to be independent of IKK activation and IκBα serine 32/36 phosphorylation, but may require activation of the p38 MAP Kinase and subsequent activation of casein kinase 2 (CK2) (Li and Karin 1998; Kato et al. 2003; Tergaonkar et al. 2003). CK2 phosphorylation of serine residues on the C-terminus of IκBα is proposed to then mediate the release of p65/p50 heterodimers from IκBα (Kato et al. 2003). Similar mechanisms of NF-κB activation have been proposed in cells treated with doxorubicin, and it remains unclear whether the initiating signal is the DNA damage itself or perhaps reactive oxygen species that are generated (Tergaonkar et al. 2003; Zhang and Chen 2004).

The activation of NF-κB by DNA damage is likely also influenced by other damage-induced signaling pathways. For example, it has been shown that p53, which is induced by DNA damage, can induce NF-κB activation, and that this induction is required for p53-mediated apoptosis (Ryan et al. 2000). These data conflict with other reports that suggest

that the activity of p53 and NF-κB is mutually antagonistic due to competition for transcriptional co-activators, and that IKK activation leads to inhibition of p53 through induction of the p53 inhibitor mdm2 (Webster and Perkins 1999; Tergaonkar et al. 2002).

It is also unclear whether NF-κB is always anti-apoptotic when activated by DNA damage. In addition to the report of NF-κB being required for p53-dependent apoptosis, other recent studies have shown that doxorubicin- or UV-C-induced NF-κB can inhibit transcription of anti-apoptotic genes through the recruitment of histone deacetylases to their promoters (Campbell et al. 2004). These studies underscore the importance of understanding the role of NF-κB in mediating the DNA damage response. A number of current cancer therapies involve DNA-damaging chemotherapeutics in combination with inhibitors of NF-κB, under the assumption that induction of NF-κB by these drugs will provide the tumor cells with protection against apoptosis (Orlowski and Baldwin 2002). If NF-κB is in fact pro-apoptotic in certain circumstances, these treatments may have undesirable effects.

Additionally, many of the inhibitors of NF-κB currently in therapeutic use may be limited in that they target key molecules in the canonical NF-κB pathway, such as IKK (Orlowski and Baldwin 2002). Given the available data, it is unclear whether this strategy will target the appropriate NF-κB pathways as other non-traditional NF-κB family members might also play an important role in oncogenesis. One family member in particular whose role in oncogenesis has not been well defined is Bcl-3. A number of recent reports have suggested that Bcl-3 may be involved in the DNA damage response (Boulton et al. 2002; Rocha et al. 2003; Watanabe et al. 2003), perhaps providing an additional link between the NF-κB family and DNA damage. However, the nature of this role has remained unclear.

II. Bcl-3

Bcl-3 function

Bcl-3 was originally identified by molecular cloning of the t(14,19) breakpoint junction occurring in a subset of B-cell chronic lymphocytic leukemias (B-CLL) (McKeithan et al. 1987). This translocation placed the Bcl-3 gene downstream of a strong Ig enhancer element, which resulted in high levels of expression of Bcl-3 protein in these cells. Sequence analysis revealed Bcl-3 to be a 446 amino acid protein with a proline rich N-terminus and proline/serine rich C-terminus flanking 7 internal ankyrin repeats (Ohno et al. 1990). Expression of Bcl-3 is high in both the liver and spleen, with lower expression in the heart, skeletal muscle, lung kidney and testes (Nolan et al. 1993). Further analysis of its expression in mouse B-cell lines shows that its levels are highest at a developmental stage just prior to Ig switch (Bhatia et al. 1991).

Early biochemical analyses revealed that Bcl-3 bound tightly to p50 and p52 homodimers both *in vitro* and *in vivo* (Wulczyn et al. 1992; Bours et al. 1993). An early model of Bcl-3 activity suggested that it aids in NF-κB dependent transcription through its ability to remove inhibitory p50 homodimers from promoters to allow activating p50/p65 heterodimers to bind (Franzoso et al. 1992; Hatada et al. 1992; Wulczyn et al. 1992). Further analysis however showed that Bcl-3 could play a more direct role in transcription when it was shown that Bcl-3, in complex with p52 homodimers, could directly activate transcription from NF-κB dependent promoters through two cooperating transactivation domains located N- and C-terminal to its ankyrin repeat domain (Bours et al. 1993). In support of the latter hypothesis, it was shown that Bcl-3 co-localizes with p50 at sites of active transcription in

the nucleus (Zhang et al. 1994). Further, it was shown that Bcl-3 is extensively phosphorylated on its C-terminus, and that this phosphorylation is critical for allowing p52/Bcl-3 complexes to bind DNA (Bundy and McKeithan 1997).

Bcl-3 Biology

While these early biochemical analyses revealed important aspects of Bcl-3 function, they shed little light on how Bcl-3 was regulated and what genes it may regulate. One early report suggested that Bcl-3 expression is induced by NF-κB and NF-κB-dependent stimuli, although it is unclear whether this occurs in all cell types (Bhatia et al. 1991). More recently, it has been shown that Bcl-3 is induced by Interleukin-1 in chondrocytes and synovial fibroblasts to activate transcription of the matrix metalloproteinase 1 (MMP1) gene (Elliott et al. 2002). It has also been shown that Bcl-3/p52 complexes can induce transcription of the anti-apoptotic Bcl-2 gene, as well as the cell cycle protein cyclin D1 (Westerheide et al. 2001; Viatour et al. 2003). Activation of target genes by Bcl-3 is likely accomplished through its interactions with histone acetylases such as Tip60 and with general transcription factors such as TFIIB, TBP and TFIIA, although the molecular details of these interactions have yet to be worked out (Na et al. 1998; Dechend et al. 1999).

Somewhat surprisingly, Bcl-3 has also been shown to be able to directly inhibit transcription at certain promoters. For example, it has been shown that interleukin 10 (IL10) induces Bcl-3 expression in macrophages and that Bcl-3 directly inhibits LPS-induced TNFα production in these cells (Kuwata et al. 2003). Its ability to interact with the histone deacetylase, HDAC1, likely contributes to this activity (Wessells et al. 2004). Thus, through

differential recruitment of co-activators and co-repressors, Bcl-3 can both activate and repress transcription of target genes.

Some functions of Bcl-3 may also be mediated through its interactions with non-NF-κB related proteins. For example, Bcl-3 has been shown to interact with the retinoic acid receptor (RXR) to activate RXR-induced transcription (Na et al. 1998). Additionally, Bcl-3 has been shown to interact with the transcription factor AP1 (Na et al. 1999). How these various interactions contribute to Bcl-3's biological function is not known.

Some insight into the biological function of Bcl-3 has been gained from studies of the Bcl-3 knockout mouse, which has been generated independently by two different labs. These mice, while developmentally normal, have numerous defects in immune function. They are unable to clear *L. monocytogenes* and are susceptible to infection by *S. pneumoniae*. Additionally, Bcl-3 knockout mice fail to generate antigen-specific antibodies and have abnormal spleen development, including the inability to form germinal centers (Franzoso et al. 1997; Schwarz et al. 1997). Consistent with the high levels of expression in lymphocytes, this phenotype suggests a critical role for Bcl-3 in immune cell function.

Bcl-3 in oncogenesis

In addition to its functions in normal physiology, Bcl-3, when dysregulated, may also play a role in certain pathologies, notably, cancer. Ever since its expression was identified in B-CLL, a role for Bcl-3 in human cancer has been proposed. More recently, high levels of Bcl-3 expression have been noted in a number of other cancers. One survey found Bcl-3 over-expressed in a high percentage of B- and T-cell lymphomas of various origins, including 26% of diffuse large B-cell lymphomas and 23% of all T- and NK-cell lymphomas

(Canoz et al. 2004). Another study showed that Bcl-3 expression was high in a number of anaplastic large cell lymphomas (ALCL) (Nishikori et al. 2003).

In addition to leukemias and lymphomas, Bcl-3 expression is increasingly found in a number of solid tumors. One study has shown that Bcl-3 is found at high levels in the nuclei of many breast cancers (Cogswell et al. 2000), and another showed that Bcl-3 is important for the development of nasopharyngeal carcinoma (Thornburg et al. 2003). However, while Bcl-3 expression is being found to be characteristic of an increasing number of cancers, the data implicating it as a direct causal factor have been limited.

The ability of Bcl-3 to activate transcription of genes such as the anti-apoptotic Bcl-2 and cell cycle activator cyclin D1 has been suggested to contribute to its oncogenicity, but direct links from these activities to oncogenesis have not been formally shown. Bcl-3 has also been shown to be required for the estrogen-independent phenotype in certain breast cancer cell lines, but again, the mechanism through which this is achieved has been elusive (Pratt et al. 2003). One potential oncogenic role for Bcl-3 that has not been explored in depth is its influence on the DNA damage response. As mentioned earlier, activated p53 has been shown to decrease levels of Bcl-3, leading to loss of cyclin D1 expression due to recruitment of HDAC1 to p52 homodimers (Rocha et al. 2003). Bcl-3 has also been shown to interact with a novel protein that has 5' polynucleotide kinase activity, suggesting a potential role in DNA repair (Watanabe et al. 2003). Additionally, a putative orthologue of Bcl-3 was identified in *C. elegans*. When this orthologue was knocked down by RNAi, these worms showed severe defects in the DNA damage response (Boulton et al. 2002).

This evidence suggests an uncovered function of Bcl-3 that could potentially be important both for its normal role in immune cell function, and its role in oncogenesis. To

begin to uncover that role, it is critical to understand what is already known about how cells respond to DNA damage.

III. The DNA damage response

DNA lesions, from both exogenous and endogenous sources, pose a serious threat to the integrity of the genome. The genetic mutations that result from these lesions have serious consequences on cellular function and viability and thus eukaryotic cells have devised myriad complex ways of sensing and dealing with this damage. Years of research in this field have revealed many of the molecular details of the cellular DNA damage response, but many holes still exist on our knowledge. While the vast literature prevents a complete discussion of these details, it is important to review some of the key aspects of this response, including the mechanisms of damage detection, the cell cycle checkpoints and apoptotic pathways elicited by the damage sensing factors, and the role of p53 in this response.

DNA Damage Detection

Normal DNA metabolism and exposure to environmental factors result in a wide variety of chromosomal lesions, and a complex surveillance system exists to recognize these many aberrant structures. In mammals, the current evidence suggests that two key protein kinases (ATM and ATR), belonging to the PI3 Kinase family, and four other key gene products (RAD17, RAD1, RAD9 and HUS1) are involved in damage recognition and initiation of the cellular response (Qin and Li 2003). ATM (ataxia telagiectasia mutated) kinase activity is activated immediately following exposure to ionizing radiation or

radiometric drugs and loss of its function results in radio-resistant DNA synthesis and erroneous entry into mitosis (Lavin and Shiloh 1997; Canman et al. 1998; Rotman and Shiloh 1998). ATM has also been shown to bind directly to DNA double strand break ends (Smith et al. 1999). It has been noted however that AT cells (cells lacking ATM function derived from ataxia telangiectasia patients) exhibit little sensitivity to UV radiation or agents that block replication (Shiloh 1997). This observation led to the cloning and identification of ATR (ATM and RAD3-related) (Keegan et al. 1996). ATR seems to respond to a broader spectrum of inducers than ATM. Thus, while ATM seems to exclusively respond to damage resulting in DNA double strand breaks, ATR responds to UV radiation, replication block, and ionizing radiation (Tibbetts et al. 1999). Loss of ATR affects the activation of key downstream targets such as p53 and leads to defective checkpoint activation following DNA damage signals (Cliby et al. 1998; Wright et al. 1998). Notably, ATM and ATR both localize to distinct nuclear foci following the appropriate DNA damage signal, implicating them both as key effectors of DNA damage signaling (Andegeko et al. 2001; Cortez et al. 2001).

The activation of ATM and ATR following DNA damage requires the formation of a crucial signaling complex consisting of the aforementioned RAD17, RAD9, RAD1 and HUS1 proteins. While the molecular details are still a matter of some debate, following DNA damage, RAD9, RAD1 and HUS form a trimeric complex (the 9-1-1 complex) that loads onto the DNA at the site of damage and resembles the PCNA trimer (which functions as a sliding clamp during DNA replication). RAD17 is critical for the recruitment of the 9-1-1 complex and collectively, these factors are referred to as the checkpoint clamp-loader proteins (Thelen et al. 1999).

How these proteins all interact to activate downstream targets remains to be fully worked out. As such ATM and ATR-mediated phosphorylation of many of the components of the clamp-loader seems to be crucial for complex formation and signal initiation, yet the precise order of events and their requirement for initiation of the checkpoint are unclear. One current model suggests that the ATR and ATM kinases and RAD17 colocalize independently to sites of DNA damage and interact with the 9-1-1 complex to originate the checkpoint signaling process (Qin and Li 2003).

BRCA1 and 53BP1 are other factors that play a role in initiating the signaling cascades downstream of DNA damage. While their exact roles are unclear, these factors interact with multiple checkpoint and repair factors and loss of their function results in multiple checkpoint deficiencies (Joo et al. 2002). Current research suggests that they may function through recruitment of key downstream targets of ATM and ATR.

Once activated, ATM and ATR phosphorylate numerous targets to initiate the broad response to DNA damage. Two serine/threonine kinases (CHK1 and CHK2) are primary targets for ATM and ATR and are thus classified as effector kinases. Activation of these kinases by ATM and ATR leads to the inititiation of the cell cycle checkpoints and phosphorylation of p53, and thus mediate the ultimate decision-making by the affected cell (Matsuoka et al. 1998; Liu et al. 2000).

Replication Checkpoints

Depending on the cell cycle stage of an affected cell, DNA damage can elicit a variety of responses. When cells in G1 are exposed to DNA damage, a key target of ATM/ATR and CHK1/CHK2 is the p53 protein. p53 is normally kept at low levels through

its interaction with the E3 ubiquitin ligase mdm2. Following DNA damage, multiple N-terminal serines of p53 are targeted either directly by ATM and ATR or their effectors CHK1 and CHK2 (Banin et al. 1998; Canman et al. 1998; Hirao et al. 2000; Shieh et al. 2000). These phosphorylation events, in addition to key phosphorylation events on mdm2 itself, lead to the disruption of the p53/mdm2 interaction and accumulation of p53 protein (Vousden and Lu 2002). A key transcriptional target of the activated p53 is the cyclin dependent kinase inhibitor p21. p21 suppresses the activity of the cyclin dependent kinase CDK2, which then prevents entry into S-phase (el-Deiry et al. 1993; Xiong et al. 1993). This delay in S-phase entry allows the cell time to repair the damage and thus prevents duplication of any damage-induced mutations.

The S-phase checkpoint is a critical one as the process of DNA synthesis often creates, or reveals, dangerous lesions. CDC25A is a key target of CHK2 in the S-phase checkpoint. CDC25A is a phosphatase that activates CDK2 by removing a key inhibitory phosphate group on Tyrosine 15 (Galaktionov and Beach 1991; Baratte et al. 1992). Upon DNA damage, ATM-activated CHK2 phosphorylates CDC25A, leading to its degradation and subsequent inhibition of CDK2 function (Falck et al. 2001). Two other proteins important for the S-phase checkpoint are NBS1 and SMC1. Both proteins are targeted directly by ATM and their loss leads to a defective S-phase checkpoint (Carney et al. 1998; Zhao et al. 2000; Yazdi et al. 2002). It is thought that they directly communicate with the replication machinery, although how they contribute to this checkpoint is not well understood.

The main target of the G2/M checkpoint is the phosphatase CDC25C. A direct target of both CHK1 and CHK2, CDC25C is responsible for removing inhibitory phosphates on

Tyrosines 14 and 15 of the cyclin dependent kinase CDC2 (Parker and Piwnica-Worms 1992), which normally complexes with cyclin B to promote entry into mitosis. However, following DNA damage, CDC25C is phosphorylated by CHK1 or CHK2 and sequestered in the cytoplasm, inhibiting its activation of CDC2 (Sanchez et al. 1997; O'Connell et al. 2000). This delays entry into mitosis and allows for repair of any accumulated damage.

Thus, activation of ATM/ATR and CHK1/CHK2 by DNA damage leads to phosphorylation events that target key components of the cell cycle machinery. In most circumstances, this allows time for the cell to repair damage and prevents the cell from passing on mutations. In some instances however, the damage is so extensive that repair is not possible. In these cases, evolution has designed a system in which cells can eliminate themselves rather than risk the possibility of multiplying with inherited mutations. This process of DNA damage-induced apoptosis is carried out primarily through the actions of p53.

p53

As described earlier, activation of ATM/ATR and CHK1/CHK2 by DNA damage leads to the stabilization of and activation of the p53 protein. Induction of p21 and initiation of the G1 checkpoint are key consequences of p53 induction, however its role in protecting cells from DNA damage and a wide variety of other stresses proves to be far more expansive. In addition to DNA damage, oncogene expression also leads to stabilization of p53 through induction of the protein ARF, which binds to and inhibits the p53 inhibitor mdm2 (Zhang et al. 1998). Thus, the actions of p53 act as a check on two of the key initiating events of tumorigenesis, oncogene activation and mutation-inducing genomic damage.

p53 is probably the most widely studied protein in biology, yet new aspects of its biology are still being uncovered. Primarily a transcription factor, active p53 tetramerizes and binds to gene promoters at a 20 bp consensus sequence that is usually present in two copies with a 7-9 bp linker sequence (Vogelstein and Kinzler 1992; Pavletich et al. 1993; Vogelstein and Kinzler 1994). The molecular details of transcription initiation by p53 are still an area of intense research, but it is thought to act through recruitment of the histone acetyl-transferase (HAT) CBP/p300 and the CBP/p300 associated factor P/CAF (Gu et al. 1997; Scolnick et al. 1997). These proteins critically modify promoter associated histone molecules to enable transcription initiation as well as modifying p53 itself to increase its stability.

p53 activity requires numerous post-translational modifications. As mentioned before, disruption of the p53/mdm2 interaction following DNA damage involves specific modifications on N-terminal serines, specifically, serines 15 and 20. Additionally phosphorylation of threonine 18 and serines 46 and 315 are thought to contribute to recruitment of CBP/p300, promoter selectivity, and inhibition of nuclear export. p53 has also been found to be acetylated, sumolated, ubiquitinated and methylated at various C-terminal residues, events that seem to be critical for transcriptional activity and stability, and that mediate the interactions between p53 and its binding partners (Bode and Dong 2004).

Numerous diverse signaling pathways converge on and modify p53 and thus provide the context in which p53 executes its life or death decision. Many of the modifications affect the specificity with which p53 binds to certain promoters and others affect its interactions with other transcription factors and co-factors. The net effect, depending on the particular context, is cell cycle arrest, mediated by targets such as p21, or apoptosis.

The ability of p53 to induce apoptosis has been shown to depend primarily on its ability to induce the transcription of pro-apoptotic target genes. The first pro-apoptotic target gene of p53 to be identified was the Bcl-2 family member Bax, which induces apoptosis by inserting into the outer membrane of the mitochondria and allowing the release of cytochrome C and subsequent activation of Caspase 9 (Miyashita and Reed 1995). However, the finding that Bax null mice were not deficient in p53-dependent apoptosis prompted the search for further targets (Knudson et al. 1995). Subsequent studies have identified a host of p53 target genes that contribute in some way to the apoptotic response. Apaf1, which collaborates with cytochrome C in the activation of Caspase 9 has been shown to be a p53 target gene, as has the FAS receptor, which is an important activator of the extrinsic apoptotic pathway (Owen-Schaub et al. 1995; Soengas et al. 1999). Additionally, Noxa and Puma, which have BH-3 domains in common with the Bcl-2 family of apoptotic factors, have been shown to be critical mediators of the p53-dependent apoptotic response (Oda et al. 2000; Nakano and Vousden 2001). Recent knockouts of these two proteins, which, like Bax, contribute to apoptosis through the release of cytochrome C from the mitochondria, exhibit similar phenotypes to the p53 knockout in their response to apoptotic stimuli (Jeffers et al. 2003; Villunger et al. 2003).

In addition to inducing pro-apoptotic target genes, p53 has also been shown to promote apoptosis in transcription-independent ways. Studies have shown that cytoplasmic pools of p53 exist that are normally bound to the anti-apoptotic protein Bcl-X_L. Upon genotoxic stress, the p53/Bcl-X_L interaction is disrupted through competitive interactions with p53-induced Puma, at which point freed p53 can bind directly to the mitochondrial membrane and contribute to its permeabilization (Chipuk et al. 2004). The importance of

this arm of the p53 pathway is not yet fully known, however it emphasizes the many overlapping ways that p53 contributes to the apoptotic response following genotoxic stress.

Another important target gene of p53 is its inhibitor, the ubiquitin ligase mdm2. Induction of mdm2 by p53 provides critical negative feedback regulation, ensuring the transience of the p53 response (Barak et al. 1993). Thus, in the many instances where apoptotic death is not warranted, p53 is induced long enough for transient growth inhibition but eventually allows for re-entry into the cell cycle.

The reputation of p53 as the guardian of the genome has been well earned. This is readily apparent from the number and type of signals that lead to its induction and the importance of the downstream pathways that it elicits. Studies of a wide spectrum of human tumors as well as analyses of p53 deficient mice have only reinforced this notion.

Studies of human tumors have found that most, if not all, tumors have inactivated p53 through some mechanism. In more than half of tumors, p53 is inactivated through a direct mutation of the p53 gene. While some of these mutations are complete or partial deletions, the majority are point mutations that result in inactivating amino acid substitutions. This leads to accumulation of the mutant protein due to the inability to up-regulate the inhibitor mdm2. It is thought that the accumulation of some of these mutant p53 variants may contribute directly to oncogenesis directly either through dominant negative activity or through the acquisition of novel function. p53 can also be inactivated through binding to the products of viral oncogenes, through the loss of the ARF gene, or through multiplication or over-expression of mdm2. Certain tumors have also been shown to mislocalize p53 to the cytoplasm, inhibiting its function by not allowing it access to target gene promoters (Vogelstein et al. 2000). The message that has been learned from these studies of p53 in

human tumors is that in order for cancer to progress, cells must in some way bypass the critical barrier that p53 has erected to prevent unregulated growth and proliferation.

The critical importance of p53 in cancer development has been also been shown by deletion of p53 in mice. p53 null mice are developmentally normal but develop spontaneous tumors, particularly lymphomas and sarcomas, with a very high frequency (Donehower et al. 1992). Lymphocytes from these mice are protected against DNA damage induced apoptosis, underscoring the importance of p53 in this process (Lowe et al. 1993).

While the importance of p53 as a tumor suppressor has been widely established, the complex regulation of its function remains an intense area of research. How various signaling pathways such as the NF-κB pathway feed into the regulation of p53 is critically important, and by investigating such pathways in the context of p53 we can better understand both p53 function and how these pathways contribute to oncogenesis.

IV. Summary

The NF-κB and p53 pathways are both critical components of cellular function and have important roles in human cancer development. Understanding how these two families interact will give us a more complete understanding of cancer biology and provide useful insights into treating this disease. How non-classical NF-κB factors contribute to this relationship, and how they contribute to oncogenesis, has not been appreciated in the past, but as more is uncovered about these factors, their importance is becoming clearer. To that end, the work described in this dissertation investigates the role of IκB family member Bcl-3 in DNA damaged induced p53 function. The work described contributes to our understanding of how Bcl-3 contributes to oncogenesis and provides a novel mechanism of

regulating p53 function. Hopefully, with this knowledge we will add another piece of the complex puzzle of understanding human cancer biology.

Chapter I

Expression of the Bcl-3 Proto-oncogene Suppresses p53 Activation

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Abstract

While Bcl-3 expression in cancer was originally thought to be limited to B-cell lymphomas with a 14;19 translocation, more recent evidence indicates that expression of this presumptive oncoprotein is significantly more widespread in cancer. However, an oncogenic role for Bcl-3 has not been clearly identified. Experiments presented here indicate that Bcl-3 is inducible by DNA damage and is required for the induction of Hdm2 gene expression and the suppression of persistent p53 activity. Furthermore, constitutive expression of Bcl-3 suppresses DNA damage-induced p53 activation and inhibits p53-induced apoptosis through a mechanism that is at least partly dependent on the upregulation of Hdm2. The results provide insight into a mechanism whereby altered expression of Bcl-3 leads to tumorigenic potential. Since Bcl-3 is required for germinal center formation, these results indicate functional similarities with the unrelated Bcl-6 oncoprotein in suppressing potential p53-dependent cell-cycle arrest and apoptosis in response to somatic hypermutation and class switch recombination.

Introduction

Bcl-3 was first identified through cloning of the t(14;19) breakpoint junction which occurs in a subset of B cell chronic lymphocytic leukemias (B-CLL) (McKeithan et al. 1987). A member of the ankyrin-repeat containing IκB family of NF-κB inhibitors (Hayden and Ghosh 2004), Bcl-3 is apparently unique in that, unlike other IκBs, its localization is nuclear and it contains a transactivation domain. Studies have shown that Bcl-3 preferentially binds to NF-κB p50 and p52 homodimers (Bours et al. 1993; Nolan et al. 1993), and through its interaction with coactivators such as CBP/p300, SRC-1, and Tip60 (Na et al. 1998; Dechend et al. 1999) and co-repressors such as HDAC1 (Wessells et al. 2004), can both activate and repress transcription of target genes. Importantly, genetic knockout of Bcl-3 leads to impaired microarchitecture in spleen and lymph nodes with associated germinal center defects (Franzoso et al. 1997; Schwarz et al. 1997).

Relatively high levels of Bcl-3 expression in certain B-CLL suggests that it may play a direct role in oncogenesis. In this regard, expression of Bcl-3 is sufficient to transform NIH3T3 cells (Viatour et al. 2004). Importantly, Bcl-3 has now been shown to be more widely expressed in cancer with expression in a significant number of breast cancers (Cogswell et al. 2000), nasopharyngeal carcinoma (Thornburg et al. 2003), lymphomas (Canoz et al. 2004), and hepatocellular carcinoma and pancreatic cancer (B. O'Neil,

unpublished). The findings that Bcl-3, in complex with p52, can promote transcription of the genes encoding the cell cycle regulator cyclin D1 and the anti-apoptotic Bcl-2 protein suggest one potential oncogenic mechanism (Westerheide et al. 2001; Viatour et al. 2003), but a more complete understanding of the role of Bcl-3 in human cancers is still lacking.

p53 is a crucial guardian of genomic integrity and its importance as a tumor suppressor is underscored by the fact that it is either mutated or otherwise dysregulated in the majority of human cancers (Vousden and Lu 2002; Vousden and Prives 2005). Normally, p53 remains at low levels through its interaction with the E3 ubiquitin ligase, Hdm2. Upon cellular stress or oncogene activation, this interaction is disrupted and p53 rapidly accumulates in the nucleus where it can activate a number of target genes, including the cyclin dependent kinase inhibitor p21 (el-Deiry et al. 1993), pro-apoptotic genes Noxa and Puma (Oda et al. 2000; Nakano and Vousden 2001; Yu et al. 2001), and its own inhibitor Hdm2 (Barak et al. 1993). Recent evidence also suggests that p53 can play a direct apoptotic role in the mitochondria, independent of its trancriptional function (Chipuk et al. 2004). Ultimately, depending on the type and intensity of the signal, as well as the cellular context, p53 stabilization results in either cell cycle arrest or apoptosis. Loss of p53 function, through either mutation or an alternative mechanism such as over-expression of Hdm2, leads to dysregulated growth, protection against apoptosis, and genomic instability, allowing for the accumulation of secondary mutations (Bond et al. 2005; Vousden and Prives 2005).

Based on the ability of NF-κB to regulate cell death pathways (Ghosh and Karin 2002; Kucharczak et al. 2003) and on recent studies that suggest a potential link between Bcl-3 and the DNA damage response (Boulton et al. 2002; Watanabe et al. 2003), we have investigated a potential role for Bcl-3 in regulating the apoptosis response to DNA damage.

We find that Bcl-3 is transiently upregulated by DNA damage and that constitutive expression of Bcl-3 in MCF-7 cells leads to a strong suppression of DNA damage-induced cell death without a significant effect on NF-κB-regulated anti-apoptotic genes. Importantly, Bcl-3 expression leads to suppression of p53 induction following DNA damage, resulting in the inhibition of expression of the p53 target genes Noxa and Puma. Analysis of Bcl-3 null MEFs or cancer cells knocked down for Bcl-3 reveals that loss of Bcl-3 leads to an enhanced p53 response. One mechanism by which this inhibition occurs is through Bcl-3 mediated induction of the p53 inhibitor Hdm2. Both stable and transient over-expression of Bcl-3 leads to increased Hdm2 expression, and siRNA-mediated knockdown of Bcl-3 blocks expression of Hdm2. Analysis of Bcl-3 null fibroblasts reveals that Bcl-3 is required for the induction of Mdm2 gene expression and for limiting the p53 activation response. Disruption of the p53-Hdm2 interaction in Bcl-3 expressing cells functions to rescue the p53 inhibition and apoptotic response. Furthermore, the data support a model whereby Bcl-3, like the unrelated oncoprotein Bcl-6, functions to suppress p53-dependent cell-cycle arrest and apoptosis in germinal center B-cells undergoing somatic mutation and class switch recombination. Our results provide significant new insight into oncogenic mechanisms associated with Bcl-3 and provide evidence that Bcl-3 is required for p53 to induce expression of Hdm2 gene expression.

Results

Elevated Bcl-3 expression inhibits DNA-damage induced apoptosis.

In addition to its relatively high expression in B-CLL carrying the t(14;19) translocation, Bcl-3 has also been found to be elevated in a number of other human cancers including nasopharyngeal carcinoma (Thornburg et al. 2003), breast cancer (Cogswell et al. 2000), lymphomas (Canoz et al. 2004), and hepatocellular carcinoma (O'Neil et al., unpublished). In order to understand the consequences of Bcl-3 constitutive expression and to investigate its potential role in driving oncogenesis, we engineered the MCF-7 breast cancer cell line to express relatively high levels of Bcl-3 (MCF-7B). Expression of Bcl-3 in the MCF-7B cells is comparable to its expression in several cancer cell lines, including Karpas 299 cells, which have been previously reported to express high levels of Bcl-3 (Nishikori et al. 2003) and in the fibrosarcoma line, HT-1080 (Figure 1.1A).

One characteristic shared by many human cancers is the inability to appropriately respond to genomic insults. To determine if Bcl-3 over-expression might contribute to this deficiency, we treated MCF-7 and MCF-7B cells with either UV-C or the DNA damage-inducing drug cisplatin and measured the apoptotic response. Intriguingly, MCF-7B cells exhibit a significant resistance to DNA damage-induced cell death (Figure 1.1B). As expected, the DNA damage-induced cell death in the parental MCF-7 line is dependent on p53 activity, as co-treatment with the p53 inhibitor Pifithrin-α (Komarov et al. 1999) inhibits the apoptotic response following UV treatment (Figure 1.1C).

Somewhat surprisingly, no increase was observed in the MCF-7B cells of expression of known anti-apoptotic genes that are reportedly regulated by NF-κB following treatment

with UV or cisplatin (D. Kashatus, Unpublished). As such we sought to investigate other potential mechanisms for this anti-apoptotic activity.

Bcl-3 inhibits the p53 response to DNA damage.

As p53 is a critical mediator of the apoptotic response following DNA damage (Vousden and Prives 2005), and given that DNA damage-induced apoptosis in MCF-7 cells is p53 dependent (Figure 1.1C), we sought to investigate whether expression or activation of this tumor suppressor is affected by constitutive expression of Bcl-3. Interestingly, Bcl-3 protein levels increase transiently between 2 and 6 hours following UV treatment in the parental MCF-7 cells, returning to basal levels by 24 hrs, suggesting that this oncoprotein may have a role in the UV response. The increase in Bcl-3 protein is likely to be at the level of protein stability as we detect no increase in Bcl-3 mRNA following UV treatment (D. Kashatus, Unpublished). The transient nature of the response is consistent with the report that p53 can negatively regulate Bcl-3 protein (Rocha et al. 2003), as the loss of Bcl-3 coincides with the accumulation of p53.

As expected, p53 is induced in the parental MCF-7 cells in response to UV treatment, with peak levels reached approximately 8 hours following treatment. Intriguingly, the p53 response in MCF-7B cells is significantly muted, with peak p53 protein levels being considerably lower than in the parental cells (Figure 1.2A). This down-regulation of the p53 response is not correlated with reduced p53 mRNA levels (Figure 1.2B). To ensure that the resulting suppression of the p53 response by Bcl-3 is not a consequence of creating and selecting stable cells, we transiently expressed flag-tagged Bcl-3 in parental MCF-7 cells and treated the cells with UV. Consistent with the results from the stable lines, increased

expression of Bcl-3 leads to a decrease in the levels of p53 protein 4 hrs following UV treatment (Figure 1.2C). Further, we transfected expression vectors for p53 plus Bcl-3 or p53 plus empty vector along with the pg13 p53 responsive reporter (el-Deiry et al. 1993) into parental MCF-7 cells to test p53 transcriptional activity in the presence of excess Bcl-3. As expected, p53 activated the luciferase reporter while co-expression of Bcl-3 partially suppressed this response (Figure 1.2D). Basal p53 activity is very low in these cells, thus any decrease in basal p53 activity upon Bcl-3 over-expression falls below the detection limit of the assay. Taken together, these results indicate that expression of Bcl-3 can suppress the activation of p53 (see Discussion).

To determine the effect of Bcl-3 expression on the induction of p53 target genes, real-time quantitative PCR was performed on cDNA generated from MCF-7 and MCF-7B cells exposed to UV. The cyclin dependent kinase inhibitor p21 is an important target of p53 that functions in p53 dependent cell cycle arrest. Real time PCR analysis of p21 mRNA indicates that Bcl-3 expression leads to an almost complete loss of p21 induction by UV (Figure 1.2E). Other important gene targets of p53 encode pro-apoptotic proteins, including Noxa and Puma, members of the BH3-only class of Bcl-2 homologues. Recent knockouts of these p53 targets indicate that they are essential effectors of p53 dependent apoptosis (Jeffers et al. 2003; Villunger et al. 2003). Analysis of these gene products indicates that, as with p21, constitutive Bcl-3 expression leads to a loss of their induction following UV treatment (Figure 1.2E). Notably, while p21 and Noxa expression are almost completely abrogated, the levels of Puma are only modestly inhibited, consistent with reports of p53-dependent and independent induction of this gene (Jeffers et al. 2003).

Knockdown or knockout of Bcl-3 expression leads to enhanced p53 activation following DNA damage.

To further analyze the role of Bcl-3 in DNA damage-induced p53 function we analyzed the outcome of knockdown of Bcl-3 in HT1080 fibrosarcoma cells since these cells express a functional form of p53 (Suzuki et al. 2003) as well as elevated Bcl-3. siRNA against Bcl-3, but not the control siRNA, leads to a near complete loss of the faster migrating form of Bcl-3 and to a significant reduction in the slower migrating form. Importantly, knockdown of expression of Bcl-3 generates enhanced basal and UV-induced p53 expression (Figure 1.3A). These results indicate that elevated expression of Bcl-3, at least in certain p53 wild-type cancer cells, represses the activation potential of p53. In order to extend these studies and to analyze the outcome of loss of Bcl-3 through a mechanism independent of siRNA knockdown, we analyzed the p53 response in Bcl-3 +/+ and Bcl-3-/- primary mouse embryonic fibroblasts. Exposure of these cells to UV leads to the expected p53 response in wild-type cells, but to elevated levels of p53 at the 24 hr time point in Bcl-3 null cells (Figure 1.3B). Consistent with the observed p53 response in Bcl-3 null fibroblasts, treatment of these cells with UV leads to an increase in apoptosis compared to the wild-type cells (Figure 1.3C). Importantly, co-treatment with pifithrin-α leads to a reduction in the apoptotic response to UV in the Bcl-3 -/- cells, indicating that this response is at least partly dependent on p53 activity (Figure 1.3C). Additionally, loss of Bcl-3 does not sensitize MEFs to TNFα-induced apoptosis (Supplemental Figure 1.1) consistent with the idea that the protection it provides is specific for p53-dependent inducers of apoptosis. These results are consistent with a role for Bcl-3 in suppressing the p53 activation response, both when Bcl-3 is constitutively expressed at high levels and when Bcl-3 is transiently induced in response to DNA damage.

Bcl-3 expression leads to an increase in Hdm2 levels.

p53 activation is controlled through modifications (such as phosphorylation) and through interactions with its inhibitor Hdm2 (Vousden and Prives 2005). Analysis of UV-induced phosphorylation of p53 (ser15 and ser20) in MCF-7B cells indicated that this modification tracked with proteins levels (D. Kashatus, Unpublished), suggesting that Bcl-3 expression does not block p53 ser15 or ser20 phosphorylation. To analyze how high levels of expression of Bcl-3 might lead to the loss of p53 induction by DNA damage, we investigated the levels of the p53 inhibitor Hdm2. Western analysis of MCF-7 and MCF-7B cells reveals that MCF-7B cells exhibit consistently higher Hdm2 levels (Figure 1.4A, 1st Panel), providing a potential mechanism for the inhibition of p53 seen in these cells. Importantly, transient over-expression of Bcl-3 in parental MCF-7 cells also leads to increased Hdm2 protein (Figure 1.4A, 2nd panel), confirming that the increase seen in the stable cells is not an artifact of clonal selection. This increase in Hdm2 in MCF-7B cells is also evident at the RNA level, as real-time quantitative PCR indicates a roughly 3-fold increase in Hdm2 levels in MCF-7B cells relative to parental MCF-7 cells (Figure 1.4B). To show that this effect is not unique to MCF-7 cells, we transiently transfected Bcl-3 into the U-2OS osteosarcoma line. Consistent with the results from MCF-7 cells, increasing levels of Bcl-3 leads to proportionately increased levels of Hdm2 protein in these cells (Figure 1.4C).

To determine whether the Bcl-3 dependent increase in Hdm2 might be due to a direct role for Bcl-3 at the Hdm2 promoter, we performed ChIP analyis using PCR primers specific for the P2 promoter region of the Hdm2 promoter, flanking the two conserved p53 binding sites. As reported for other p53 inducible genes (Espinosa et al. 2003), there are relatively

low levels of p53 at the promoter under basal conditions, with a several fold increase following UV treatment (Figure 1.4D). Notably, the levels of p53 at the promoter 4 hrs post UV are lower in the MCF-7B cells when compared to the parental MCF-7 cells, consistent with the lower levels of stabilized p53 in these cells. Intriguingly, Bcl-3 is also present at the Hdm2 promoter at levels consistent with its expression in the cells. In MCF-7 cells, Bcl-3 is not detectable basally but increases as the protein levels increase, while in the MCF-7B cells it is present both basally and after UV treatment (Figure 1.4D). As a control, it is shown that neither Bcl-3 nor p53 are present at significant levels at the β-actin promoter.

The observed recruitment of Bcl-3 to the Hdm2 promoter led us to investigate potential binding sites for Bcl-3 in the Hdm2 P2 promoter region. We identified a putative NF-κB binding site 275 bp upstream of the exon 2 start site that is conserved in the mouse Mdm2 promoter (Supplemental Figure 1.2A). Intriguingly, gel shift analysis using a 32P-labeled oligonucleotide probe specific for this site shows increased binding to the probe in the MCF-7B cells compared to the parental MCF-7 cells. Further analysis using antibodies specific for various NF-κB subunits shows that the complex binding this site contains both p50 and p52, known binding partners of Bcl-3 (Supplemental Figure 1.2B).

Additionally, we performed reporter assays using Hdm2 P2 luciferase reporters containing various portions of the P2 promoter. The two reporters containing the region of the promoter that includes the NF-κB binding site (Hdm2-Luc-01 & Hdm2-Luc-02) consistently exhibit increased activity in the MCF-7B cells compared to the parental MCF-7 cells. Conversely, the reporter construct that lacks the region containing the NF-κB site (Hdm2-Luc-03) exhibits no difference between the two cell types (Supplemental Figure 1.2C). While we can't rule out the existence of other important regulatory sites in this

deleted region, these data are consistent with the importance of this binding site for Bcl-3-induced Hdm2 transcription.

Bcl-3 is required for Hdm2 gene expression.

Given that Bcl-3 is recruited to the Hdm2 promoter following UV treatment (Figure 1.4D), and that its elevated expression leads to increased levels of Hdm2, we sought to determine whether Bcl-3 is required for Hdm2 expression. Knockdown of Bcl-3 by RNAi in both MCF-7 and Karpas 299 cells leads to a near complete loss of Hdm2 expression, consistent with Bcl-3 being required for basal Hdm2 expression in these cells (Figure 1.5A). Note that Karpas 299 cells are reported to express mutant p53 (Hubinger et al. 2001), suggesting a lack of involvement of p53 in control of Hdm2 gene expression in these cells. To further test our hypothesis, primary fibroblasts from mice lacking Bcl-3 and their wild-type littermates were analyzed following DNA damage. When wild type or Bcl-3 null MEFs are treated with either UV or the DNA damage inducing drug cisplatin, cells lacking Bcl-3 fail to induce mdm2 mRNA to the levels seen in wild-type cells (Figure 1.5B). Consistent with this, the induction of Mdm2 protein levels is severely repressed in Bcl-3 -/- cells as compared to their wild-type counterparts (Figure 1.5C). In order to determine if suppression of Bcl-3 inhibits the induction of Hdm2 in another cell type, siRNA was utilized to suppress Bcl-3 expression in HT-1080 cells, which express functional p53 (Suzuki et al. 2003). Knockdown of Bcl-3 in HT-1080 cells also leads to a suppression of the induction of Hdm2 following UV treatment (Figure 1.5D). These results demonstrate an important role for Bcl-3 in controlling Hdm2/mdm2 gene expression in several cell types.

Disruption of the p53-Hdm2 interaction in MCF-7B cells rescues the DNA damage-induced p53 response.

In order to determine if Bcl-3-mediated repression of DNA damage-induced p53 stability involves Hdm2, we analyzed whether disruption of the p53-Hdm2 interaction is able to rescue p53 responsiveness in MCF-7B cells. The recently described compound RITA (reactivation of p53 and induction of tumor cell apoptosis) has been shown to bind to p53 both *in vitro* and *in vivo* and to prevent its interaction with Hdm2 (Issaeva et al. 2004). MCF-7 and MCF-7B (Bcl-3 expressing) cells were exposed to RITA or to diluent, and to UV treatment. The results demonstrate the expected p53 induction in MCF-7 cells and the strong suppression of p53 activation in MCF-7B cells. Importantly, exposure of the MCF-7B cells to RITA leads to a rescue of the Bcl-3-controlled repression of p53 as evidenced by higher levels of p53 stabilization than in cells treated with UV and vehicle control (Figure 1.6A). To test the Hdm2 dependence in another way, we transfected MCF-7B cells with either a control siRNA or an siRNA specific for Hdm2. Consistent with what we see following RITA treatment, knockdown of Hdm2 in MCF-7B cells leads to a rescue of the p53 response following UV treatment (Figure 1.6B). Collectively, these data indicate that the repression of p53 induction seen in MCF-7B cells is dependent on the p53-Hdm2 interaction, consistent with the model that increased Hdm2 levels in MCF-7B cells are responsible for the repression.

To determine if the protection against apoptosis observed in MCF-7B cells (see Figure 1.1) is dependent on Hdm2-mediated repression of p53, MCF-7 and MCF-7B cells were either treated with UV alone or with UV plus RITA and the apoptotic response was measured. Consistent with the data from figure 1.1, MCF-7B cells are significantly protected

against apoptosis when treated with UV alone. However, cotreatment with RITA rescues the apoptotic defect in MCF-7B cells. Importantly, cotreatment with UV and RITA results in equivalent levels of apoptosis in MCF-7 and MCF-7B cells, demonstrating that the interaction between p53 and Hdm2 is critical for the observed apoptotic differences in the two cell lines (Figure 1.6C). Similarly, siRNA-mediated knockdown of Hdm2 is able to restore the UV-induced apoptotic response in MCF-7B cells (Supplemental Figure 1.3), albeit to a lesser extent than treatment with RITA. This is likely due to a less than 100% transfection efficiency in these cells leading to an incomplete knockdown of Hdm2. These data further demonstrate the importance of the p53-Hdm2 interaction the antiapoptotic effects of Bcl-3. Collectively, these data indicate that the Bcl-3-mediated protection against DNA damage-induced apoptosis involves the regulated interaction between p53 and Hdm2 and support the hypothesis that Bcl-3-controlled induction of Hdm2 is responsible for the observed repression of p53.

Discussion

The data presented here reveal a surprising pro-oncogenic role for the oncoprotein Bcl-3, namely its ability to suppress the activation of the p53 tumor suppressor protein in response to DNA damage. A major proposed mechanism in this regulatory circuit is the ability of Bcl-3 to induce the expression of Hdm2, the inhibitor of p53. The experiments demonstrate that expression of Bcl-3 leads to an increase in Hdm2 levels and to a subsequent loss of DNA damage induced p53 protein stability. The consequence of the Bcl-3 mediated inhibition of p53 is a loss of induction of p53 target genes and protection against DNA damage-induced apoptosis. Interestingly, the loss of Bcl-3 leads to the inability of p53 to induce hdm2 transcription and, correspondingly, to enhanced p53 protein stability, suggesting that a normal physiological role of Bcl-3 is to limit the p53 activation response.

Bcl-3 over-expression is characteristic of a growing number of human cancers (Cogswell et al. 2000; Thornburg et al. 2003; Canoz et al. 2004), yet a specific oncogenic role for Bcl-3 in these cancers has been elusive. Recently we and others (Westerheide et al. 2001; Rocha et al. 2003) demonstrated that Bcl-3 can contribute to cyclin D1 transcription, a key component in driving cell cycle progression. The finding that Bcl-3 can inhibit p53 function provides a novel mechanism for how aberrant Bcl-3 expression can fulfill an oncogenic function. The importance of p53 as a tumor suppressor is illustrated by the fact that it is mutated or lost in over half of all human cancers tested (Vousden and Lu 2002; Vousden and Prives 2005) and presumably cancer cells with wild-type p53 have compromised its function through different mechanisms. Loss of the tumor suppressor ARF and high level expression of Hdm2 have been thought to be two of main ways that this is

achieved. Consistent with this, it has been reported (Bond et al. 2004) that an Hdm2 promoter polymorphism increases affinity for the transcription factor Sp1 promoting high level transcription and subsequent attenuation of the p53 response. Additionally, N-Myc (Slack et al. 2005) and estrogen receptor-α (Phelps et al. 2003) can regulate Hdm2 in a p53-independent fashion. Similarly, Bcl-3, through induction of Hdm2, would provide a level of inhibition to p53 that tumor cells need to progress, bypassing the need for mutation or deletion of p53.

Current models of p53 activation by DNA damage suggest that post-translational modification of p53 disrupts its interaction with Hdm2 and thus it might be suggested that any increase in Hdm2 protein levels would have a minimal effect on p53 protein stability following DNA damage. However, it is likely that the dynamics of the DNA damage response are more complex. First, it has been reported that modification of both p53 and Hdm2 are important for the disruption of the interaction (Mayo et al. 1997a; Hay and Meek 2000), suggesting that a consistent supply of newly transcribed Hdm2 provided by Bcl-3 would provide a steady stream of unmodified Hdm2 to re-engage p53. Further, the fact that p53-induced Hdm2 provides negative feedback regulation of p53 suggests that at some point following the initial damage event, the newly transcribed Hdm2 is able to rebind to p53 and turn off the response. Consistent with this, our data indicate that Bcl-3-induced Hdm2 will indeed have the effect of dampening the p53 response. This conclusion is further supported by the many reports of Hdm2 over-expression in human cancers (Momand et al. 1998).

Furthermore, while our data indicate that Bcl-3 can regulate p53 through induction of Hdm2, we cannot rule out additional roles for Bcl-3 in regulating p53 function. For example, certain p53-dependent genes have NF-κB consensus sites in their promoters and Bcl-3,

presumably through interactions with the p50 or p52 NF-κB subunits, may play a direct role in the regulation of those genes. In fact, we have found recruitment of Bcl-3 as well as other NF-κB subunits to a number of those promoters and are currently investigating those mechanisms (D. Kashatus, Unpublished). Additionally, higher levels of Bcl-3 may affect NF-κB dimer composition and indirectly affect NF-κB regulation of those promoters. It is also possible that Bcl-3 regulates p53 protein levels or functional activity through additional mechanisms. While we find no evidence that Bcl-3 has a role in direct transcriptional regulation of p53, it may affect p53 through control of stability or translational efficacy.

The finding of this novel function for Bcl-3 raises intriguing questions regarding a role for Bcl-3 in development, immune and inflammatory function, and in the stress response. An indication of what that role may be comes from a the recent finding that Bcl-6, a transcriptional repressor also found aberrantly expressed in B-cell lymphomas which is unrelated to Bcl-3, directly inhibits p53 transcription in germinal center B-cells (Phan and Dalla-Favera 2004). This negative regulation of p53 is presumed to be important to allow these cells to tolerate the DNA damage required for immunoglobulin class switch recombination and somatic hypermutation without mounting an apoptotic response. Our new data, plus the fact that Bcl-3 knockout mice, like the Bcl-6 knockouts, fail to develop germinal centers (Franzoso et al. 1997), suggests that Bcl-3 may function to block p53 activation in germinal center B-cells, but through a different mechanism than that reported for Bcl-6. It has also been shown that expression of Bcl-3 correlates with survival in adjuvant-induced T-cells (Mitchell et al. 2001). Activated T-cells require a survival signal following antigen stimulation in order to avoid apoptosis and to undergo clonal expansion. The apoptotic signal has been shown to be mediated by the p53 family member p73 (Wan

and DeGregori 2003). Given its role in p53 inhibition, Bcl-3 may protect activated T-cells through down-regulation of this apoptotic response by inhibiting p73 through a similar mechanism. The failure of Bcl-3 null mice to mount an effective T-cell response to influenza virus supports this role for Bcl-3 (Franzoso et al. 1997). Finally, we observe that Bcl-3 is activated by UV, potentially at the level of protein stabilization (see Fig. 1.2). Given that loss of Bcl-3 leads to enhanced p53 activation and to enhanced apoptosis (Fig. 1.3), it is likely that the induction of Bcl-3 by UV (or by other DNA damaging agents) functions normally to limit p53 induction and apoptosis.

Given the previous intriguing results regarding both cooperative (Ryan et al. 2000) and antagonistic (Webster and Perkins 1999) relationships between the NF-κB and p53 pathways, it would be expected that the findings reported here relate to these original findings. However, the direct relationship between Bcl-3 expression and the classic NF-κB pathway remains unclear. In this regard, it has been reported that Bcl-3 gene expression can be induced in an NF-κB-dependent manner (Brasier et al. 2001). Our analyses of p65 null MEFs, however, do not reveal a similar defect in mdm2 expression or p53 stabilization (D. Kashatus, Unpublished), suggesting the Bcl-3 mediated regulation is p65 independent. Further analysis of cells lacking other NF-κB subunits, specifically p50 and p52, should allow us to determine their requirement in this regulation. The fact that loss of IKKβ leads to a loss of mdm2 induction suggests that this kinase may function upstream of Bcl-3 in this response (Tergaonkar et al. 2002). IKKβ may act through regulation of p50 or p52, or it could potentially be involved in the DNA damage-induced stabilization of Bcl-3 through direct phosphorylation.

In summary, our findings provide a critical understanding of the functional consequences of Bcl-3 expression in human cancer cells and could potentially have direct clinical applications for cancer treatment. Unlike Bcl-3, p53 and Hdm2 are popular targets for drug development and these findings may provide rationale for a potential new intervention pathway for certain cancers. These data also provide an additional link in the expanding relationships between the NF-κB/IκB family and p53 and open new avenues for exploration into how these two important protein families interact to control oncogenesis.

Figure 1.1

Over-expression of Bcl-3 inhibits DNA damage induced apoptosis **(A)** Expression of Bcl-3 in MCF-7, MCF-7B, Karpas 299 and HT-1080 cells. Western blots of extracts from indicated cell lines were probed with antibodies against Bcl-3 and β-tubulin. **(B)** MCF-7B cells are protected against DNA damage-induced apoptosis. MCF-7 and MCF-7B cells were left untreated, or treated with 40 J/m^2 UV-C or 10 μg/ml cisplatin as indicated. 18 hrs following treatment apoptosis was measured by flow cytometric analysis of Annexin-V staining. **(C)** UV-induced apoptosis in MCF-7 cells is p53 dependent. MCF-7 cells were treated with DMSO, 10 μM pifithrin-α, 40 J/m^2 UV-C plus DMSO or 40 J/m^2 UV-C plus 10 μM pifithrin-α as indicated. 18 hrs following treatment apoptosis was measured by flow cytometric analysis of Annexin-V staining.

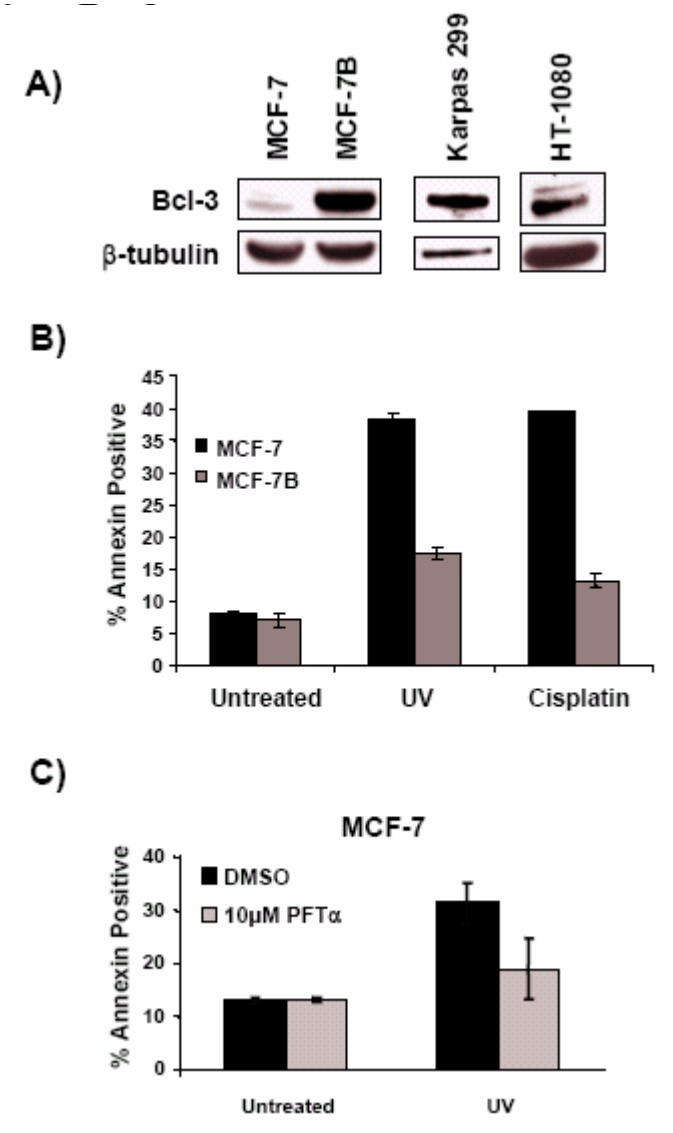
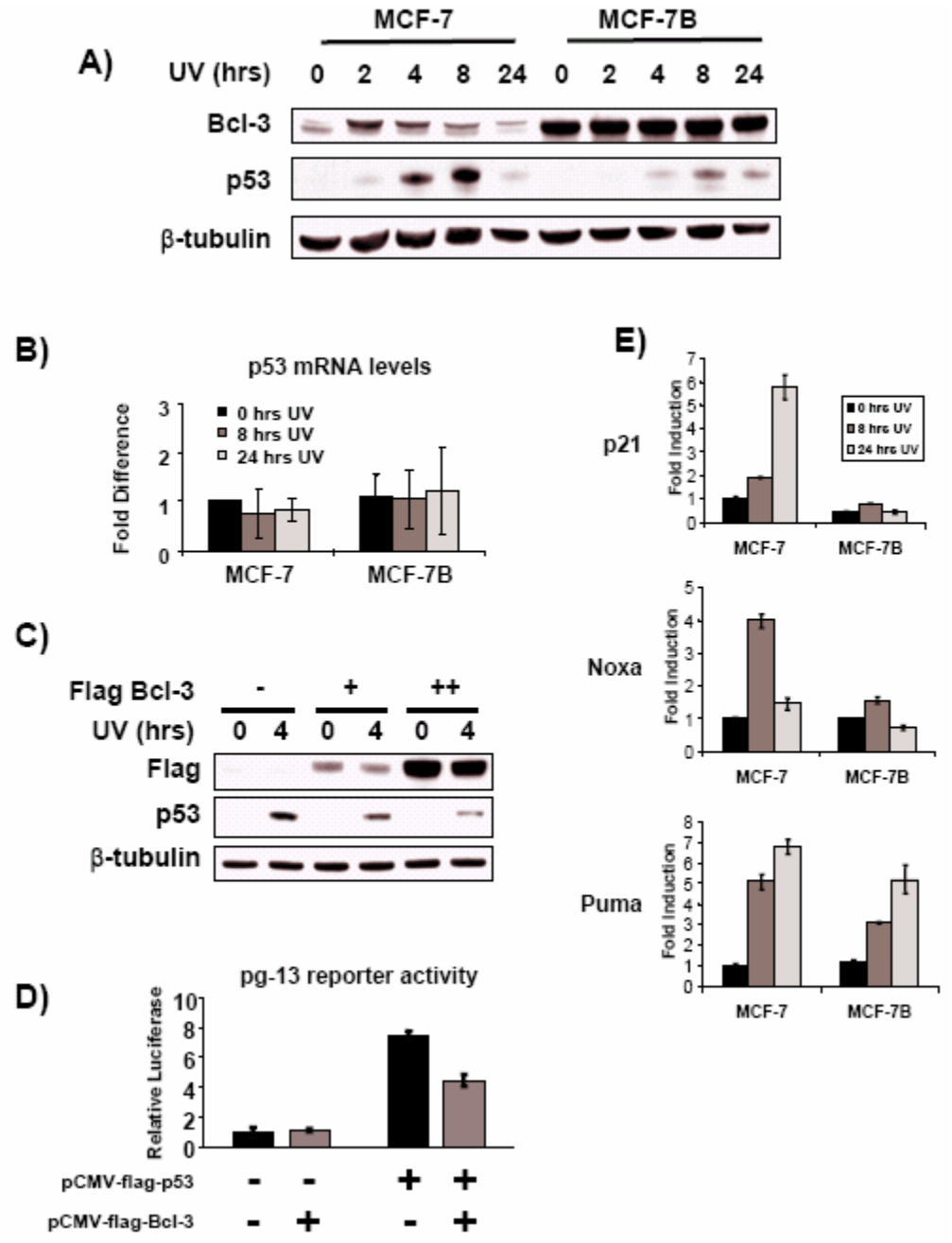

A)
MCF-7
MCF-7B
Karpas 299
HT-1080
Bcl-3
β-tubulin
B)
% Annexin Positive
MCF-7
MCF-7B
Untreated
UV
Cisplatin
C)
MCF-7
% Annexin Positive
DMSO
10μM PFTα
Untreated
UV

Figure 1.2

Bcl-3 over-expression inhibits DNA damage-induced p53 activity. **(A)** UV-induced p53 protein levels are reduced in MCF-7B cells. MCF-7 and MCF-7B cells were treated with 40 J/m^2 UV-C for the indicated times and western analysis was performed on whole cell extracts using antibodies against Bcl-3, p53 and β-tubulin. **(B)** Over-expression of Bcl-3 does not affect p53 mRNA levels. MCF-7 and MCF-7B cells were treated with 40 J/m^2 UV-C for the indicated times and relative expression of p53 was measured by quantitative real-time PCR. Expression levels were normalized to expression of glucuronidase-β and the values represent the fold increase or decrease relative to untreated MCF-7 cells (lane 1). **(C)** Transient expression of Bcl-3 leads to decreased p53 protein levels following UV treatment. MCF-7 cells were transfected with either empty vector or 2 μg or 4 μg of pCMV-flag-Bcl-3. Two days following transfection, the cells were left untreated or treated with 50 J/m^2 UV-C for 4 hrs and western analysis was performed with antibodies against the flag epitope, p53 or β-tubulin. **(D)** Transient expression of Bcl-3 inhibits p53 transcriptional activity. MCF-7 cells were transfected with 50 ng of pg-13-luciferase and 5 ng renilla luciferase plus 100 ng of pCMV-flag-Bcl-3 and pCMV-flag-p53 where indicated. Firefly luciferase activity was measured and normalized to renilla luciferase. Values represent fold increase over basal activity (lane 1). **(E)** DNA damage-induced expression of p53 target genes is lost in MCF-7B cells. MCF-7 and MCF-7B cells were treated with 40 J/m^2 UV-C for the indicated times and relative expression of p21, Noxa and Puma was measured by quantitative real-time PCR. Expression levels were normalized to expression of glucuronidase-β and the values represent the fold increase or decrease relative to untreated MCF-7 cells (lane 1).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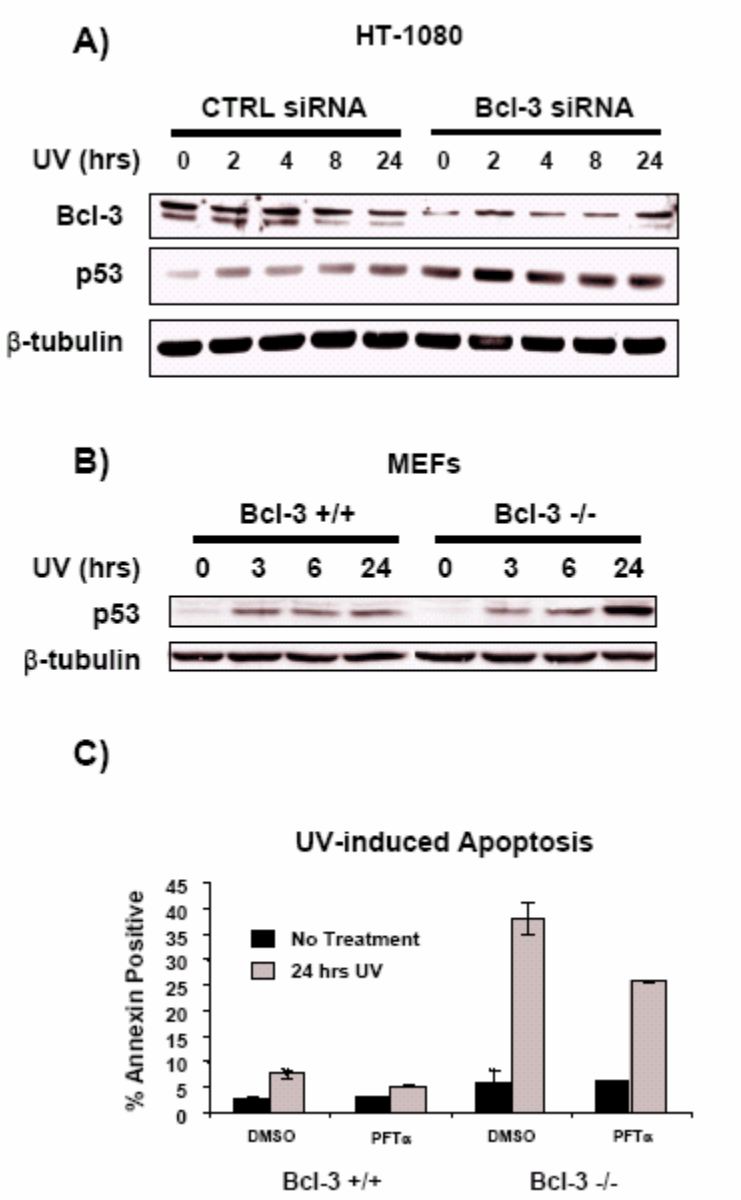
MCF-7
MCF-7B
UV (hrs)
0 2 4 8 24 0 2 4 8 24
Bcl-3
p53
β-tubulin
B)
p53 mRNA levels
Fold Difference
0 hrs UV
8 hrs UV
24 hrs UV
MCF-7
MCF-7B
C)
Flag Bcl-3
- + ++
UV (hrs)
0 4 0 4 0 4
Flag
p53
β-tubulin
D)
pg-13 reporter activity
Relative Luciferase
pCMV-flag-p53
pCMV-flag-Bcl-3
E)
p21
Noxa
Puma
Fold Induction
0 hrs UV
8 hrs UV
24 hrs UV
MCF-7
MCF-7B

Figure 1.3

Loss of Bcl-3 leads to increased levels of p53 following DNA damage and sensitivity to DNA damage-induced apoptosis. **(A)** Knockdown of Bcl-3 results in an increase of UV-induced p53 protein. HT-1080 cells were transfected with a control siRNA or an siRNA targeting Bcl-3. 48 hrs following the transfection, cells were treated with 40 J/m^2 UV-C for the indicated timepoints. Western analysis was performed on whole cell extracts using antibodies against Bcl-3, p53 and β-tubulin. **(B)** Bcl-3 null MEFs have increased p53 levels following UV treatment. Wild-type and Bcl-3 deficient mouse embryonic fibroblasts were treated with 40 J/m2 UV-C for the indicated timepoints. Western analysis was performed on whole cell extracts using antibodies against p53 and β-tubulin. **(C)** Bcl-3 null MEFs are sensitized to p53 dependent UV-induced apoptosis. Bcl-3+/+ and Bcl-3-/- MEFs were treated with DMSO, 10 μM pifithrin-α, 40 J/m^2 UV-C plus DMSO or 40 J/m^2 UV-C plus 10 μM pifithrin-α as indicated. 18 hrs following treatment, apoptosis was measured by flow cytometric analysis of Annexin-V staining.


A)
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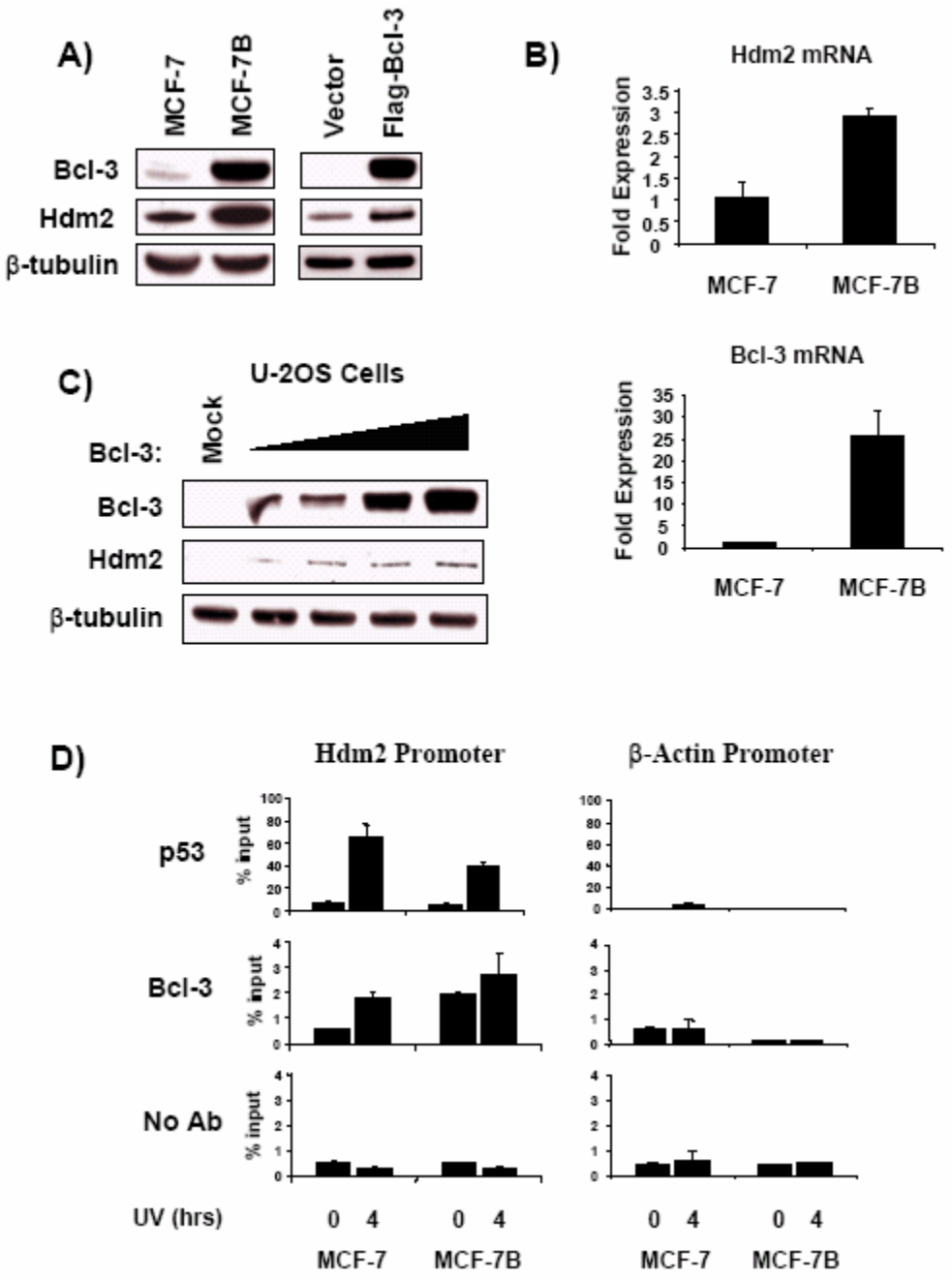
Bcl-3 siRNA
UV (hrs)
0 2 4 8 24 0 2 4 8 24
Bcl-3
p53
β-tubulin
B)
MEFs
Bcl-3 +/+
Bcl-3 -/-
UV (hrs)
0 3 6 24 0 3 6 24
p53
β-tubulin
C)
UV-induced Apoptosis
% Annexin Positive
45
40
35
30
25
20
15
10
5
0
No Treatment
24 hrs UV
DMSO
PFTα
DMSO
PFTα
Bcl-3 +/+
Bcl-3 -/-

Figure 1.4

Over-expression of Bcl-3 leads to an increase in Hdm-2 expression. **(A)** Both transient and stable over-expression of Bcl-3 lead to higher basal levels of Hdm-2 protein. Whole cell extracts were prepared from MCF-7 and MCF-7B cells as well as MCF-7 cells transfected with 8 μg of either empty vector or pCMV-flag-Bcl-3 and western analysis was performed using antibodies against Bcl-3, Hdm-2 and β-tubulin. **(B)** MCF-7B cells have higher basal levels of Hdm-2 RNA. Real-time quantitative PCR was performed on cDNA prepared from MCF-7 and MCF-7B cells using primers specific for Bcl-3 and Hdm-2. Expression levels were normalized to expression of glucuronidase-β and values represent fold difference relative to MCF-7 (lane 1) for each gene tested. **(C)** Transient over-expression of Bcl-3 leads to an increase in Hdm-2 levels in U-2OS cells. U-2OS cells were transfected with 0-200 ng of pCMV-flag-Bcl-3 in 50 ng increments. Total DNA content was brought to 200 ng with pCMV-flag vector. 48 hrs following transfection, western analysis was performed on whole cell extracts using antibodies against Bcl-3, Hdm-2 and β-tubulin. **(D)** Bcl-3 is present at the Hdm-2 promoter at higher levels in MCF-7B cells. MCF-7 and MCF-7B cells were treated with 40 J/m2 UV-C for either 0 or 4 hours and Chromatin immunoprecipitation was performed using antibodies specific for p53, Bcl-3 or no antibody. Real-time quantitative PCR was performed on precipitated DNA using primers specific for the p2 promoter region of the Hdm-2 gene and for the promoter region of the β-Actin gene. Values are normalized against the input DNA and are represented as percent of input for each given sample. Each value represents the mean of three independent measurements of the precipitated DNA and

the error bars represent one standard deviation. The experiment was repeated three times with identical results.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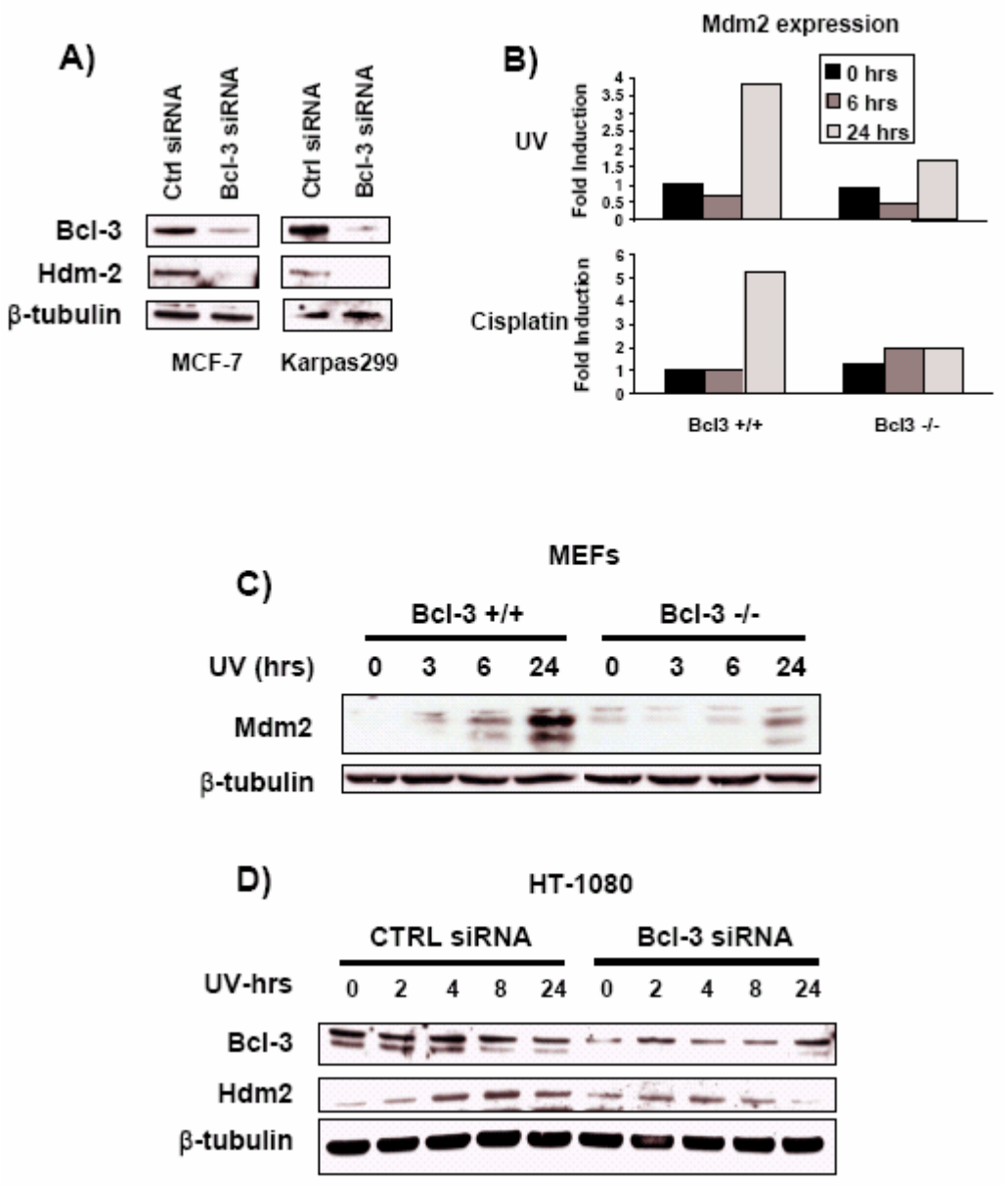
MCF-7B
Vector
Flag-Bcl-3
Bcl-3
Hdm2
β-tubulin
B)
Hdm2 mRNA
Fold Expression
3.5
3
2.5
2
1.5
1
0.5
0
MCF-7
MCF-7B
C)
U-2OS Cells
Mock
Bcl-3:
Bcl-3
Hdm2
β-tubulin
Bcl-3 mRNA
Fold Expression
35
30
25
20
15
10
5
0
MCF-7
MCF-7B
D)
Hdm2 Promoter
β-Actin Promoter
p53
Bcl-3
No Ab
% input
UV (hrs)
0 4 0 4
MCF-7 MCF-7B
0 4 0 4
MCF-7 MCF-7B

Figure 1.5

Loss of Bcl-3 leads to a decrease in basal and DNA damage-inducible Hdm2 expression. **(A)** Knockdown of Bcl-3 in human cancer cells leads to loss of Hdm-2 expression. MCF-7 and KARPAS 299 cells were transfected with either control siRNAs or siRNAs specific for Bcl-3. 48 hours following transfection, western analysis was performed on whole cell extracts using antibodies specific for Bcl-3, Hdm-2 and β-tubulin. **(B)** DNA damage fails to induce Mdm-2 RNA in Bcl-3 deficient fibroblasts. Wild type and Bcl-3-null MEFs were treated with either 40 J/m2 UV-C or 10 μg/ml cisplatin for the indicated times and Mdm-2 gene expression was measured by quantitative real-time PCR. Expression levels were normalized to expression of GAPDH and the values represent the fold increase or decrease relative to untreated wild-type MEFs (lane 1). **(C)** DNA damage fails to induce Mdm-2 protein in Bcl-3 deficient fibroblasts. Wild type and Bcl-3-null MEFs were treated with 40 J/m2 UV-C for the indicated times and western blots were performed on whole cell extracts using antibodies specific for Mdm-2 and β-tubulin. **(D)** Knockdown of Bcl-3 impairs the ability of DNA damage to induce Hdm-2 in HT-1080 cells. HT-1080 cells were transfected with either control siRNAs or siRNAs specific for Bcl-3. 48 hours following transfection, cells were treated with 40 J/m^2 UV-C for the indicated times and western analysis was performed on whole cell extracts using antibodies specific for Bcl-3, Hdm-2 and β-tubulin.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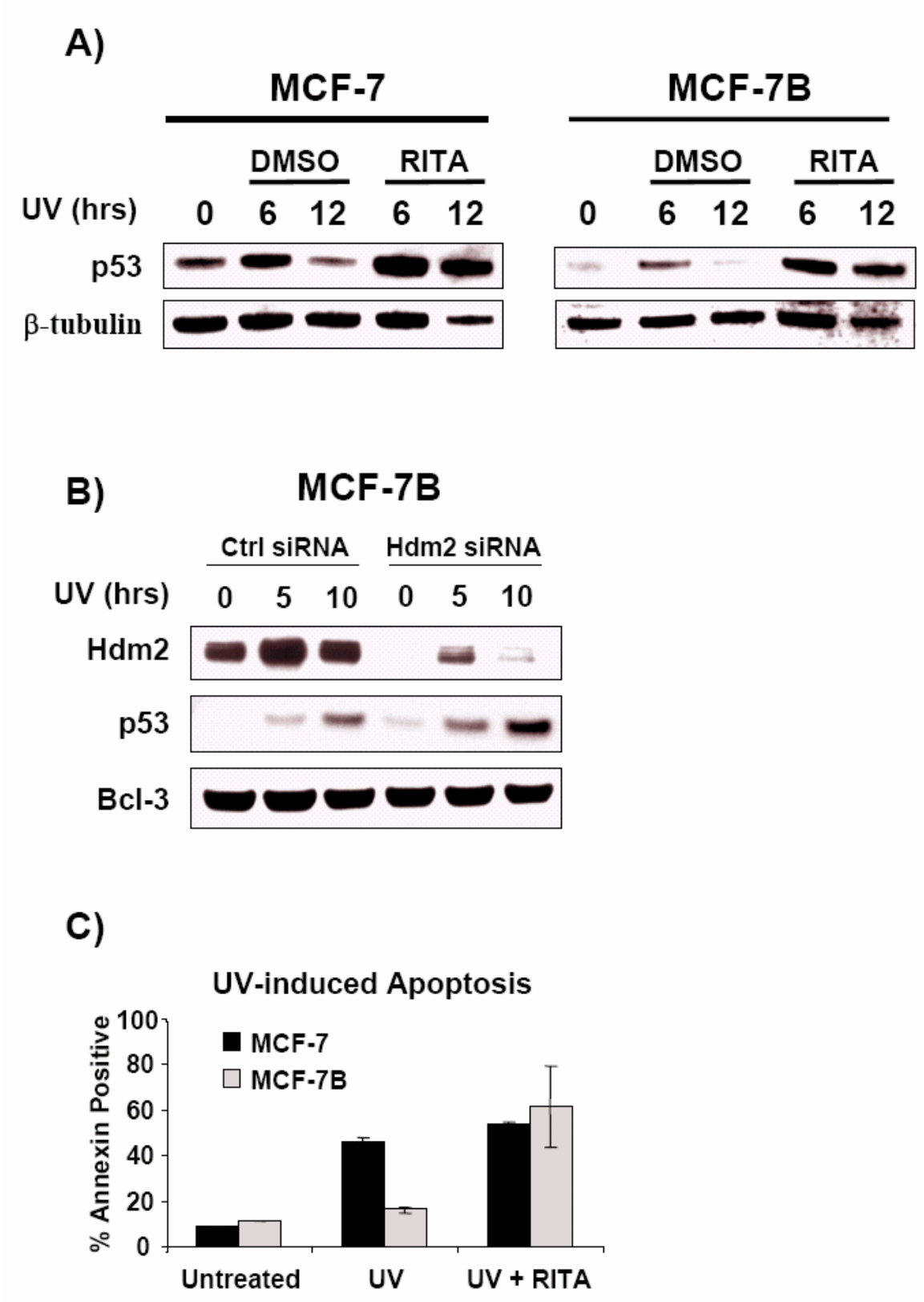
Ctrl siRNA
Bcl-3 siRNA
Ctrl siRNA
Bcl-3 siRNA
Bcl-3
Hdm-2
β-tubulin
MCF-7
Karpas299
B)
Mdm2 expression
0 hrs
6 hrs
24 hrs
UV
Fold Induction
Cisplatin
Fold Induction
Bcl3 +/+
Bcl3 -/-
C)
MEFs
Bcl-3 +/+
Bcl-3 -/-
UV (hrs)
0 3 6 24 0 3 6 24
Mdm2
β-tubulin
D)
HT-1080
CTRL siRNA
Bcl-3 siRNA
UV-hrs
0 2 4 8 24 0 2 4 8 24
Bcl-3
Hdm2
β-tubulin

Figure 1.6

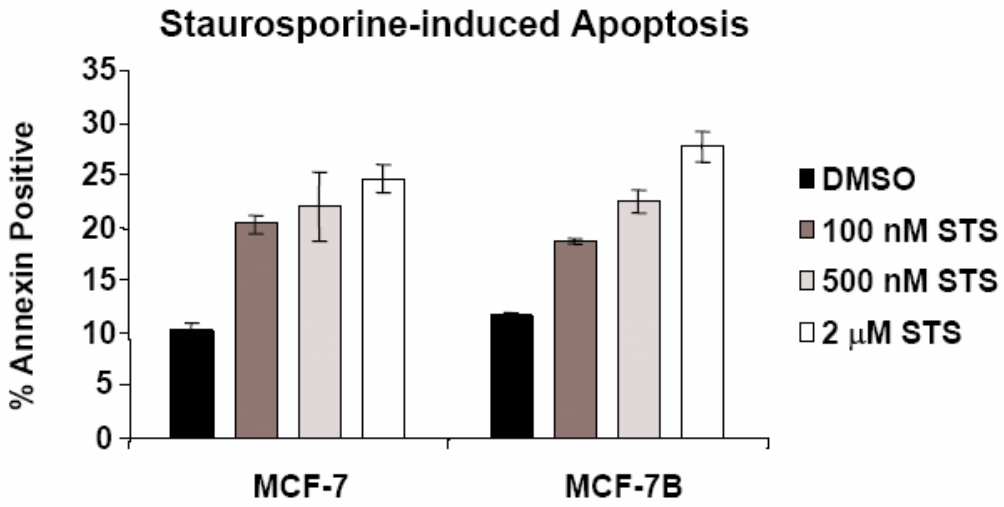
Disruption of the p53-Hdm2 interaction rescues the effects of Bcl-3 over-expression. (A) Disruption of the p53-Hdm-2 interaction restores the ability of UV to induce p53 in MCF-7B cells. MCF-7 and MCF-7B cells were pretreated for 30 minutes with either DMSO or 2μM RITA then treated with 40 J/m^2 UV-C for the indicated times. Whole cell extracts were prepared and subjected to western blot analysis using antibodies specific for p53 and β-tubulin. **(B)** Knockdown of Hdm2 restores the ability of UV to induce p53 in MCF-7B cells. MCF-7B cells were transfected with either a control siRNA or an siRNA specific for Hdm2. 48 hours following transfection, cells were treated with 40 J/m2 UV-C for the indicated times. Whole cell extracts were prepared and subjected to western blot analysis using antibodies specific for p53, Hdm2 and β-tubulin. **(C)** Disruption of the p53-Hdm-2 interaction restores the ability of UV to induce apoptosis in MCF-7B cells. MCF-7 and MCF-7B cells were left untreated, treated with 40 J/m^2 UV-C or treated with 40 J/m^2 UV-C plus 2 μM RITA. 18 hrs following treatment, apoptosis was measured by flow cytometric analysis of Annexin-V staining.


A)
MCF-7
MCF-7B
DMSO
RITA
DMSO
RITA
UV (hrs)
0 6 12 6 12
0 6 12 6 12
p53
β-tubulin
B)
MCF-7B
Ctrl siRNA
Hdm2 siRNA
UV (hrs)
0 5 10 0 5 10
Hdm2
p53
Bcl-3
C)
UV-induced Apoptosis
% Annexin Positive
100
80
60
40
20
0
MCF-7
MCF-7B
Untreated
UV
UV + RITA

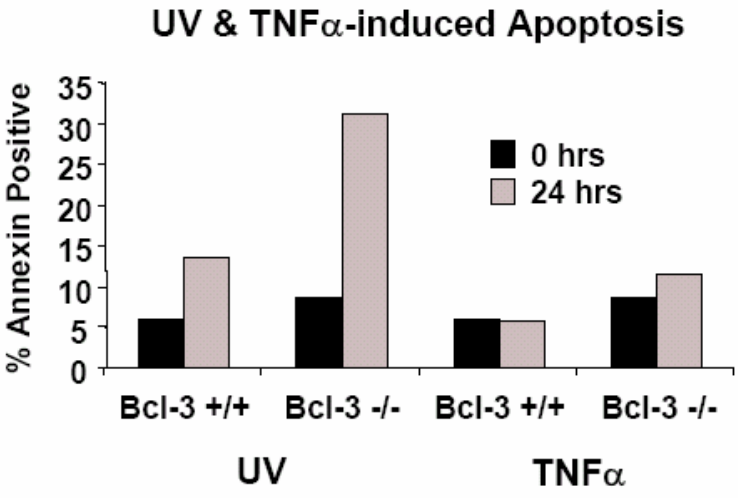
Supplemental Figure 1.1

The anti-apoptotic effects of Bcl-3 are specific for p53-dependent inducers of apoptosis. **(A)** Staurosporine induces similar levels of apoptosis in both MCF-7 and MCF-7B cells. MCF-7 and MCF-7B cells were treated with DMSO or a 100 nM, 500 nM or 2 μM dose of Staurosporine. 24 hrs following treatment, apoptosis was measured by flow cytometric analysis of Annexin-V staining. **(B)** Loss of Bcl-3 sensitizes MEFs to UV-induced, but not TNFα-induced apoptosis. Bcl-3 +/+ and Bcl-3 -/- mouse embryo fibroblasts were left untreated, treated with 40 J/m^2 UV-C or treated with 10 ng/ml TNFα. 24 hrs following treatment, apoptosis was measured by flow cytometric analysis of Annexin-V staining.

A)

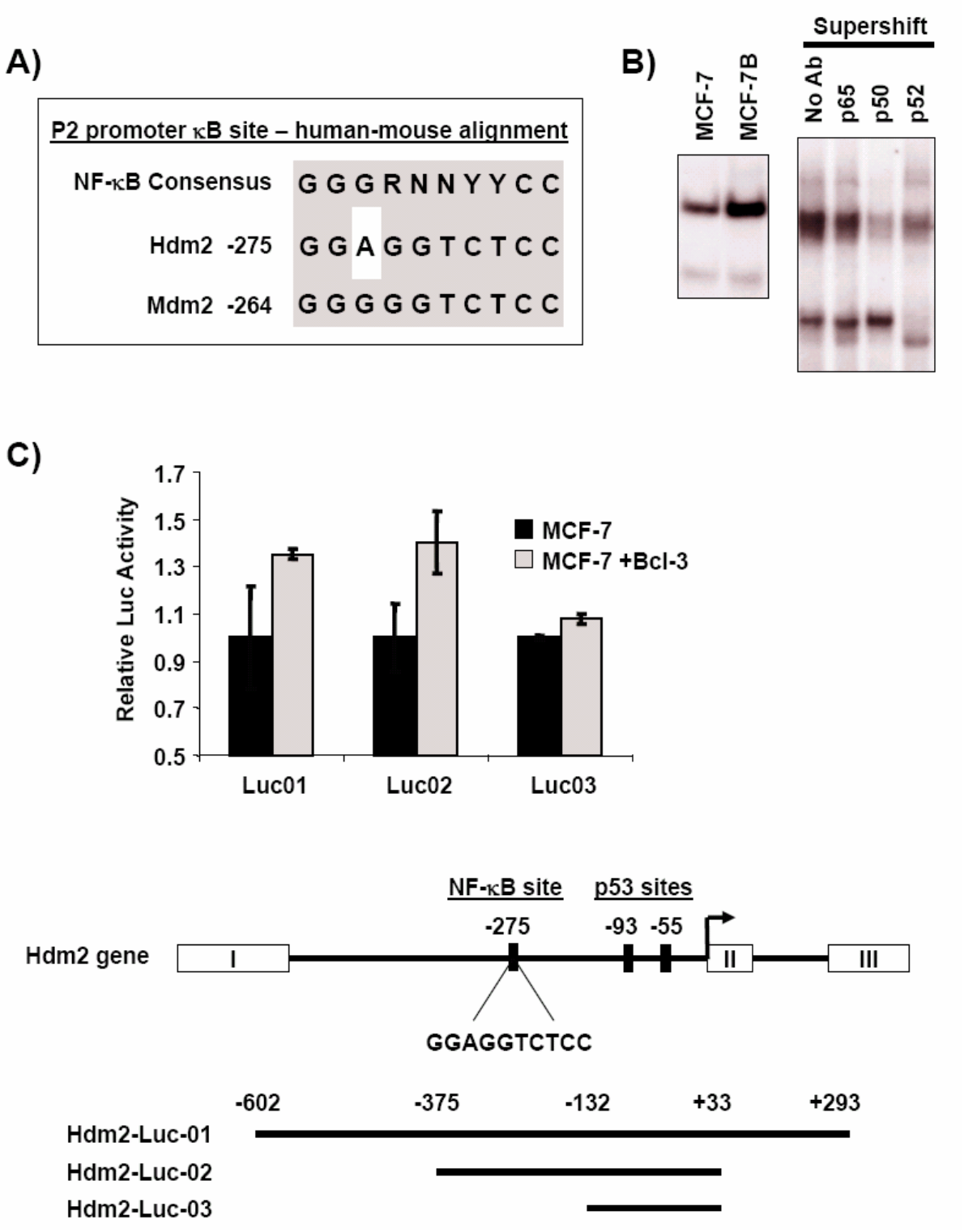

Staurosporine-induced Apoptosis
% Annexin Positive
35
30
25
20
15
10
5
0
MCF-7
MCF-7B
DMSO
100 nM STS
500 nM STS
2 μM STS

B)


UV & TNFα-induced Apoptosis
% Annexin Positive
35
30
25
20
15
10
5
0
0 hrs
24 hrs
Bcl-3 +/+
Bcl-3 -/-
Bcl-3 +/+
Bcl-3 -/-
UV
TNFα

Supplemental Figure 1.2

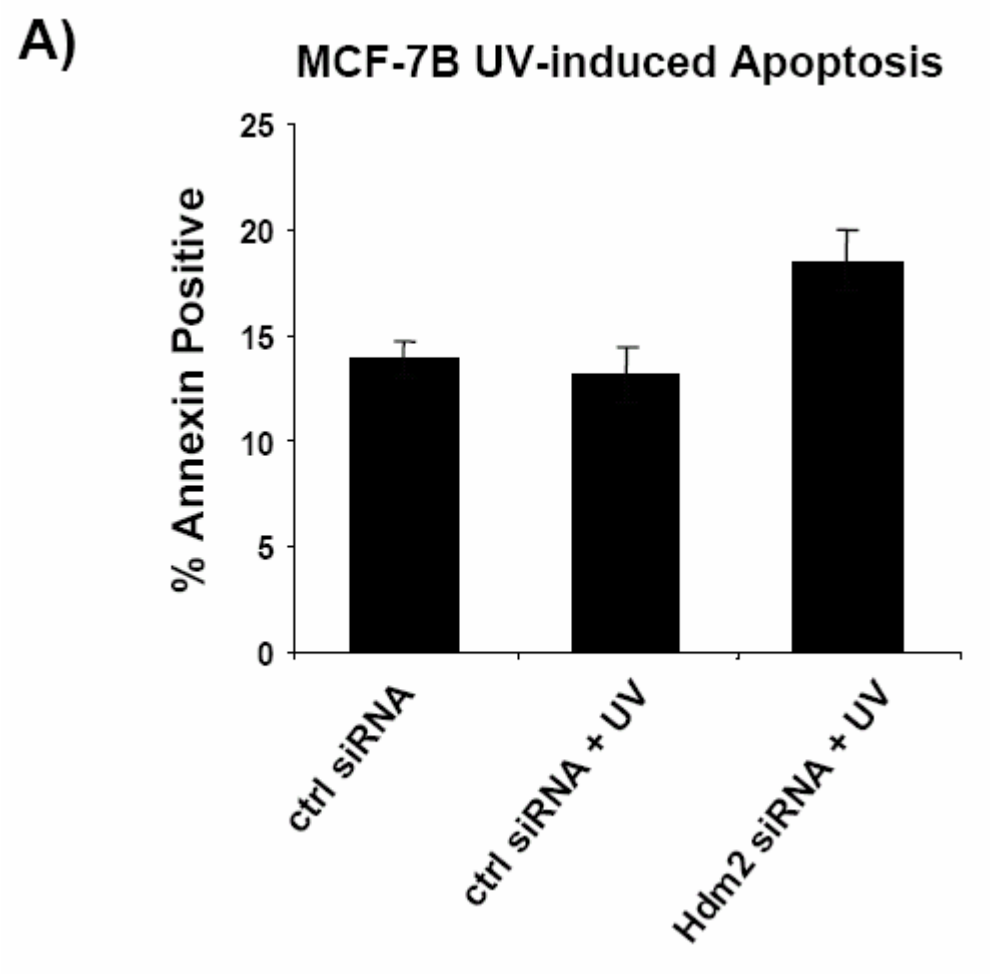
MCF-7B cells exhibit higher NF-κB binding and increased promoter activity at the Hdm-2 P2 promoter. (A) A putative NF-κB site in the Hdm2/Mdm2 promoter. Promoter analysis reveals a potential NF-κB binding site 275 bp upstream of the exon 2 start site in the Hdm2 P2 promoter. The sequence is aligned to the NF-κB consensus sequence and the corresponding site in the mouse Mdm2 promoter. **(B)** Increased NF-κB binding to a DNA probe corresponding to the NF-κB site in the Hdm2 P2 promoter. Nuclear extracts were prepared from MCF-7 and MCF-7B cells and incubated with a 32P labeled double stranded oligonucleotide probe corresponding to the putative NF-κB site in the P2 promoter region. The complexes were run on a non-denaturing gel and visualized by autoradiography. The experiment was repeated in MCF-7B cells with the addition of antibodies against p65, p52 and p50 to identify the subunits present in the bound complexes. **(C)** MCF-7B cells exhibit increased promoter activity from Hdm-2 P2 promoter constructs that include the region containing the NF-κB binding site. 50 ng each of three Hdm2 P2 promoter constructs containing the indicated portions of the P2 promoter were transfected into MCF-7 and MCF-7B along with 5ng of renilla luciferase. Firefly luciferase activity was measured and normalized to renilla luciferase. Values represent fold increase over basal activity (lane 1).


A)
P2 promoter κB site – human-mouse alignment
NF-κB Consensus G G G R N N Y Y C C
Hdm2 -275 G G A G G T C T C C
Mdm2 -264 G G G G G T C T C C
B)
MCF-7
MCF-7B
Supershift
No Ab
p65
p50
p52
C)
Relative Luc Activity
1.7
1.5
1.3
1.1
0.9
0.7
0.5
MCF-7
MCF-7 +Bcl-3
Luc01
Luc02
Luc03
NF-κB site
p53 sites
-275
-93 -55
Hdm2 gene
I
II
III
GGAGGTCTCC
-602
-375
-132
+33
+293
Hdm2-Luc-01
Hdm2-Luc-02
Hdm2-Luc-03

Supplemental Figure 1.3

siRNA-mediated knockdown of Hdm2 partially restores the apoptotic response to UV in MCF-7B cells. Knockdown of Hdm2 restores the ability of UV to induce apoptosis in MCF-7B cells. MCF-7B cells were transfected with either a control siRNA or an siRNA specific for Hdm2. 48 hours following transfection, cells were treated with 40 J/m2 UVC for the indicated times. 24 hrs following UV treatment, apoptosis was measured by flow cytometric analysis of Annexin-V staining.

A)


MCF-7B UV-induced Apoptosis
% Annexin Positive
25
20
15
10
5
0
ctrl siRNA
ctrl siRNA + UV
Hdm2 siRNA + UV

Experimental Procedures:

Cell Culture

Primary murine embryo fibroblasts were isolated from day 13 embryos and grown in DMEM supplemented with 10% FBS (Sigma) and 1X penicillin/streptomycin (Gibco). MCF-7 cells (ATCC) were grown in MEMα supplemented with 10% FBS, 10 ug/ml Insulin (Gibco), 1mM Sodium Pyruvate (Gibco) and 1X penicillin/streptomycin. Karpas 299 cells (DSMZ, Braunschweig, Germany) were grown in RPMI supplemented with 10% fetal bovine serum and 1X penicillin/streptomycin. HT1080 cells (ATCC) were grown in DMEM supplemented with 10% FBS and 1X penicillin/streptomycin. U-2OS cells (ATCC) were grown in McCoys 5A supplemented with 15% fetal bovine serum and 1X penicillin/streptomycin. MCF-7B cells were generated by transfecting the expression construct pFlag-Bcl-3 into MCF-7 cells. Stable clones were generated in medium containing 1 μg/ml puromycin (Sigma). Clones were verified by Western blotting with a Bcl-3-specific antibody. Cisplatin (Sigma) was resuspended in DMSO and stored at –20°C. RITA was resuspended in DMSO to a concentration of 10mM and stored in the dark at –20°C. Pifithrin-α (A.G. Scientific) was resuspended in DMSO to a concentration of 10mM and stored in the dark at –20°C. For UV treatments, cells were placed under a UV lamp and dosage was measured with a UV-X radiometer (Ultra Violet Products, Upland, CA).

Antibodies

For western blots, ChIP analysis and supershift analysis we used antibodies against p53 (FL393, Santa Cruz), Bcl-3 (Upstate), Hdm2 (Ab-1, Calbiochem), mdm2 (2A10, from

A. Levine), flag (M2, sigma), p65 (5192, Rockland), p52 (From N. Rice), p50 (H-119, Santa Cruz) and β-tubulin (H-235, Santa Cruz). HRP-conjugated anti-mouse and anti-rabbit IgG secondary antibodies were from Promega.

Western Blot

Typically, cells were plated in a 100 mm or 6-well tissue culture dish and treated as indicated. After the indicated timepoints, cells were wash with PBS and lysed in modified RIPA buffer (1% NP40, 20mM Tris, 137 mM NaCl, 10% glycerol, 2mM EDTA, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 1 mM PMSF). 10 or 15 μg protein were loaded onto 4-12% Bis-Tris gels (Invitrogen). After electrophoresis, gels were transferred to nitrocellulose (Biorad) and blocked for 1 hr in TBS containing 0.5% Tween-20, 4% milk and 1% BSA. Primary and secondary antibody incubations were performed in blocking buffer at 4°C (primary) or room temp (secondary).

Apoptosis Detection

Cells were plated in 100mm dishes and treated with the indicated dose of UV or cisplatin. At the indicated timepoints, cells were washed with PBS and collected by trypsinization. Cells were washed again in PBS and resuspended in 100 μL annexin binding buffer (10mM HEPES, 140mM NaCl, 2.5mM $CaCl_2$, pH 7.4)) containing 5 μL Alexa-488 conjugated Annexin-V (Molecular Probes) plus 1 μg/ml propidium iodide (Sigma). Cells were incubated for 15 minutes at room temperature and then analyzed on a Facscalibur (Becton Dickinson). Apoptotic cells were measured as positive for Annexin-V staining but negative for propidium iodide.

Plasmid Constructs

pCMV2-flag Bcl-3 (Westerheide et al. 2001), pCMV-flag-p53 (Zhang et al. 1998), pg-13-luciferase (el-Deiry et al. 1993) and Hdm2-Luc01, Hdm2-Luc02, and Hdm2-Luc03 (Phelps et al. 2003) were described previously.

ChIP Assay

ChIP analysis was performed using a chromatin immunoprecipitation kit (Upstate Biotechnology) and a modified version of the manufacturers protocol. Following the indicated treatment, cells were fixed for 5 minutes in 1% formaldehyde, washed with PBS and lysed for 10 min in lysis buffer. Chromatin was sheared by sonication to an average size of approximately 1 kilobase and pre-cleared for 2 h at 4 °C with salmon sperm DNA-saturated protein G Sepharose beads. Chromatin solutions were precipitated overnight at 4°C using 10 µl of the indicated antibodies. Immune complexes were collected with salmon sperm DNA-saturated protein G Sepharose beads for 1 h and washed extensively following the manufacturer's protocol. Input and immunoprecipitated chromatin were incubated at 65 °C overnight to reverse crosslinks. After proteinase K digestion, DNA was extracted with phenol/chloroform and precipitated with ethanol. Precipitated DNAs were analysed by Real Time PCR on an ABI 7100 using SYBR green master mix (ABI). Each sample was normalized to input using the $2^{-\Delta\Delta C(T)}$ method (Livak and Schmittgen 2001). Hdm2 P2 promoter specific primers used were: 5'-GAGGTCCGGATGATCGCAGG-3' and 5'-

GTGGCGTGCGTCCGTGCCCA-3' and β-Actin promoter specific primers used were 5'-CCTCCTCCTTCTTCCTCAATCT-3' and 5'-GGGGAGAGGGGAGGAAA-3'.

Luciferase Assays

Typically, cells were plated in 24-well dishes and allowed to grow to ~70% confluency. Cells were transfected with the indicated plasmids using polyfect reagent (Qiagen) following the manufacturers protocol. 48 hours post-transfection, extracts were prepared using the Dual Luciferase Assay System (Promega, Madison, WI) following the manufacturers protocol and luciferase activity was measured on an LMax luminometer (Molecular Devices, Sunnyvale, CA).

Real Time PCR

Cells were plated in 100 mm dishes and treated with the indicated doses of UV or cisplatin. At the indicated timepoints, cells were washed in PBS, lysed in Trizol Reagent (Invitrogen) and RNA was collected following the manufacturers protocol. cDNA was generated using the M-MLV reverse transcriptase kit (Invitrogen) and quantitative PCR was performed on an ABI Prism 7000 (Applied Biosystems, Foster City, CA) using gene specific TAQman primer/probe sets (Applied Biosystems).

RNAi

Synthetic dsRNA oligonucleotides targeting Bcl-3 and Hdm-2 were purchased from Xeragon and Ambion, respectively. The targeted sequence for Bcl-3 is 5'-AATGGTCTTCTCTCCGCATCA-3'. Cells were plated in 6-well dishes and allowed to

grow to ~70% confluency. Transfection of the gene specific siRNA plus a control siRNA (Ambion) was performed using the transmessenger transfection reagent (Ambion) according to the manufacturers protocol. 48 hours post-transfection, cells were treated for the indicated time points, lysed and western analysis was performed as described.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear and cytoplasmic extracts were obtained from MCF-7 and MCF-7B cells, and gel shift analysis was performed as previously described (Mayo et al. 1997b). Briefly, an oligonucleotide corresponding to the putative NF-κB site in the *Hdm2* P2 promoter (5'-GGTGGTTCGGAGGTCTCCGCGGGAGT-3') was radiolabeled using [α-32P]dCTP (Perkin Elmer). The probe was incubated with 4μg of nuclear extract and 0.1μg/μl poly dIdC in DNA binding buffer (50mM NaCl, 10mM Tris pH7.6, 10% glycerol, 1mM DTT, 0.5mM EDTA) for 15 minutes at room temperature. For antibody supershift analysis, extracts were preincubated for 15 minutes at room temperature with 1 μg of antiserum before the addition of the radiolabeled gel shift probe. Reactions were separated using non-denaturing PAGE and visualized by autoradiography.

Chapter II

Primary Human Hepatocellular Carcinomas Exhibit High Levels of Nuclear Bcl-3 Expression.

Contributions to this chapter were made by Bert O'Neil, Petra Bůžková, Hillary Farrah, Hanna Kelly and Bill Funkhouser.

Abstract

The transcription factor NF-κB has been shown to be activated in hepatocellular carcinoma (HCC), but how it contributes to the oncogenic process is not clear. Recently, studies have identified the IκB family member Bcl-3 as a possible mediator of NF-κB activation in other inflammation-associated cancers. We set out to determine whether high levels of Bcl-3 are characteristic of HCC. Further, we sought whether Bcl-3 expression is associated with particular NF-κB subunit activation and whether expression of Bcl-3 correlates with expression of the Bcl-3 target genes cyclin D1 and Hdm2 in HCC. Archived HCCs from 30 patients were evaluated by immunohistochemistry for all NF-κB subunits, Bcl-3, Hdm2, cyclin D1, Bcl-xL and p53. Also, frozen tissue that was available was analyzed by western blot for expression of Bcl-3, Hdm2, and p65. Results were correlated with clinico-pathologic features. We find that the p50 and p52 NF-κB subunits were frequently localized to tumor cell nuclei, indicative of activation (40% and 48%, respectively), whereas p65 expression was infrequent. Also, high levels of nuclear Bcl-3 were found in the majority of cases (90%) and p52 expression was strongly correlated with Bcl-3 expression. While Bcl-3 expression did not correlate with either mdm2 or cyclin D1 expression as measured by immunohistochemistry, there was a positive correlation between Bcl-3 expression and mdm2 expression in the limited number of samples available for western blot analysis, suggesting that further investigation of the mechanisms of Bcl-3 activation and the role of this activation in hepatocarcinogenesis are warranted.

Introduction

Hepatocellular carcinoma is a common killer worldwide, with increasing incidence in the United States caused by large numbers of both recognized and unrecognized cases of hepatitis C virus infection (Davila et al. 2004). The discovery of effective therapies for HCC is an urgent priority, but is hampered by our limited understanding of the molecular pathogenesis of HCC. A growing body of literature has implicated the transcription factor NF-κB in hepatocarcinogenesis (Tai et al. 2000a; Tai et al. 2000b; Chiao et al. 2002; Liu et al. 2002; Wang et al. 2003; Waris et al. 2003; Cavin et al. 2004; Chan et al. 2004; Girard et al. 2004). The NF-κB family of transcription factors is comprised of five subunits, p65/RelA, p50, p52, RelB and c-rel that homo- or heterodimerize to form active transcriptional complexes (Baldwin 2001). The function of the various NF-κB subunits with regards to neoplasia remains an active field of investigation. NF-κB is expressed ubiquitously, but sequestered in the cytoplasm by inhibitory protein IκB. IκB family members include IκBα, IκBβ, IκBε and the atypical IκB protein Bcl-3 (Ghosh and Karin 2002). Activation of NF-κB involves IκB kinase (IKK)-mediated phosphorylation of IκBα on serines 32 and 36 followed by ubiquitination and subsequent proteasomal degradation, resulting in the release of NF-κB and its translocation to the nucleus. There, NF-κB mediates the transcription of a wide variety of target genes, including potent anti-apoptotic effector proteins such as Bcl-xL (Khoshnan et al. 2000), TRAFs, IAPs (Wang et al. 1998) and others.

The most abundant and widely studied NF-κB dimer is the p50/p65 heterodimer. Strong rationale for investigating the role of p50/p65 heterodimers in human HCC is

provided by a recent study in an mdr2 knockout mouse model in which loss of mdr2 results in a phenotype of peri-portal inflammation that in turn results in multiple peri-portal HCCs (Balkwill and Coussens 2004; Pikarsky et al. 2004). In this model, inflammation (and presumably resultant TNF-α secretion) results in activation of p50/p65 heterodimers in hepatocytes adjacent to inflamed portal areas. Specific blockade of NF-κB effected by crossing the mdr2 knockout mouse with a mouse expressing a dominant-negative IκBα abrogated formation of hepatocellular tumors in this model. This implicates NF-κB activation as an important initiating event in hepatocarcinogenesis, and suggests a potential preventative role of NF-κB inhibition for patients with chronic hepatitis. Several studies of NF-κB in HCC cell lines (Tai et al. 2000b; Chiao et al. 2002; Wang et al. 2003) and patients with HCC (Tai et al. 2000a) have been reported in which p50 and p65 were detected by immunohistochemistry or electromobility shift assay (EMSA), but few if any studies of HCC have focused on the other NF-κB subunits.

More recently, the "atypical" IκB protein Bcl-3 has emerged as a potentially important oncogene in several solid tumors, most notably nasopharyngeal cancer and endometrial cancer, both of which are mediated by chronic viral infection (Thornburg et al. 2003; Pallares et al. 2004). Bcl-3 was first identified as the product of a chromosomal translocation in certain lymphomas (McKeithan et al. 1987; Ohno et al. 1990), but little is known about its normal physiologic functions outside the immune system, where it seems to have a role in several aspects of immune responses (Franzoso et al. 1997; Schwarz et al. 1997). Bcl-3, which has a C-terminal transactivation domain, functions as a transcriptional co-activator in complex with homodimers of p50 (Fujita et al. 1993), and p52 (Bours et al. 1993), neither of which has intrinsic trans-activation function. Recently, it was demonstrated

that Bcl-3 overexpression occurs in a proportion of human breast cancer samples (Cogswell et al. 2000), and subsequently demonstrated that over-expression of Bcl-3 and p52 in breast cancer cell lines *in vitro* leads to increased expression of cyclin D1, suggesting a possible oncogenic mechanism (Westerheide et al. 2001). Also, we previously demonstrated that Bcl-3 is capable of regulating the expression of mdm2, with resultant inhibition of the pro-apoptotic function of p53 (Kashatus et al. 2006). This is a potentially interesting finding with regards to hepatocellular carcinoma, as p53 is known to be mutated in only 10-40% of HCCs (Murakami et al. 1991; Kubicka et al. 1995; Tannapfel et al. 2000; Anzola et al. 2004). These data raise the possibility that Bcl-3 activation in HCC may function as a previously unrecognized mechanism of p53 inactivation.

We undertook the current study with the goal of determining: 1) which NF-κB subunits are most frequently active (as measured by nuclear localization) in HCC and whether nuclear localization of any individual NF-κB subunits is negatively associated with clinical outcome (particularly survival); 2) whether Bcl-3 over-expression and nuclear localization are common and relevant in terms of disease characteristics and survival in HCC; 3) whether there is correlation between Bcl-3 over-expression and NF-κB subunit activation, cyclin D1 expression and/or mdm2 expression. Our principal hypotheses were that tumors with nuclear NF-κB expression would have worse clinical outcomes, and that Bcl-3 expression would correlate with p50/p52 activation and with increased expression of mdm2 and cyclin D1.

Results

Patient characteristics

The clinical characteristics of the 30 patients whose tumors comprised the tissue microarray are summarized in Table 2.1. Patients with negative serology for hepatitis B and C virus were over-represented compared with what would be expected for an unselected population of U.S. HCC patients, likely because patients without chronic viral hepatitis are more likely to be candidates for partial hepatectomy. Four of the patients had wedge biopsies without attempt at formal resection because of more advanced disease, in some cases identified at the time of laparotomy. One patient who underwent transplant as therapy had only tumor tissue available in our pathology archives. These five samples were incorporated into the TMA without corresponding adjacent non-neoplastic liver. At the time of data analysis 14 patients were alive, with a median follow-up of 45 months (range 27-84 months). 16 patients had died, with a median survival of 20 months (range 2-62 months).

Bcl-3 is frequently overexpressed in hepatocellular carcinoma

In our patient sample, nuclear Bcl-3 staining was observed in 27 of 30 cases of HCC (present in $\geq$ 1+/50% of cells in 15 of the 27 positive cases), compared with only 9/25 positive (to any degree) in adjacent non-neoplastic liver (Figure 2.2). The difference in staining intensities between tumor and non-tumor samples was highly statistically significant (student's paired t-test $p < 0.001$), and also comprised an internal negative control as normal and tumor cores were mixed on the same TMA slides. Representative positive and negative stains are shown in Figure 2.1. Because nearly all of the tumors were Bcl-3 positive, there

was no detectable association between Bcl-3 expression and survival, nor did we detect an association between intensity (on a continuous scale) of Bcl-3 staining and survival (Figure 2.2). Cytoplasmic staining was present in most samples, but only nuclear staining was scored for purpose of analysis.

In seven cases, frozen tissue was available in our Tissue Procurement Facility: five cases with paired tumor and non-neoplastic liver and two cases with tumor only. Western blotting with anti-Bcl-3 antibody confirmed our visual IHC results with the exception of one case where the IHC and Western blot results appeared to be discordant (Figure 2.3). The Western blots also confirmed that the antibody was recognizing a protein of the expected molecular weight.

To explore the question of whether Bcl-3 over-expression occurs at the level of transcription or protein stability, we assayed the five available pairs of frozen tumor and non-tumor liver samples (all from patients included in this study) by quantitative PCR. As shown in Figure 2.4, 2/4 of the tumors with Bcl-3 over-expression had equal tumor and normal liver Bcl-3 message and the other 2 appeared to over-express message compared with adjacent liver. A larger sample size of frozen tissue would be necessary to resolve whether Bcl-3 over-expression is more likely to occur at the transcriptional, translational or protein stability level. Search of the ONCOMINE^{TM} (Rhodes et al. 2004) microarray database revealed one study (Patil et al. 2005) in which gene expression in 104 HCCs was compared with that in 74 matched non-neoplastic liver samples. No statistically significant differences in gene expression of Bcl-3 were seen, at least by oligonucleotide microarray assay methodology. In fact, there was a trend toward lower Bcl-3 expression in the normal (or cirrhotic) liver tissue.

p50 and p52 but not other NF-κB subunits are frequently localized to the nucleus in HCC

In contradistinction to other reported series of HCC patients in which p65 was frequently localized to tumor cell nuclei (a surrogate for activation), we found p65 to be visibly present in the nucleus of tumor cells in only 4/30 cases, using both an antibody against unmodified p65 and an antibody specific to p65 that has been phosphorylated at serine 536. We also found little evidence of phospho-p65 expression by more sensitive Western blotting of whole-cell extracts in seven frozen cases (Figure 2.3), including in cases where p50 was over-expressed (data not shown). p50 and particularly p52 were much more frequently visualized in tumor nuclei than other NF-κB subunits. Staining was less intense but also present in matched non-neoplastic liver samples. While there appeared to be differences in both frequency and intensity of staining in tumors compared with non-neoplastic liver (Table 2.2 and Figure 2.2), these differences did not reach statistical significance for either p50 or p52 (student's t test, for p50: $p = 0.09$, for p52: $p = 0.34$). NF-κB subunits rel-B and cRel were expressed in the nuclei of only one (3%) and none of the tumors, respectively. Interestingly, higher p50 nuclear expression trended strongly toward worsening survival, with the HR for death increasing by 8% for every decade increase in IHC score (95% CI 1% to 17%, $p = 0.075$), but the relationship was of borderline statistical significance. Our sample did not have adequate power to detect a statistical correlation between p52 nuclear expression level and survival (HR 1.047, 95% CI 0.976, 1.122; $p = 0.2$).

Cyclin D1 and mdm2

Two of our hypotheses regarding Bcl-3 were that mdm2 and cyclin D1 would be more frequently over-expressed in Bcl-3 expressing tumors. Mdm2 was over-expressed in

the cytoplasm of 7 tumors, and the nuclei of 3 tumors (all of which were also cytoplasm positive). There was no apparent correlation between intensity of Bcl-3 staining and positivity for mdm2 by IHC. Interestingly, the degree of nuclear staining for cyclin D1 was actually negatively correlated with Bcl-3 staining intensity (R= -0.28, 95% CI -0.537, -0.071) (not shown). In our limited sample of frozen tumors analyzed by western blotting, there appeared to be a strong visible association between strength of bcl-3 expression and strength of mdm2 expression (Figure 2.3), suggesting that IHC on paraffin-embedded samples may be too insensitive for examining this association compared with western blotting. More frozen samples would be necessary to corroborate this, and we are currently in the process of collecting these samples for analysis.

Discussion

Hepatocellular carcinoma is an example of a neoplasm that arises in the setting of chronic inflammation, a setting in which NF-κB may play a very significant role. Once activated in tumor cells, NF-κB controls the expression of a number of potent inhibitors of apoptosis. The main purpose of this study was to explore the frequency of NF-κB subunit nuclear expression in HCC, and whether evidence of activation correlated with patient survival. While our results revealed a trend toward an association between p50 activation and survival, our sample size was constrained by tissue availability and thus limited in answering this question definitively. We also set out to determine whether Bcl-3 activation was common in HCC based on prior observations in endometrial cancer and nasopharyngeal cancer, two virally-mediated malignancies (Thornburg et al. 2003; Pallares et al. 2004). Our study is the first to comprehensively analyze the activation status of all characterized NF-κB subunits in human hepatocellular carcinoma. Our results differ from previously published studies in that there is little suggestion in our data that the canonical p50/p65 heterodimer is the dominant form of NF-κB expressed in HCC. What our data do suggest is that HCCs nearly always over-express Bcl-3, likely in association with p52 and/or p50 homodimers in the nucleus. Based on the strong trend toward correlation of p50 nuclear localization with survival, it is possible that this activity of Bcl-3 and NF-κB is important for initiation and/or progression of HCC.

At present there is relatively little understanding of the regulation of Bcl-3 expression. Bcl-3 is degraded by the proteasome upon phosphorylation by GSK-3β in one model, which would suggest protein stability as a major determinant of cellular levels of Bcl-

3 (Viatour et al. 2004). Very recently, however, a group has published evidence of Bcl-3 gene amplification in anaplastic large cell lymphoma lines (Mathas et al. 2005). In our limited-sized sample of frozen tumors, we found that 2 of 4 Bcl-3 over-expressing cancers had mRNA levels that were no different than that found in the adjacent liver, but in the other two cases there appeared to be significantly higher message level. A much larger sample of frozen tissue would be required to study this question in a rigorous manner. As mentioned previously, examination of an oligonucleotide microarray database revealed no evidence of Bcl-3 upregulation at the message level when compared with non-neoplastic liver tissues from the same patients. These findings would also tend to support the model that Bcl-3 levels are more dependent upon protein stability than they are upon transcription. If it is the case that Bcl-3 is regulated by phosphorylation, a more complete understanding of the kinases that target Bcl-3 might potentially lead to therapies or preventive therapies for HCC.

As stated previously, based on a Bcl-3 over-expression model we predicted that mdm2 would be over-expressed in concert with Bcl-3. This did not appear to be the case in our group of patients, at least when examining mdm2 expression by immunohistochemistry. Of course, regulation of the mdm2/p53 system is exceedingly complex (Vousden and Lu 2002). Immunohistochemical measurement of mdm2, therefore, is likely inadequate to answer the question of whether Bcl-3 independently regulates mdm2 expression. Supporting this argument, we saw higher expression of mdm2 by western blot in at least one case than we did by IHC, suggesting a more sensitive assay than IHC would be necessary to establish a link between Bcl-3 and mdm2 protein levels in human tumors. We found that a large fraction of our samples over-expressed nuclear p53 by IHC, a finding which can have a number of explanations not limited to mutation (Anzola et al. 2004). However, if regulation

of p53 in HCC were dominated by mdm2 over-expression, we would have expected to see lower levels of over-expression of p53 in these samples.

Cyclin D1 plays an important role in the cell cycle, and is under the control of a large number of cellular factors, as evidenced by the fact that the protein was frequently expressed in the nuclei of non-neoplastic liver cells in patients who mostly had underlying inflammatory processes. One of the first described functions of Bcl-3 was the transcriptional regulation of cyclin D1 levels (Westerheide et al. 2001). Our study results do not strongly implicate Bcl-3 as a primary driver of cyclin D1 expression, and in fact uncovered a negative association between Bcl-3 and cyclin D1 expression in our sample of HCC patients.

In summary, in this set of experiments we have established that HCCs are characterized by nuclear Bcl-3 expression in the vast majority of cases, and that the NF-κB family members involved in HCC appear to be p50 and p52. Due to lack of other subunits, and to the fact that p50 and p52 do not heterodimerize with each other, we surmise that in HCC, over-expression of Bcl-3 results in recruitment of p50 and p52 homodimers to the nucleus. Our future studies will concentrate on elaborating mechanisms by which Bcl-3 over-expression might be oncogenic in hepatocytes, and in seeking a link between chronic inflammation and the nuclear over-expression of Bcl-3. An important potential clinical implication of this finding is that treatment of HCCs with proteasome inhibitors as a means of inhibiting NF-κB might not have the intended consequence if the primary means of regulation of NF-κB is via Bcl-3. In fact, since Bcl-3 is degraded by the proteasome, such inhibition could lead to further accumulation with potential for a stronger anti-apoptotic signal.

Table 2.1 – Patient Characteristics

Male sex	19 (63)	
Cirrhosis (cause)	19 (63)	
HCV	7	
HBV	4	
HBV/HCV	1	
ETOH	4	
Auto	2	
PSC	1	
Ascites	5 (17)	
Total Bilirubin (n =29)	1.71 mg/dl	(0.2 to 6.1)
Albumin (n = 27)	3.14 g/dl	(1.6 to 4.1)
Child's class		
B	8	
C	3	
AFP mean (n = 26)	3830 ng/ml	(5- 69540)
AFP median	18 ng/ml	
Tumor #		
one	17	
two to five	10	
> 5	3	
Surgery type		
Biopsy	4	
Resection	16	(3 R1, 2 R2)
Transplant	10	

Figure 2.1

Representative Immunohistochemistry for Bcl-3

(A & B) Paired tumor (A) and normal (B) tissues from patient 6 were analyzed on the same TMA slide stained simultaneously for expression of Bcl-3. The average scores across 3 separate cores were 100 for tumor and 5 for normal tissue. **(C & D)** Paired tumor (C) and normal (D) tissues from patient 11 were analyzed on same TMA slide stained simultaneously for expression of Bcl-3. The average scores across 3 separate cores were 160 for tumor and 53 for normal tissue. Note the lack of any Bcl-3 staining in endothelial cell nuclei in the tumor sample (C). **(E)** Tumor tissue from patient 9 (with no paired normal tissue) was analyzed for expression of Bcl-3. The average score across 3 separate cores was 35. **(F)** Simultaneously stained negative primary antibody control.

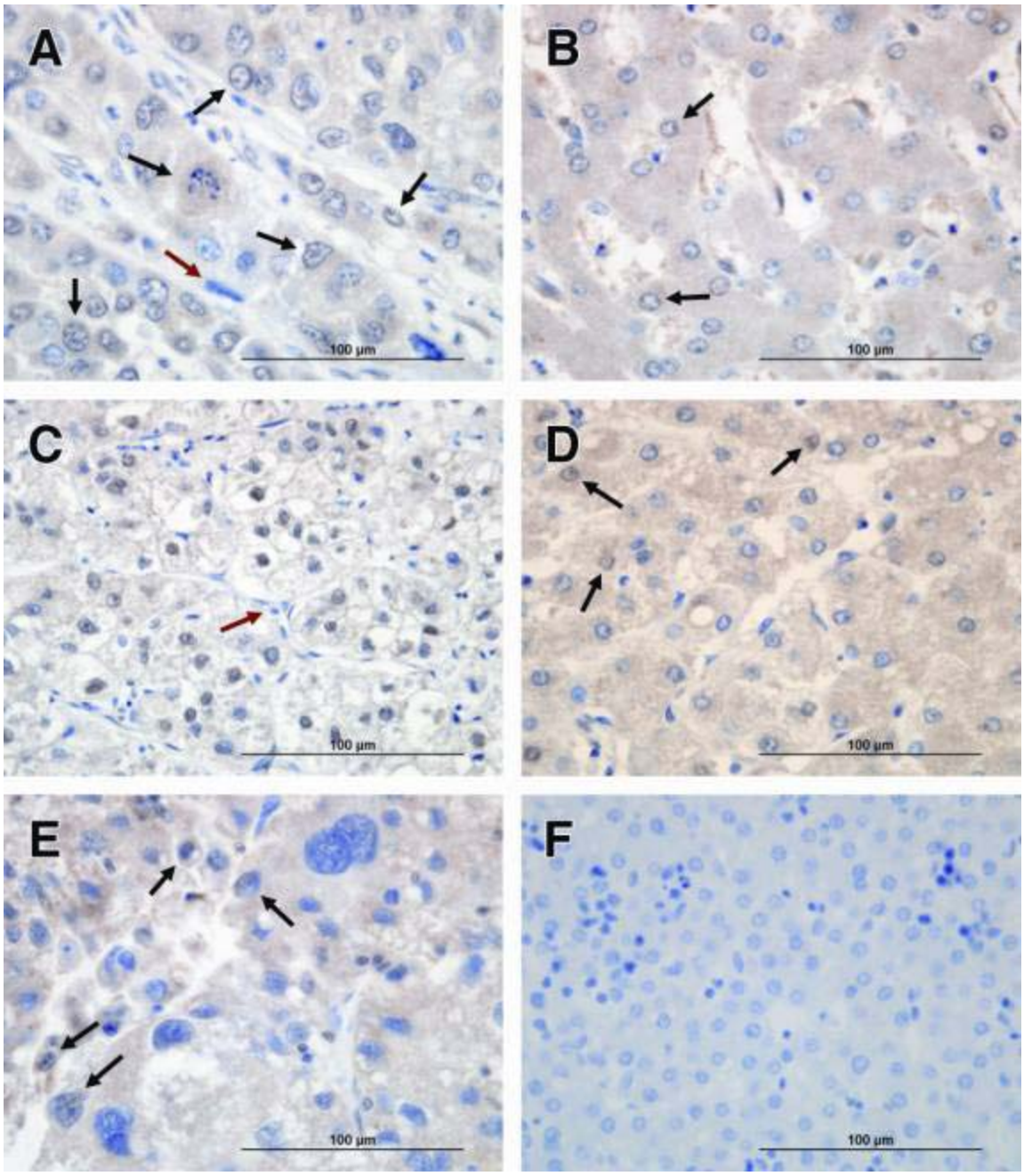

A
100 µm
B
100 µm
C
100 µm
D
100 µm
E
100 µm
F
100 µm

Figure 2.2

Immunohistochemical analysis of 31 hepatocellular carcinoma tumors.

The immunostain intensity was sorted by duration of survival (as of April 2005). White indicates no expression. Green represents tumors, blue represent adjacent non-neoplastic liver. Lightest color represents an IHC "score" of 10-49, medium color represents a score of 50-99, darkest color represents a score of >100. Gray represents a missing data point. Note that intense p50 staining appears to occur preferentially in patients in the upper half, representing the shortest survival. (T = tumor, Tc = tumor cytoplasm, N = non-neoplastic, Nc = non-neoplastic cytoplasm. Only the nuclear score is represented for p50, p52, Bcl-3, Bcl-X_L, p53 and Ki-67). * indicates that only tumor sample was available (no normal tissue).

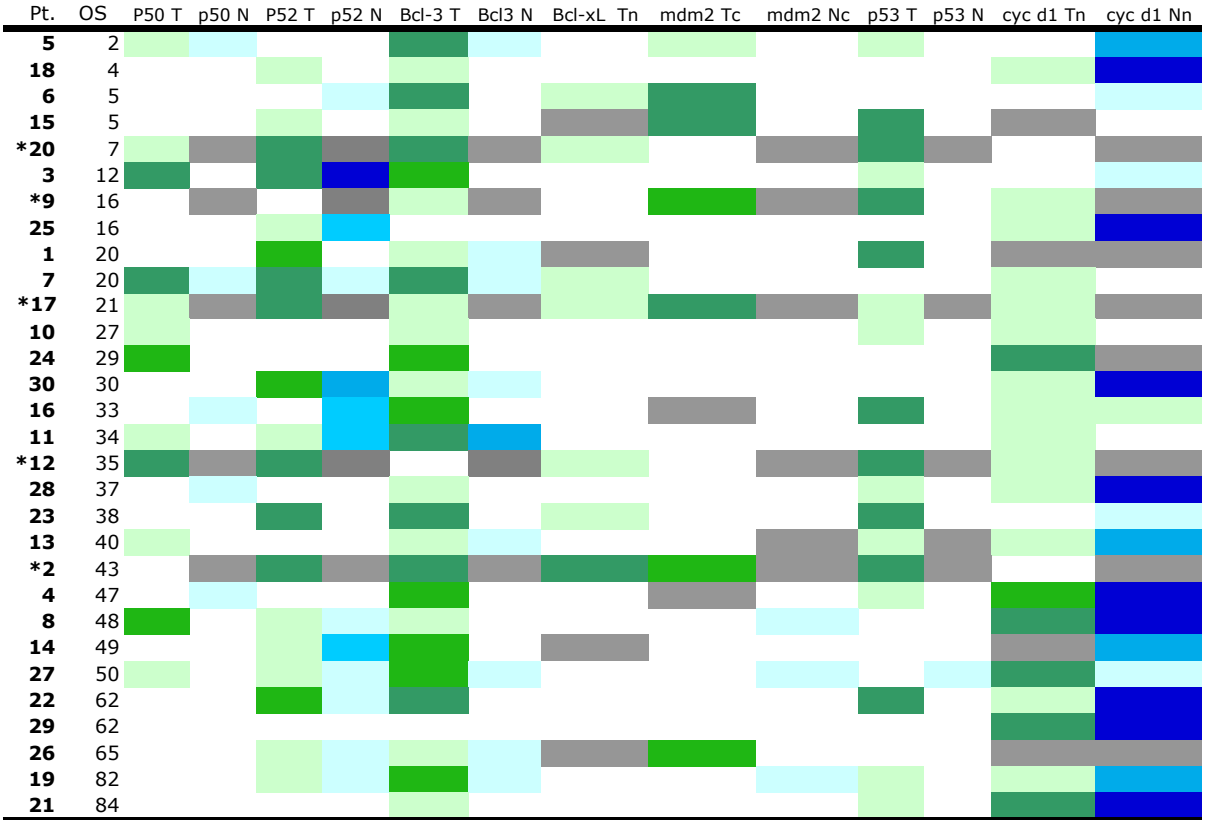
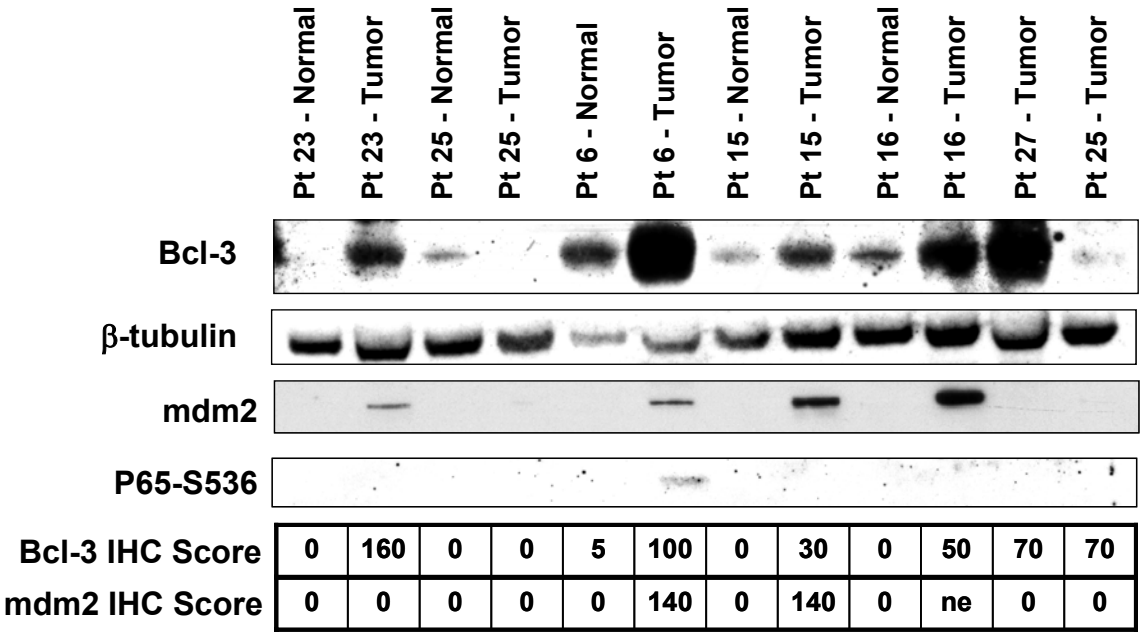

Pt.
OS
P50 T
p50 N
P52 T
p52 N
Bcl-3 T
Bcl3 N
Bcl-xL Tn
mdm2 Tc
mdm2 Nc
p53 T
p53 N
cyc d1 Tn
cyc d1 Nn
5 2
18 4
6 5
15 5
*20 7
3 12
*9 16
25 16
1 20
7 20
*17 21
10 27
24 29
30 30
16 33
11 34
*12 35
28 37
23 38
13 40
*2 43
4 47
8 48
14 49
27 50
22 62
29 62
26 65
19 82
21 84

Figure 2.3

High levels of Bcl-3 expression in HCC tumor samples

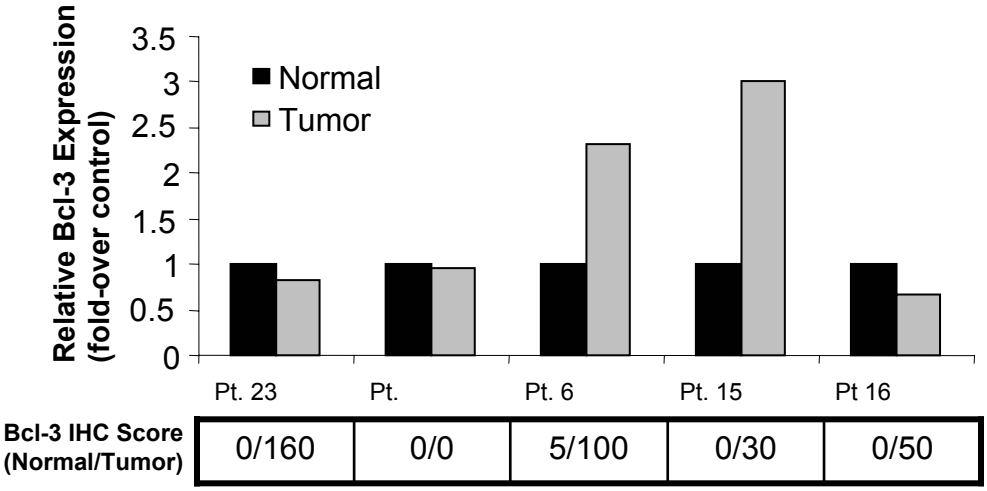
Western blots were performed on whole cell lysates in five paired and two unpaired cases and stained with antibodies against Bcl-3, mdm2, β-tubulin and phospho-p65 (S536). IHC scores for Bcl-3 and mdm2 are shown at bottom for each sample. There was generally good concordance between apparent protein abundance on Western and blinded IHC score.



Bcl-3 IHC Score	0	160	0	0	5	100	0	30	0	50	70	70
mdm2 IHC Score	0	0	0	0	0	140	0	140	0	ne	0	0

Figure 2.4

High levels of Bcl-3 protein do not necessarily correlate with high levels of mRNA. Quantitative RT-PCR was performed on five paired samples of tumor and non-neoplastic liver for expression of Bcl-3. Expression data for each sample are normalized to expression of glucuronidase β. For each pair, expression of Bcl-3 in normal tissue is set at 1 and expression in tumor is represented as fold difference to the normal. Of four tumors that overexpressed Bcl-3 by immunohistochemistry and on western blot, 2 showed higher tumor expression of mRNA and two did not. The one tumor in which Bcl-3 was not overexpressed demonstrated no evidence of higher expression in tumor compared to the baseline in adjacent liver.


Relative Bcl-3 Expression
(fold-over control)
3.5
3
2.5
2
1.5
1
0.5
0
Normal
Tumor
Pt. 23
Pt.
Pt. 6
Pt. 15
Pt 16
Bcl-3 IHC Score
(Normal/Tumor)
0/160
0/0
5/100
0/30
0/50

Experimental Procedures

Antibodies

Antibodies used for immunohistochemistry were p50 (p50NLS), p52 (C-5), RelB (C-19), p65 (F-6), Bcl-3 (H-146), Bcl-XL (H-5) and c-Rel (c-Rel) from Santa Cruz, and p53 (DO-7) and Bcl-2 (124) from DAKO. Antibodies used for western blot were p53 (FL393, Santa Cruz), Bcl-3 (Upstate), mdm2 (2A10, from A. Levine), phospho-p65 (Upstate), and β-tubulin (H-235, Santa Cruz).

Tissue Microarray creation

We identified 30 patients who had undergone resection or wedge biopsy of HCCs between 1998 and 2002 in the UNC Cancer Registry. Clinical data including tumor stage, underlying liver disease and survival were abstracted from patient records. 59 sequential tumor samples were identified in the UNC Department of Pathology, 30 of which were of suitable size and quality for creation of a tissue microarray (TMA). A TMA was created using triplicate cores of fixed and paraffin-embedded tumors and adjacent non-neoplastic liver (available in 25 of the 30 cases) which were cored with a 1 mm needle and inserted into a recipient paraffin block (Beecher Instruments, Sun Prairie, WI). Samples from the same tumor were staggered in the array and a map created for later identification of the identity of individual cores.

Clinical data was abstracted from UNC medical records and outside records under a HIPAA-compliant, IRB-approved protocol. Because time of disease recurrence was not always available (e.g. patients had followed up out of the area), only overall survival was

recorded. All survival data were censored as of the last time of survival data collection in April 2005.

Immunohistochemical staining

Unstained 5 micron thick sections were baked at 60º C for 15 min to 1 hour. Baked sections were soaked twice in fresh xylene for 5 min each, then soaked in 100% ethanol for 3 min, then blocked for endogenous peroxidase with 3% hydrogen peroxide in methanol for 10 min. Slides were then soaked in 95% ethanol for 3 min, soaked in 70% ethanol for 3 min, rinsed in distilled water, and soaked in Dako wash buffer (Dako, cat. no. S3006) for 5 min. Slides were then steamed in a Black & Decker steamer for 25 minutes using antigen retrieval buffers (Dako) for each primary antibody to be studied, and then allowed to cool for at least 20 minutes. Sections were transferred to Dako wash buffer for 5 min. The TMA was circled using a PAP pen (Zymed). Endogenous biotin was neutralized by incubating the slides in a biotin blocking system (Dako X0590) for 10 min at RT in each of the two solutions. Sections were then exposed to the primary antibodies at the titers specified in Table 1 for 30 min at RT. After rinsing in Dako wash buffer, slides were incubated with the Dako LSAB2 biotinylated link for 10 minutes at RT, rinsed in Dako wash buffer, and then incubated with the Dako LSAB2 streptavidin-HRP for 10 minutes at RT. After again rinsing in Dako wash buffer, detection of the antibody/antigen complex was visualized using 3-3' diaminobenzidine (DAB) for 5 min. Slides were then rinsed in water, lightly counterstained in filtered Mayer's hematoxylin, rinsed, dehydrated, cleared, and mounted. The cells of interest in each core were scored for percentage reactivity and signal strength in both the

cytoplasm and the nuclei. Simultaneously stained normal (non-cirrhotic) liver served as a negative control in each experiment.

Scoring of TMAs

One surgical pathologist blinded to clinical information scored all TMA cores reporting the most intense staining seen in a given core on an intensity scale of 0 to 3+, then estimated the percentage of cells that were positive (to any degree). Nuclear and cytoplasmic scores were reported separately. For NF-κB subunits, Bcl-X_L, and Bcl-3, only nuclear scores were used for analysis. Triplicate cores were staggered in the TMA to reduce the chance of bias. A second surgical pathologist, also blinded to clinical data, reviewed all cores to confirm tumor versus normal/cirrhotic liver. For the determination of final scores, if three samples were evaluable (occasional cores will loosen and come off the slides creating missing data points, or necrotic tissue will be present in the majority of a core), the scores were averaged, rounding intensity to the nearest whole number and percent positive to the nearest whole number. If two cores were considered positive (1+ or greater/any %) and one core negative (0+), the negative value was thrown out. If only two cores were evaluable, the negative core was averaged with the positive core.

Western Blot Analysis

Western blot analysis was performed by preparing whole cell extracts and then separating 30 to 50 μg of total protein by SDS-PAGE. After transferring the separated proteins to nitrocellulose, blots were blocked in Tris-buffered saline-Tween (TBST) with 5% milk and then were incubated in primary antibody for either 1 to 2 hours at room temperature

or overnight at 4°C. The membranes were then washed in TBST and were incubated for 1 hour in antirabbit horseradish peroxidase-conjugated secondary antibody (Promega, Madison, WI) and were washed again. Protein bands were visualized with enhanced chemiluminescence detection (Amersham Life Science).

Quantitative real-time PCR

Total RNA was isolated with Trizol (Invitrogen) according to the manufacturer's protocol. Two μg of RNA was reverse transcribed with random hexamers and MMLV-RT (Invitrogen) according to the manufacturer's protocol. Total RNA was then diluted 1:5 and was used for quantitative PCR. Bcl-3 and Glucuronidase-β-specific TAQman primer/probe sets were ordered from Applied Biosystems (Foster City, CA). Each PCR reaction contained 1 μL of cDNA, 1 μL of primer/probe, and 10 μL 2X ABI TAQman master mix in a 20-μL total volume. PCR was performed in an ABI Prism 7900 sequence detection system (Applied Biosystems). All of the reactions were performed in triplicate and Bcl-3 levels were normalized to the number of copies of Glucuronidase-β detected in each sample.

Statistical Analysis

For the purpose of statistical analysis, IHC scores were converted to a single "intensity score" by multiplying maximum intensity (0 -3 +) times percent of cells positive (to any degree) with a possible range of 0 - 300 units possible. Scores of 10 or more were considered positive for purposes of analysis when a dichotomous variable was necessary. Wherever possible, the continuous variable was used. Cox proportional hazard models were used for comparisons of IHC scores and survival data. Associations between immunostains were estimated and tested using a nonparametric Kendall correlation. A student's t-test was

used for comparisons of staining intensities between neoplastic and non-neoplastic tissue samples. The reported p-values are two-sided and not adjusted for multiple testing that would preserve the overall type I error.

CONCLUSIONS

The role of Bcl-3 in normal and pathological cellular processes is becoming increasingly apparent as more is learned about its cellular function. In these studies, I set out to determine the biological function of Bcl-3 as it pertains to the cellular response to DNA damage and to investigate its potential function in hepatocellular carcinoma. I have identified Bcl-3 as a critical inhibitor of p53 function downstream of genotoxic stress, a finding that sheds light on its potential oncogenic function and provides a new model for its role in B-cell development. In addition, I show that disregulated Bcl-3 expression is characteristic of hepatocellular carcinoma.

While the presence of high levels of Bcl-3 in certain hematopoietic malignancies was established early on, it is becoming increasingly clear that Bcl-3 expression is characteristic of a much wider range of human tumors (McKeithan et al. 1987; Ohno et al. 1990; Cogswell et al. 2000; Thornburg et al. 2003; Pallares et al. 2004). However, how high levels of Bcl-3 contribute to these malignancies, if at all, has remained a mystery. A member of the IκB family, Bcl-3 preferentially binds to p50 and p52 homodimers, and through interactions with co-activators and repressors, can induce or inhibit expression of a number of target genes (Bours et al. 1993; Nolan et al. 1993; Na et al. 1999). Presumably, the oncogenic functions of Bcl-3 are mediated through its control of transcription. As such, a number of putative Bcl-3 target genes have been identified that can be considered candidate effectors of its oncogenic function.

Cyclin D1 is an important cell cycle regulatory protein that binds to and activates the cyclin dependent kinases CDK4 and CDK6 to promote the G1 to S transition. Expression of Bcl-3, along with p52, has been shown to induce expression of cyclin D1 and to promote cell cycle entry in cultured cells (Westerheide et al. 2001). It has been further shown that loss of Bcl-3 protein leads to an increased interaction of HDAC1 and p52 at the cyclin D1 promoter and subsequent inhibition of cyclin D1 transcription (Rocha et al. 2003). A potent oncogene, cyclin D1 is an attractive candidate effector of Bcl-3. Cyclin D1 has been shown to be important for breast cancer, colon cancer, lymphoma, melanoma and prostate cancers, among others (Fu et al. 2004), and aberrant Bcl-3 expression may contribute to its activity in some of these cases.

Bcl-2 is another target of Bcl-3 with potential oncogenic function. It was recently shown that Bcl-3, in complex with p52 homodimers, directly activates Bcl-2 transcription in breast cancer cells (Viatour et al. 2003). A potent inhibitor of apoptosis, Bcl-2 expression is characteristic of a number of malignancies as well as popular target for anti-cancer therapeutics (Droin and Green 2004).

The finding that Bcl-3 induces mdm2 to inhibit p53 function provides yet another important link between Bcl-3 and oncogenesis. We have found that high levels of Bcl-3 expression lead to an increase in mdm2 and a subsequent loss of p53 induction following DNA damage. The consequence of this p53 inhibition is protection against DNA damage induced apoptosis. Importantly, loss of Bcl-3 leads to a loss of mdm2 expression and an increase in p53 levels following DNA damage induction, leading to an increase in apoptosis.

This novel role for Bcl-3 has important implications for cancer development. All cancers must disable p53 in order to progress. While most cancers have mutations or

deletions of p53, many bypass its function through deletion of ARF or upregulation of mdm2 (Bond et al. 2005). Our data suggest that the upregulation of Bcl-3 seen in many cancers may function to inhibit p53 through the upregulation of mdm2, thus bypassing the need for p53 mutation. This model is supported by the fact that many of the cancers in which Bcl-3 expression is characteristic, such as hepatocellular carcinoma and nasopharyngeal carcinoma, generally exhibit lower percentages of p53 mutations (Spruck et al. 1992; Chang et al. 2002; Anzola et al. 2004).

Further support of this model will come from more in depth analyses of human tumor samples. Our western blot analysis on the limited hepatocellular carcinoma samples available to us revealed a correlation between Bcl-3 over-expression and mdm2 over-expression. Analysis of a large number of tumor samples will be necessary to know whether those data are significant. Furthermore, our model would predict that tumors with high levels of Bcl-3 should negatively correlate with p53 mutation status. Extensive p53 mutation analysis on a larger tumor set will reveal whether that prediction holds.

Our findings also provide novel strategies for combating cancers in which Bcl-3 expression is high. While Bcl-3 itself has not traditionally been target for drug discovery, and most drugs targeting the NF-κB pathway do not affect Bcl-3 function, a number of drugs that are currently in development target the p53-mdm2 interaction in an effort to restore p53 function to cells in which it has been lost through upregulation of mdm2 or deletion of ARF (Issaeva et al. 2004; Vassilev 2004). Intriguingly, the use of one of these drugs, nutlin-3, has recently been shown to be effective in inducing apoptosis in cells derived from B-cell chronic lymphocytic leukemia (Coll-Mulet et al. 2006), the cancer from which Bcl-3 was originally cloned (McKeithan et al. 1987).

The importance of Bcl-3-mediated p53 inhibition for cancer development is clear, but how this novel function of Bcl-3 impacts normal physiology is less so. Early analysis of Bcl-3 expression patterns showed that its expression is highest in the spleen and liver, suggesting a role in immunological function (Nolan et al. 1993). Intriguingly, further analyses in mouse cell lines derived from different stages of B-cell development showed that Bcl-3 expression is highest in B-cells just prior to the Ig switch, perhaps indicating a vital role in mediating that process (Bhatia et al. 1991). Further indication of its role comes from the fact that the Bcl-3 knockout mice, while developmentally normal, have compromised immune function. They are unable to clear *L. monocytogenes* and are susceptible to infection by *S. pneumoniae*. Additionally, they fail to generate antigen-specific antibodies and have abnormal spleen development, including the inability to form germinal centers (Franzoso et al. 1997; Schwarz et al. 1997).

We propose that inhibition of p53 may be a critical function for Bcl-3 in mediating a number of processes in immunological development, particularly B-cell maturation. B-cell maturation involves two processes, class switch recombination and somatic hypermutation, that are important for generating antibody diversity. However, these processes generate lesions that under different circumstances would be detrimental to the cell and likely elicit a DNA damage response (Xu et al. 2005). The high levels of Bcl-3 expression present in B-cells just prior to Ig switch will inhibit the p53 response generated by these processes, allowing the cells to survive.

This model presents a number of testable predictions. For example, if the defect in germinal center development seen in the Bcl-3 knockout mice is due to increased p53-dependent apoptosis, crossing these mice with p53 null animals should rescue the defect.

Furthermore, germinal center B-cells isolated from wild-type animals should exhibit higher levels of mdm2 expression and lower levels of p53 activity than those from Bcl-3 null mice. These studies are currently ongoing in the lab and should reveal whether the model is correct.

It is interesting to note that this model is similar to one recently proposed for the unrelated protein Bcl-6. Like Bcl-3, Bcl-6 is often translocated in leukemias and lymphomas and the Bcl-6 knockout phenotype is remarkably similar to that of Bcl-3 (Ye et al. 1993; Ye et al. 1997). Intriguingly, Bcl-6 has also been shown to inhibit p53 function, but unlike Bcl-3, it inhibits p53 directly at the levels of transcription. A recent report concludes that this inhibition of p53 is critical to allow germinal center B-cells to undergo somatic hypermutation and class switch recombination without eliciting an apoptotic response (Phan and Dalla-Favera 2004).

It has also been shown that expression of Bcl-3 correlates with survival in adjuvant-induced T-cells (Mitchell et al. 2001). Activated T-cells require a survival signal following antigen stimulation in order to avoid apoptosis and to undergo clonal expansion. The apoptotic signal has been shown to be mediated by the p53 family member p73 (Wan and DeGregori 2003). Given its role in p53 inhibition, Bcl-3 may protect activated T-cells through down-regulation of this apoptotic response by inhibiting p73 through a similar mechanism. Supporting this notion, mdm2 has been shown to bind and inhibit p73, although not through induction of proteosomal degradation. Instead, mdm2 disrupts the interaction of p73 with critical coactivators and inhibits its ability to promote transcriptional activation (Zeng et al. 1999).

This model also presents testable predictions. For example, if Bcl-3-mediated mdm2 induction is critical for T-cell survival, one would predict that an increase in mdm2 levels

would follow treatment of cells with adjuvant. Further, knockdown of Bcl-3 in the context of T-cell activation should lead to an increase in p73-dependent apoptosis. Further experimentation should clarify these issues.

Our data also suggest a more general role for Bcl-3 in modulating the p53 response given the fact that fibroblasts and other non-hematopoietic cells depend on Bcl-3 for this process. These data support a model in which the levels of Bcl-3 present in a given cell determine the extent of the p53 response following DNA damage. It has been proposed that the choice between cell cycle arrest and apoptosis following DNA damage is determined in part by the timing of p53 induction because the promoters of the genes mediating these two processes have different affinities for p53 (Vousden and Lu 2002). Bcl-3 may be a critical determinant of this decision based on its ability to modulate p53. As such, cells with relatively high Bcl-3 levels will experience a transient p53 response, allowing only for the transcription of p53 targets with high affinity binding sites, such as p21. The result will be a transient cell cycle arrest. Conversely, p53 will remain active longer in cells with relatively low levels of Bcl-3, allowing it to induce a wider range of targets, including Noxa, Puma and other pro-apoptotic effectors. Creating a panel of cell lines expressing variable levels of Bcl-3 should allow us to test this model. We would predict that as Bcl-3 levels increase, the duration of p53 induction will decrease and a lower percentage of cells will undergo apoptosis following DNA damage.

The data presented here, when placed in context of previous work on Bcl-3, support a model in which control of p53 is a critical function of Bcl-3 in immune development and oncogenesis. As Bcl-3 expression is found in an ever-growing list of human malignancies, it is imperative that we continue to uncover the details of its cellular function. This work has

answered a number of questions about Bcl-3, but has revealed many more. When these new questions are answered, we will be left with a clearer picture of Bcl-3 and hopefully be able to use this knowledge to combat disease.

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