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Regulation of Bax Activation and Apoptosis by Src and Acetylated Mutant p53

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Regulation of Bax Activation and Apoptosis by Src and Acetylated Mutant p53

By

Nicholas Taylor Woods

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Cancer Biology
College of Graduate Studies
University of South Florida

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Note to the Reader

The original of this document contains color that is necessary for understanding the data.

The original dissertation is on file with the USF library in Tampa, Florida.

Dedication

I would like to dedicate this dissertation to all my family friends who have supported me throughout the years. I owe the entirety of my success in the pursuit of my doctorate degree to my wife Neha. She has been a constant source of love and support, and our countless conversations on science have immeasurably enhanced my growth as a researcher. This work is also dedicated to my parents who have taught me the value of hard work and perseverance that has driven me to accomplish my goals, my friends who have provided needed laughter and distraction, and to my grandfather Royal who has been a source of unlimited inspiration. Words cannot begin to express my gratitude to all of you.

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Nicholas T. Woods

ABSTRACT

Apoptosis is an inherent suicide mechanism that cells invoke for a variety of reasons including embryo cavitation, tissue homeostasis, excessive DNA damage and aberrant oncogene activation. Apoptosis is regulated by a diverse set of proteins including, but not limited to, the Bcl-2 family. This family set is comprised of both pro-death and pro-survival proteins whose relative expression, localization and/or modifications regulate the balance between life and death for each cell. The keystones to this system are the proapoptotic proteins Bax and Bak, which are regulated by their conformation and localization. However, the exact mechanisms by which Bax and Bak become activated remains to be resolved. Similarly, research focusing on the cancer cell's ability to deregulate apoptosis by preventing the activation of Bax or Bak will provide further insight into the development of targeted therapies for cancer that will hopefully contribute to the cure of this formidable disease.

Src, the classic oncogenic kinase, has been found to deregulate Bax activation in response to the detachment of a cell from its substratum support thereby preventing anoikis, the Bax-dependent apoptotic response involved in the impairment of metastatic dissemination of cancer. Our findings indicate that Src deregulates this response by altering the relative expression of Bcl-2 family members Mcl-1 and Bim through the PI3-K/Akt and Erk1/2 pathways. However, Src retains its ability to prevent anoikis even in

the setting of Akt and Erk1/2 signaling inhibition. Further evaluation of the role of Src in this process revealed that Bif-1, a protein known to associate with and activate Bax, could be directly phosphorylated by Src which prevented the association of Bax with Bif-1 and impaired the anoikis response.

In addition, our studies have also found that Bax activation in response to treatment with type I and II histone deacetylase inhibitors is dependent on the expression of the tumor suppressor p53. Acetylation of p53 at carboxy-terminal lysine residues enhances its transcriptional activity associated with cell cycle arrest and apoptosis. Here, we demonstrate that p53 acetylation at K320/K373/K382 is also required for its transcription-independent functions in Bax activation, ROS production, and apoptosis in response to the histone deacetylase inhibitors (HDACi) SAHA and LAQ824. Knockout of p53 in HCT116 cells markedly reduced HDACi-induced apoptosis. Unexpectedly, expression of transactivation-deficient p53 variants sensitized p53-null cancer cells to HDACi-mediated Bax-dependent apoptosis, whereas knockdown of endogenous mutant p53 inhibited HDACi-induced apoptosis. Evaluation of the mechanisms controlling this response led to the discovery of a novel interaction between p53 and Ku70. The association between these two proteins was acetylation independent, but acetylation of p53 could prevent and disrupt the Ku70/Bax complex and enhance apoptosis. These results suggest a new mechanism of acetylated p53 transcription-independent regulation of apoptosis.

Chapter One: Introduction

Cancer

Cancer is a complex group of diseases that exhibit similar cellular traits such as uncontrolled growth, invasiveness, and sometimes metastasis of abnormal cells. When the spread of cancer is uncontrolled the result is often death. The lifetime risk of developing cancer in men is 1 in 2, and for women it is 1 in 3. Cancer can originate at many different sites but the cancer with the highest mortality rate is lung and bronchus in both men and women. Cancer associated mortalities remain alarmingly high accounting for 1 in every 4 deaths and averaging over half a million per year in the United States alone. Although cancer is the second leading cause of death in the United States after heart disease, over the years little advancement in improving survival rates has occurred compared to that for heart disease (Jemal et al., 2007). The diversity and heterogeneity of cancer has been the largest obstacle to advanced clinical therapies. It has become clear that the path to a cure will likely be a long-term research endeavor culminating in personalized therapeutics that will incorporate the lessons learned in a variety of different fields.

Hallmarks of Cancer

The causes of cancer are diverse in nature and include environmental factors such as chemical carcinogens, ionizing radiation and viruses as well as internal factors such as inherited genetic mutations, hormones and the immune system. Therefore, the cause of

cancer is generally cryptic in nature with the exception of those caused by cigarette smoking or alcohol consumption, but even in these cancers it appears that the combination of genetic predisposition and carcinogen consumption determines cancer formation and progression (Sellers et al., 1992). Nonetheless, all cancer cells exhibit specific traits termed the ‘Hallmarks of Cancer’ that dictate malignant progression (Hanahan and Weinberg, 2000). These hallmarks are self-sufficiency in growth signals, resistance to anti-growth signals, evasion of cell-death, limitless replicative potential, angiogenesis, and tissue invasion and metastasis. The multiplicity of these hallmarks reflects the progressive nature cancer must exhibit to overcome biological barriers to their formation and dissemination. Multiple molecular pathways have been identified that control each one of these hallmarks and current research has focused on targeting these different pathways to eliminate cancer.

Oncogenes

Oncogenes are protein encoding genes that drive malignant transformation and progression when mutated, overexpressed or hyperactive. There have been a number of oncogenes identified since the initial discovery of Src in the 1970’s (Erikson et al., 1980). The normal cellular counterparts are termed proto-oncogenes to indicate their potential to drive oncogenesis if activated. Proto-oncogenes can be activated by qualitative or quantitative mechanisms. Chromosomal translocations can cause oncogenes to be driven by promoters that are constitutively active in the target cells, such as the well known Philadelphia chromosome translocation t(9:22)(q34:q11) resulting in the oncogenic fusion product BCR-Abl found in chronic myelogenous leukemia and acute leukemia

(Kurzrock et al., 1987). Gene amplifications of oncogenes can also lead to aberrant signaling. An example of this is the dihydrofolate reductase gene (*DHFR*) amplification found in methotrexate-resistant acute lymphoblastic leukemia (Alt et al., 1978). Qualitative alteration of BRAF through the mutation V599E results in a constitutively active protein that drives the MAP kinase pathway (Davies et al., 2002). Since oncogenes are well studied and their role in cancer is generally pronounced, many therapeutic agents have been derived that specifically target them. One of the most successful inhibitors of oncogenes developed has been imatinib (Gleevec) targeting BCR-Abl, which has been highly effective in the treatment of Philadelphia chromosome positive leukemias.

Src

The discovery of the first oncogene, Src, was set in motion by the discovery of the cell-free transmission of a malignant avian virus by Peyton Rous in 1910, for which he was awarded the Nobel Prize (1966). This retrovirus was later found to encode for the viral oncogene v-Src which shared sequence homology with DNA found in normal avian genetic material (Stehelin et al., 1976). Src was determined to exhibit protein kinase activity required for its transformation potential (Collett et al., 1979; Levinson et al., 1978). The original v-Src was later determined to have a deletion of the c-terminal negative regulatory domain and was therefore constitutively active. The full-length mammalian homolog, c-Src, is often overexpressed, hyperactivated or mutated in a number of cancers of epithelial origin (Frame et al., 2002; Irby et al., 1999; Jacobs and Rubsamen, 1983; Rosen et al., 1986). Infrequent Src mutation in cancers results in a truncated and activated protein (Irby et al., 1999; Sugimura et al., 2000). Given Src's well

established role in tumorigenesis, therapeutic disruption of its mechanisms of action has been aggressively pursued.

Structure and Regulation of Src

Src is a non-receptor tyrosine kinase that localizes in the cytoplasm, but can also be recruited to membranes via N-terminal myristolation. Its three key domains are the SH3 domain, SH2 domain and the kinase domain (Figure 1). The SH2 domain recognizes pTyr motifs and is responsible for the auto inhibition of c-Src as it binds phosphorylated Tyr530 (*Homo sapiens* nomenclature; Tyr527 in chicken) in the c-terminal regulatory domain. Binding of the SH2 domain to pTyr530 also promotes the interaction of the SH3 domain with the polyproline tract located within the linker region between the SH2 and kinase domains of Src. These coordinated effects lead to structural changes in Src that inactivate the kinase activity of the protein (Yamaguchi and Hendrickson, 1996). Phosphorylation of Tyr530 can be accomplished by proteins such as CSK or its homolog CHK. Alternatively protein tyrosine phosphatases SHP1, SHP2, PTP α , or PTP1B can dephosphorylate Src resulting in an open active conformation of the kinase (Yeatman, 2004). Competitive binding of the SH2 domain by focal adhesion kinase (FAK), epidermal growth factor receptor (EGFR) or erbB2 can also promote the active conformation of Src (Cobb et al., 1994; Schaller et al., 1994). Likewise, SH3 binding by the HIV associated Nef protein can promote Src family kinase activity (Moarefi et al., 1997). Full activation of Src is accompanied by autophosphorylation of tyrosine 419 within the kinase domain. These multiple modes of Src regulation intricately regulate downstream signaling that control cell survival, proliferation, angiogenesis and

metastasis.

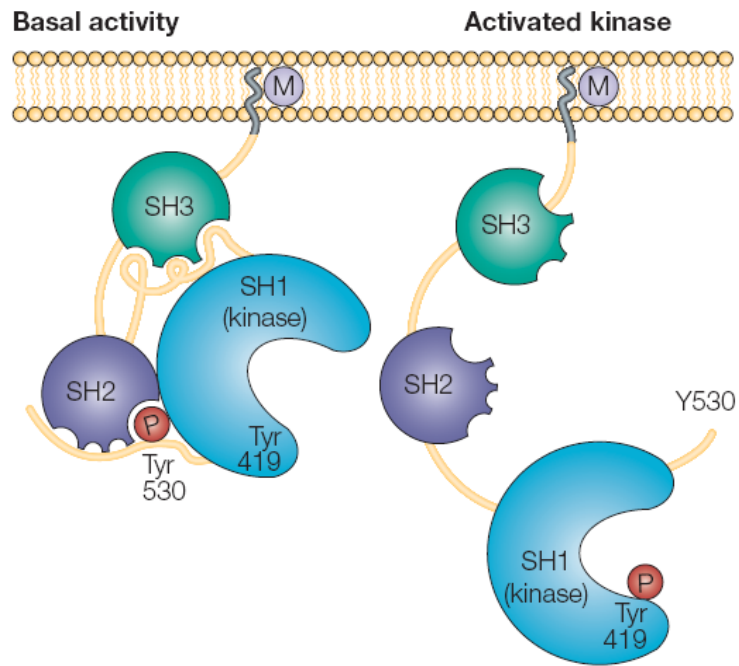


Figure 1. Domain structure of c-Src and v-Src. (Yeatman, 2004)

Src Signaling

Src is involved in diverse and numerous signaling pathways. Src family kinases (SFKs), including Lck, Lyn, Fyn, Blk, Fgr, Hck, Yrk, and Yes, are also involved in a broad spectrum of cellular signaling, but for the sake of brevity this introduction will only attempt to summarize important Src signaling cascades relevant to the research outlined below. For an excellent comprehensive review on SFK signaling in different biological pathways please refer to Thomas and Brugge, 1997.

One of the most studied functions of Src is its role in the signaling cascade of focal adhesions. These structures regulate cell morphology, attachment and mobility.

Many focal adhesion associated proteins are also substrates for v-Src such as focal adhesion kinase (FAK), p130^{CAS}, paxillin, talin, vinculin, tensin, and $\beta 1$ integrin subunit, which are phosphorylated on tyrosine residues when localized to these structures (Thomas and Brugge, 1997). Specifically, Src directly phosphorylates FAK which controls FAK kinase activity and creates binding sites for recruitment of proteins to FAK complexes thereby perpetuating downstream signaling that promote cell migration and survival (Calalb et al., 1996; Cobb et al., 1994; Schlaepfer and Hunter, 1996). The role of Src in cell migration was also demonstrated in src^{-/-} fibroblasts which show reduced cell motility that could be rescued by kinase-active but not inactive Src (Hall et al., 1996). However, there is also evidence that the SH2 and SH3 domains of Src act as a scaffold responsible for assembly of FAK signaling complexes independent of Src's kinase activity (Brunton et al., 2005; Schlaepfer et al., 1997).

Src is also an upstream mediator of such pathways as PI3-K, Erk1/2, and STAT3 (Fincham et al., 2000; Park et al., 1999; Yu et al., 1995). Like Src, these cell signaling pathways are responsible for regulating a diverse set of biological functions. The phosphatidylinositol 3-kinase (PI3-K) and extracellular regulated kinase (Erk1/2) pathways are often deregulated in cancer by overexpression or mutation of key regulatory elements such as Src. The PI3-K regulatory subunit p85 can interact with Lck and Abl which directly phosphorylate the subunit at tyrosine 688 allowing the catalytic domain of p110 of PI3-K to become activated (von Willebrand et al., 1998). The PI3-K pathway is also deregulated by mutation or loss of phosphatase with tensin homology (PTEN). When PTEN is disrupted, the cell loses the ability to negatively regulate PI3-K dependent formation of the second messenger lipid phosphatidylinositol (3,4,5) triphosphate (PIP₃),

which results in constitutive activation of the Akt pathway responsible for apoptosis inhibition and cell cycle progression (Cully et al., 2006). Overactive Src signaling can also promote aberrant signaling through the Erk1/2 pathway by promoting phosphorylation of the adaptor protein Shc which then leads to enhanced signaling through the Grb2-SOS-Ras pathway (Blake et al., 2000; Ravichandran, 2001; Salcini et al., 1994). Ras activation can also lead to the activation of PI3-K through direct binding to the p110 subunit (Sjolander et al., 1991; Walker et al., 1999). These convoluted pathways are essential to the progression of cancer as they collectively promote the tumor phenotype. However, for the purposes of this report the focus of their signaling outcomes will be their antiapoptotic potentiation.

Anoikis

The development of a metastatic phenotype is a late event in tumor progression. Metastases are an indicator of poor prognosis. In fact, 90% of the mortalities associated with cancer arise from metastatic dissemination (Weigelt et al., 2005). In order for a tumor cell to acquire the metastatic phenotype it must first overcome or bypass the intrinsic cellular response of anoikis, detachment induced apoptosis. When normal cells are detached from the extracellular matrix (ECM) and deprived of integrin signaling they die by a process termed anoikis (Frisch and Francis, 1994). Anoikis is essentially a normal apoptotic reaction in response to the specific stimulus of improper integrin engagement. The execution of a cell by anoikis stimulation can rely on several different mechanisms of apoptosis. For instance, inhibition of the extrinsic apoptotic cascade results in the inhibition of apoptosis (Frisch, 1999). However, regulation of the intrinsic

pathway of apoptosis can also effectively prevent anoikis in a variety of systems.

Anoikis is essential for common biological processes involved in homeostasis, morphogenic changes, and inhibition of cancer metastasis. Integrins not only function as structural adhesive support between the cell and the ECM, they also serve as a scaffold for intracellular signaling proteins (Hynes, 2002). This signaling activates pathways involved in survival and proliferation which prevents anoikis. Cells of different origins express unique sets of integrins and preferentially adhere to specific complements of ECM ligands. Therefore, when a cell comes into contact with the improper complement of ECM ligands, signaling becomes insufficient and anoikis soon follows (Pullan et al., 1996). However, upon mobilization some metastatic cells can change their complement of integrin receptors to adapt to a new ECM environment to prevent anoikis (Montgomery et al., 1994). Therefore, it is essential to understand integrin function and their signaling pathways to decipher the mechanisms of anoikis initiation.

Integrin Signaling

The ECM provides adhesive support to normal tissues and controls numerous signals that regulate diverse cellular processes such as survival, growth, and differentiation (Hynes, 1999). These signaling mechanisms are regulated by integrins. Integrins are heterodimeric proteins that consist of two α and β type-I transmembrane subunits. There are 18 α and 8 β subunits that can be expressed in mammals, which can combine to form 24 recognized integrins. These integrins bind specific components of the ECM, but some overlapping is found between substrate recognition of different integrins (Guo and Giancotti, 2004). Integrin engagement of extracellular matrix substrates leads

to the formation of focal adhesions, as mentioned previously. These focal adhesions are membrane attachment plaques that couple the ECM attached transmembrane integrins to the intracellular actin cytoskeleton and other cellular structural components (Jockusch et al., 1995). Integrin signaling affects many different pathways such as the Erk1/2, PI3-K/Akt, NF- κ B and others through Src family kinase mediated signal propagation (Figure 2). In this way, integrins regulate biological functions such as cell survival, proliferation and migration.

Integrin activation of the aforementioned signaling pathways is highly reminiscent of extracellular growth factor receptors. In fact, growth factor signaling by epidermal growth factor receptor (EGFR) can suppress the anoikis response in mammary epithelial cell line MCF-10A (Reginato et al., 2003). Detachment of this cell line leads to a downregulation of EGFR expression, repressed Erk1/2 signaling, and enhanced Bim expression leading to apoptosis. Further crosstalk between growth factor receptors and integrins has been documented in both primary oligodendrocytes and mammary epithelial cells that enhances growth factor signaling and survival (Gilmore, 2005). Given the high interconnectedness between integrin and growth factor receptor pathways, it will be important to determine the individual contributions of inhibited growth factor and integrin signaling in anoikis.

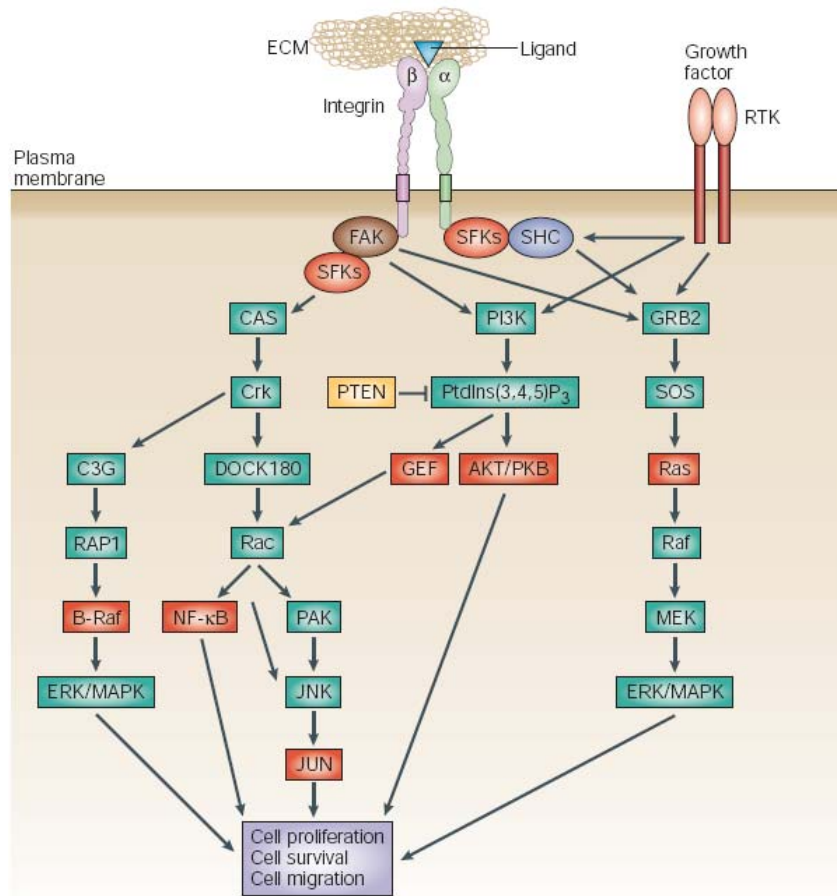


Figure 2. Integrin signaling networks (Guo and Giancotti, 2004).

Src Mediated Suppression of Anoikis

When cells are deprived of integrin signaling due to ECM detachment these pro-survival pathways are ablated which triggers the anoikis response. Therefore, in order for a cell to suppress anoikis it must acquire the means to compensate for the lost signaling and prevent apoptosis. Aggressive breast, colon, and lung malignancies with a propensity to metastasize have been shown to lack the normal apoptotic response after detachment from the supporting matrix (Shanmugathan and Jothy, 2000; Wei et al., 2001; Yawata et al., 1998). Moreover, cancer cells that are more apt to resist anoikis due to genetic

mutation or overactive survival signaling are increasingly likely to initiate distal metastases (Douma et al., 2004).

Src has been implicated in processes that increase the metastatic potential of cancer cells including cell adhesion (Kaplan et al., 1995), migration (Rahimi et al., 1998), and invasion (Zhang et al., 2004). Transient increases in Src activity upon detachment in both normal and cancer cells is critical to inhibition of anoikis (Loza-Coll et al., 2005; Wei et al., 2004). Active c-Src likely promotes survival once the cell is detached from the ECM by compensating for the loss of integrin and growth factor signaling. However, the direct mechanisms that potentiate Src mediated anoikis suppression are poorly understood. Two of the projects that have been included in this report attempt to delineate specific mechanisms of Src action in the prevention of anoikis and the promotion of metastatic dissemination of cancers (Woods et al., 2007; Yamaguchi et al., 2008). The findings presented herein provide insights into Src mediated disruption of Bax activation required for anoikis.

Apoptosis

Apoptosis is a multi-step process that culminates in the self-destruction of an individual cell. This self-killing mechanism proceeds through a highly regulated set of events that lead to the activation of cellular cysteine proteases known as caspases (Wolf and Green, 1999). Apoptosis is characterized by specific biochemical and morphological characteristics. This process is essential to counteract normal proliferation resulting in relatively steady state in an organism's total cell number. The deregulation of apoptosis has been characterized in many different diseases. Neurodegenerative disorders are often

the result of increased apoptosis of neuronal lineage cells. Alternatively, inhibition of the normal apoptotic response often results in the development of cancer when combined with sustained proliferation (Thompson, 1995). Luckily, there are many built-in regulatory mechanisms that maintain apoptosis at acceptable levels.

Morphological and biological features that distinguish apoptosis from other forms of cell death such as necrosis or autophagy include cell shrinkage, membrane blebbing, nuclear condensation, activation of cellular caspases, DNA fragmentation, mitochondrial membrane disruption, and exposure of phosphatidylserine (PS) on the outer leaflet of the plasma membrane (Fadok et al., 1992; Green and Evan, 2002; Reed, 1995). Unlike necrosis, apoptosis is a rather 'clean' process in that there is limited or no release of inflammatory intracellular components. The exposure of PS results in the recruitment of macrophages that recognize the signal and engulf the apoptotic cell.

Apoptosis can be triggered by a number of different stimuli. Depending on the specific stimulus, apoptosis can be initiated through one of two independent pathways referred to as intrinsic or extrinsic based on the origin of the initiating apoptotic signal. The intrinsic pathway of apoptosis is regulated by the integrity of the outer mitochondrial membrane (OMM) and the Bcl-2 family members that control it. Alternatively, the extrinsic pathway of apoptosis is regulated by transmembrane receptors that are activated by ligand specific binding. Whereas, the intrinsic apoptotic response is determined by the integrity of the OMM, extrinsic apoptosis may or may not require OMM disruption for efficient apoptosis.

Extrinsic Apoptotic Pathway

The extrinsic pathway of apoptosis is initiated upon binding of ligand to their cognate receptors. These receptors, ominously dubbed “death receptors” such as TRAIL, TNFR1-2, DR4, DR5 and Fas/CD95 all contain cysteine rich extracellular domains that allow specific binding of ligand resulting in trimerization and activation (Naismith and Sprang, 1998). This clustering of death receptors results in the recruitment of cytoplasmic adapter proteins FADD or TRADD to the complexes, which interact with the death domains (DD) of the receptor via their own DD. FADD also contains a death effector domain (DED) which recruits initiator caspase-8 and/or caspase-10, via their own DED domain, to the receptor resulting in the formation of the “death inducing signaling complex” (DISC). The enhanced proximity of these initiator caspases promotes their proteolytic processing into mature proteases (Ashkenazi, 2002) (Figure 3A). Apoptosis resulting from death receptor signaling can be classified as type I or type II. Type I extrinsic apoptosis occurs when activation of initiator caspases alone is sufficient to activate effector caspases-3 and 7 which then promote the enzymatic destruction of the cell. However, the much more common type II extrinsic apoptosis relies on the involvement of the intrinsic apoptotic pathway and outer mitochondrial membrane permeabilization through caspase-8 mediated enzymatic cleavage of Bid into two fragments. The C-terminal 15 kDa fragment, tBid, translocates to the mitochondria and acts as a potent inducer of apoptosis through its interaction with Bcl-XL and Bax (Luo et al., 1998). This mechanism allows for the amplification of the initial apoptotic insult and the efficient execution of apoptosis.

Intrinsic Apoptotic Pathway

Bcl-2 was originally identified in follicular lymphoma based on the common translocation t(14:18) in this tumor type (Tsujimoto et al., 1985). Originally classified as an oncogene, it was later found that expression of Bcl-2 had no effect on cell cycle progression and instead specifically prevented cell death in response to stimuli (Thompson, 1995). Subsequent identification of the rest of the Bcl-2 family members and several non-Bcl-2 proteins has led to our current understanding of the basic functions of these proteins in the regulation of apoptosis.

The Bcl-2 family is composed of opposing subsets of proteins such as antiapoptotic members Bcl-2, Bcl-XL, and Mcl-1 which protect the integrity of the OMM through inhibition of the multi-domain proapoptotic proteins Bax and Bak. Activation of proapoptotic molecules Bax and Bak is associated with the loss of OMM integrity and the release of apoptogenic proteins such as cytochrome c, AIF, endonuclease G, HtrA2/Omi and Smac/Diablo (Green, 2005). Release of cytochrome c into the cytoplasm allows it to bind Apaf-1 culminating in the formation of the apoptosome with the recruitment of procaspase-9 (Figure 3A). The clustering of procaspase-9 results in autolytic processing and maturation of the initiator caspase-9 and subsequent activation of downstream effector caspases and the resultant apoptosis. Release of HtrA2/Omi and Smac/Diablo prevent the inhibition of cellular caspase activity by inactivating the inhibitors of apoptosis (IAP) proteins such as cIAP and XIAP. Endonuclease G and AIF are both nucleases that once released, translocate to the nucleus and participate in DNA fragmentation (Daugas et al., 2000; van Loo et al., 2001).

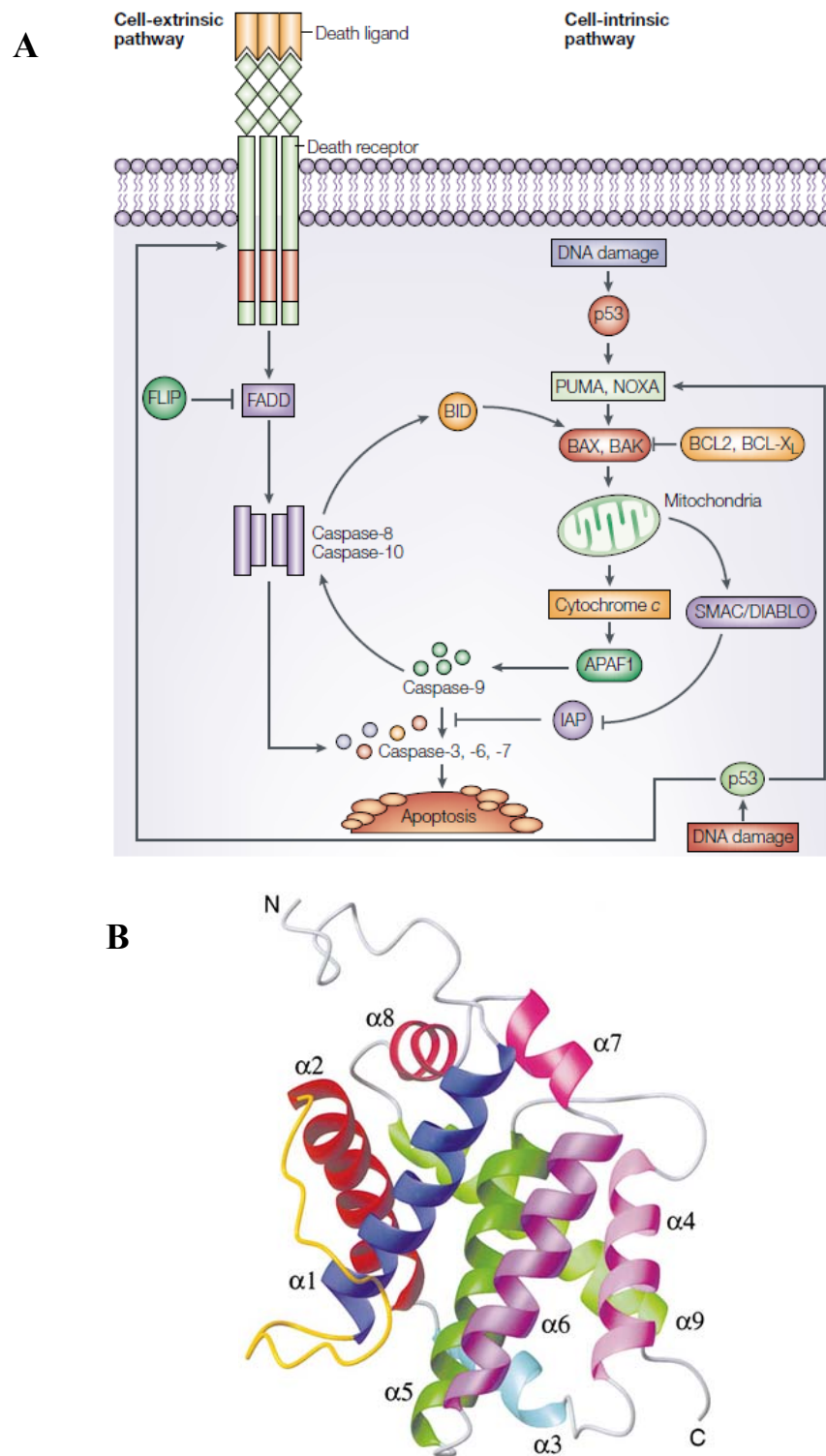


Figure 3. Apoptotic Pathways and Bax Organization. (A) Extrinsic and Intrinsic Apoptotic Pathways (Ashkenazi, 2002). (B) Native conformation of inactive Bax (Suzuki et al., 2000).

Bax Activation

Bax activation is a multi-step process that converts cytoplasmic monomers into pore forming membrane bound oligomers and is a key initiating step in the execution of intrinsic apoptosis and anoikis (Gilmore et al., 2000; Lindsten et al., 2000). Activation involves a conformational rearrangement, recruitment to the mitochondrial membrane, insertion into the membrane, oligomerization and pore formation. In aqueous solution Bax exists as a globular protein similar to other Bcl-2 family members (Petros et al., 2004). Bax is composed of 9 α helices separated by amorphous loop regions (Figure 3B). The two core helices $\alpha 5$ and $\alpha 6$ are largely hydrophobic and are surrounded by the seven remaining amphipathic helices. These seven helices maintain their hydrophobic regions toward the center of the protein and their hydrophilic regions toward the exterior, which promotes the solubility of the protein and its localization to the cytoplasm. The BH1-3 domains of Bax form a hydrophobic pocket that is occupied by the C-terminal helix $\alpha 9$. Bax conformational activation involves the dissociation of helix $\alpha 9$ from the hydrophobic pocket. This C-terminal helix of Bax is responsible for the anchoring of Bax to intracellular membranes. Akt mediated phosphorylation of S184 in helix $\alpha 9$ or mutation to a charged residue prevents Bax localization to the mitochondria even under apoptotic conditions (Gardai et al., 2004). Similarly, S184 deletion or mutation to valine results in constitutive association of Bax with the mitochondria, but without another stimulus this alone does not stimulate the apoptotic response (Nechushtan et al., 1999). Rather, the core hydrophobic helices $\alpha 5$ and $\alpha 6$, which strongly resemble the pore forming domain of diphtheria toxin, are required to be exposed in order for pore formation to occur. Exposure of these two helices is responsible for insertion of Bax into the OMM and as an

isolated region they are able to insert into synthetic membrane lipids and form pores (Annis et al., 2005; Garcia-Saez et al., 2006). However, Bax localization to the OMM is independent of helices $\alpha 5$ and $\alpha 6$ and instead requires the exposure of the normally restricted N-terminal domain consisting of amino acids 20-37 (Cartron et al., 2003). Exposure of this N-terminal domain can be assessed using the conformation specific Bax 6A7 antibody. As evidenced by these previous findings, in order for efficient Bax mediated apoptosis this protein must undergo extensive rearrangement of its three-dimensional structure for proper targeting, insertion and pore formation. Although much is already known about the changes in Bax required for OMM permeabilization, the exact mechanism by which Bax is persuaded to undergo such gross rearrangement remains to be resolved.

Bcl-2 Family Regulation of Bax

Bax activation status is regulated by other Bcl-2 proteins consisting of both pro- and antiapoptotic molecules with opposing functions. The antiapoptotic Bcl-2, Bcl-XL, Mcl-1, and Bcl-w proteins sequester proapoptotic BH3-only proteins as well as Bax and Bak. Currently two models have been proposed to explain the function of BH3-only molecules in the activation of Bax and Bak. In the displacement model, BH3-only proteins bind to antiapoptotic proteins and cause the release of Bax and Bak allowing them to become activated. In this model, BH3-only proteins Bim, tBid, and Puma are the most potent inducers of Bax activation presumably for their broad affinity for the majority of the antiapoptotic proteins. Indeed, the different BH3-only proteins exhibit drastically different affinities to antiapoptotic molecules (Certo et al., 2006).

Contrastingly, in the direct binding model Bim, tBid and Puma are classified as activators because they can directly activate Bax (Desagher et al., 1999; Harada et al., 2004; Kuwana et al., 2005; Letai et al., 2002; Wei et al., 2000). The remaining BH3-only proteins, such as Noxa, Bmf, Hrk, Bik, Bad, Blk, and Bnip3, are classified as sensitizers and bind to antiapoptotic proteins and prevent their inhibition of the BH3-only activators as well as Bax and Bak. Although both models adequately account for the regulation of Bax and Bak activation, definitive experimental evidence is still needed to resolve discrepancies in both models.

Although BH3-only proteins may affect Bax activation through several different mechanisms as hypothesized in the direct and indirect models, problems with both models still remain to be resolved. For instance, evidence that BimEL can directly associate with Bax is lacking. Co-immunoprecipitation of the two proteins has only been successfully tested in exogenous expression systems and this model is highly contested due to the lack of interaction seen by others (Harada et al., 2004; Marani et al., 2002). Interestingly, the BimS isoform is able to induce apoptosis without interacting with antiapoptotic proteins (Weber et al., 2007). This accumulating evidence suggests that the multitude of different Bim isoforms can display proapoptotic functions in a mutually exclusive manner. Our lab has also determined that Bcl-XL can protect against Bax activation and cytochrome c release independent of its direct interaction with Bax and Bim (Yamaguchi and Wang, 2002). Also, chemical inhibition of Bcl-2 and Bcl-XL by ABT-737 does not promote substantial apoptosis when Mcl-1 is expressed. Concomitantly, when Mcl-1 is knocked down, apoptotic induction still requires another stimulus. Interaction between the Bcl-2 family members is highly dependent on

conformation. Therefore, it is essential to critically analyze experimental methods used to probe the molecular interactions between these proteins as detergents used to make cell lysates have a large impact on the findings. Future work is needed to determine how the molecular interactions occur, where they occur and their biological significance.

Regulation of Bax by non-Bcl-2 Family Proteins

Non-Bcl-2 proteins Ku70, Bif-1, humanin peptide, p53, 14-3-3, cardiolipin, Tom22, and ASC have been identified as potential mediators of Bax activation and it is likely that their individual role is determined by several factors such as the apoptotic insult and cell lines tested (Chipuk et al., 2004; Cohen et al., 2004a; Guo et al., 2003; Kuwana et al., 2002; Nomura et al., 2003; Ohtsuka et al., 2004; Ott et al., 2007b; Takahashi et al., 2005). Ku70, 14-3-3 and humanin control apoptosis through their inhibition of Bax activation by sequestering inactive Bax away from the mitochondria and preventing its activation (Guo et al., 2003; Nomura et al., 2003; Subramanian et al., 2005). These retention factors may explain how Bax is kept in the inactive state when localized to the cytoplasm without direct interaction with Bcl-XL which resides on the mitochondria. Although less well characterized than Bcl-2 proteins, these retention factors add another layer of control into the scheme of Bax activation and provide additional prospective therapeutic targets to potentiate an apoptotic response.

Non-Bcl-2 family membrane proteins are also required for Bax activation, as pretreatment of purified mitochondrial membranes with protease K prevents tBid induced Bax oligomerization (Roucou et al., 2002). Similarly, tBid and Bax can efficiently release pre-loaded dextran from outer mitochondrial vesicles compared to chemically defined

protein-free liposomes (Kuwana et al., 2002). However, controversy persists as to which mitochondrial proteins are required for Bax activation (Ott et al., 2007b). The research and discussion found in the following sections will explore the mechanisms of Mcl-1, Bim and Bif-1 regulation and their impacts on Bax activation in response to detachment from adhesive support, as well as the novel mechanism of acetylated p53 regulation of Bax activity through a Ku70 dependent manner.

Bim

As mentioned previously, the two competing hypotheses of direct and indirect BH3-only protein regulation of Bax activation remain to be resolved. One of the proteins at the center of this conflict is Bim. Although the exact mechanism is debated, the fact remains that there is a positive correlation between Bim expression and apoptosis which can be prevented by overexpression of antiapoptotic proteins or the absence of Bax and Bak. The expression of Bim is regulated at both the transcriptional and posttranslational levels. Transcription is controlled by several transcription factors, most notably FKHRL1, Foxo3a (Dijkers et al., 2000; Gilley et al., 2003). Akt directly phosphorylates Foxo3a to promote its interaction with 14-3-3 and retention in the cytoplasm (Brunet et al., 1999). There have been many different isoforms of Bim identified but the most predominantly expressed are BimEL, L and S (EL, extra long; L, long; S, short) (Miao et al., 2007). At the posttranslational level, Bim can be regulated by phosphorylation events. Erk1/2 phosphorylates BimEL on Ser69 thereby targeting the protein for ubiquitination and degradation via the proteasome, which leads to inhibition of apoptosis as a result of repressed expression (Akiyama et al., 2003; Luciano et al., 2003). Akt can also

phosphorylate Bim at Ser87 and mutation of this site is associated with enhanced proapoptotic activity (Qi et al., 2006). Mouse models have also identified phosphorylation of Bim at Thr112 to be important for Bcl-2 binding and repression of apoptosis (Hubner et al., 2008). Bim is also maintained in an inactive state by binding to the dynein motor complex or by sequestration to the mitochondria by antiapoptotic Bcl-2 family members, such as Bcl-XL and Mcl-1 (Puthalakath et al., 1999; Yamaguchi and Wang, 2002). One of the reasons Bim is thought to be such an efficient activator of apoptosis is its ability to interact with most antiapoptotic Bcl-2 proteins. However, Mcl-1 has been shown to have a higher affinity for Bim compared to Bcl-2 and Bcl-XL (Gomez-Bougie et al., 2005).

Mcl-1

Mcl-1 is a short-lived protein with a half-life of approximately 30 minutes. It sequesters BH3-only proteins such as Bim and tBid to the mitochondria and prevents activation of Bax and Bak (Clohessy et al., 2006; Opferman et al., 2003). Mcl-1 is regulated transcriptionally by the Src/STAT3 pathway (Niu et al., 2002), and posttranslationally by Mule, Mcl-1 ubiquitin-ligase E3, which is able to ubiquitinate Mcl-1 and promote its degradation (Zhong et al., 2005). GSK-3 β phosphorylation of Ser159 on Mcl-1 enhances its ubiquitination and degradation via the proteasome (Maurer et al., 2006). The resulting decrease in Mcl-1 expression levels promotes apoptosis. Mcl-1 is also a substrate for cellular caspases. Truncation of Mcl-1 may result in the acquisition of a proapoptotic function that can enhance the apoptotic response similar to that observed for caspase cleaved Bcl-2 and Bcl-XL, but there is still some uncertainty as to what

impact this truly has (Clohessy et al., 2004; Weng et al., 2005).

In relation to metastasis, it was determined in a Lewis lung carcinoma model that the expression of Mcl-1 was directly related to metastatic potential of the tumor as these cells were more resistant to hypoxia-induced apoptosis (Koshikawa et al., 2006).

Therefore, the selection of tumor cells with high levels of Mcl-1 may also give rise to anoikis resistant metastatic cells. Indeed, our research has demonstrated Mcl-1 to be a critical regulator of anoikis. Specifically, Src activity can prevent detachment induced Mcl-1 depletion through Akt mediated suppression of GSK-3 β activity. This results in decreased phosphorylation of Mcl-1 at S159 which promotes its stabilization and inhibition of Bax activation after detachment from the ECM.

In this study, we have identified the stabilization of Mcl-1 and suppression of Bim as critical events during oncogenic suppression of anoikis. The transition of cells to a metastatic phenotype correlates not only with increased Mcl-1 expression, but also to the altered regulation of its degradation profile in response to detachment. Inhibition of survival signals mediated by active Src or downstream Akt and Erk1/2 kinases that control Mcl-1 degradation and Bim induction is able to restore Bax activation and anoikis susceptibility. Furthermore, Mcl-1 repression, but not Bcl-2 or Bcl-XL inhibition, is capable of initiating anoikis in metastatic cancer cells. This study is the first to characterize Mcl-1 degradation in anoikis and the oncogenic signaling that can disrupt this essential mechanism in human cancers.

Bif-1

Bax interacting factor-1 (Bif-1), also known as SH3GLB1 or Endophilin B1, was

initially identified by two independent groups as a Bax interacting molecule (Cuddeback et al., 2001; Pierrat et al., 2001) even though it lacks homology with the Bcl-2 family. Bif-1, like other members of the Endophilin family, contains a carboxy-terminal SH3 domain, binds to membranes through their N-BAR domain and promotes membrane curvature (Gallop et al., 2006; Masuda et al., 2006; Peter et al., 2004). Bif-1 localizes on the membranes of intracellular organelles such as the Golgi and mitochondria (Karbowski et al., 2004; Takahashi et al., 2005; Yang et al., 2006). Importantly, Bif-1 directly interacts with Bax and enhances the kinetics of apoptosis induction by promoting conformational activation of Bax and Bak in response to intrinsic apoptotic signals (Cuddeback et al., 2001; Takahashi et al., 2005).

Bax pore formation in large unilamellar vesicles is accompanied by structural changes in the lipid bilayer caused by monolayer curvature (Terrones et al., 2004), and Bax has a tendency to accumulate at sites of fission and fusion on the OMM where lipids are likely to deviate from a bilayer structure (Karbowski et al., 2002). Together, these previous findings suggest that Bax pore formation can be regulated by mitochondrial proteins and those that alter membrane curvature, such as Bif-1, are increasingly likely to affect Bax conformational activation. However, the mechanism by which Bif-1 accomplishes this regulation of Bax remains unclear.

The data presented below illustrates that Src signaling can prevent the initiation of anoikis by inhibiting both Mcl-1 degradation and Bim induction. However, depletion of Mcl-1 along with induction of Bim by blocking the Akt and Erk1/2 signaling is unable to illicit a robust anoikis response in the presence of Src activity. Therefore, Src must be acting at multiple levels to prevent Bax activation during detachment. In this study, we

found that Src directly interacts with Bif-1 and phosphorylates Bif-1 on tyrosine 80. This phosphorylation event has a direct impact on the ability of Bif-1 to bind Bax which correlates with a repression of Bax activation during anoikis.

Ku70

Ku70 was originally identified as an essential regulator of nonhomologous end joining (NHEJ) DNA double strand break repair and is also essential for V(D)J recombination (Lieber et al., 2003). Ku70 associates with Ku80 to form the heterodimeric protein Ku that binds to both fragment ends of DNA double strand breaks to maintain them in close proximity. Recruitment of other NHEJ proteins such as DNA-PK_{CS}, Artemis and DNA-ligase IV results in the repair of these DNA lesions. The Ku70/Ku80 complex has also been shown to be an essential regulator of telomere maintenance (Tuteja and Tuteja, 2000). Ku70 knock-out mice are viable but smaller than their wild type counterparts, exhibit increased apoptosis in neuronal cells during development, are immune compromised and are hypersensitive to ionizing radiation (Gu et al., 2000; Manis et al., 1998; Ouyang et al., 1997). Ku70 is an abundant protein in the nucleus but also has been found to localize to the cytoplasm and may play a role in cell-cell or cell-matrix adhesion (Muller et al., 2005; Subramanian et al., 2005).

In addition to its role in DNA damage repair, Ku70 forms an inhibitory complex with Bax that impairs apoptosis initiation. This complex is disrupted by acetylation of the carboxy-terminus of Ku70 mediated by CBP/p300, PCAF, SirT1 and HDACi treatment (Cohen et al., 2004a; Cohen et al., 2004b; Subramanian et al., 2005). The interaction between Ku70 and Bax also leads to decreased levels of ubiquitinated Bax but does not

affect the total pool of unmodified Bax (Amsel et al., 2008). This may lead to the enrichment of unmodified Bax that is more efficient at initiating apoptosis. Ku70/Bax association may serve a dual purpose of both inhibiting Bax translocation to the mitochondria while ensuring that adequate pools of functional Bax are available when needed. Specifically, we have found that acetylated p53 can also have a profound effect on the association between Ku70 and Bax.

p53

The tumor suppressor p53 is a highly regulated transcription factor that has a fundamental role in the prevention of tumorigenesis and response to chemotherapy, making it one of the most comprehensively studied molecules in cancer research. Normal cells exhibit low levels of p53 in resting conditions to maintain cellular homeostasis and prevent aberrant apoptosis. Cellular stress leads to rapid increases in p53 protein levels as a result of combined increases in transcription, translation and posttranslational modifications that repress degradation and enhance its activity. The characterization of p53 function is often accompanied by specific posttranslational modifications such as acetylation, methylation, ubiquitination, neddylation, and sumoylation (Figure 4) (Brooks and Gu, 2003; Toledo and Wahl, 2006). These modifications regulate diverse aspects of p53 function ranging from cellular localization, DNA binding, protein-protein interactions, and transcriptional regulation.

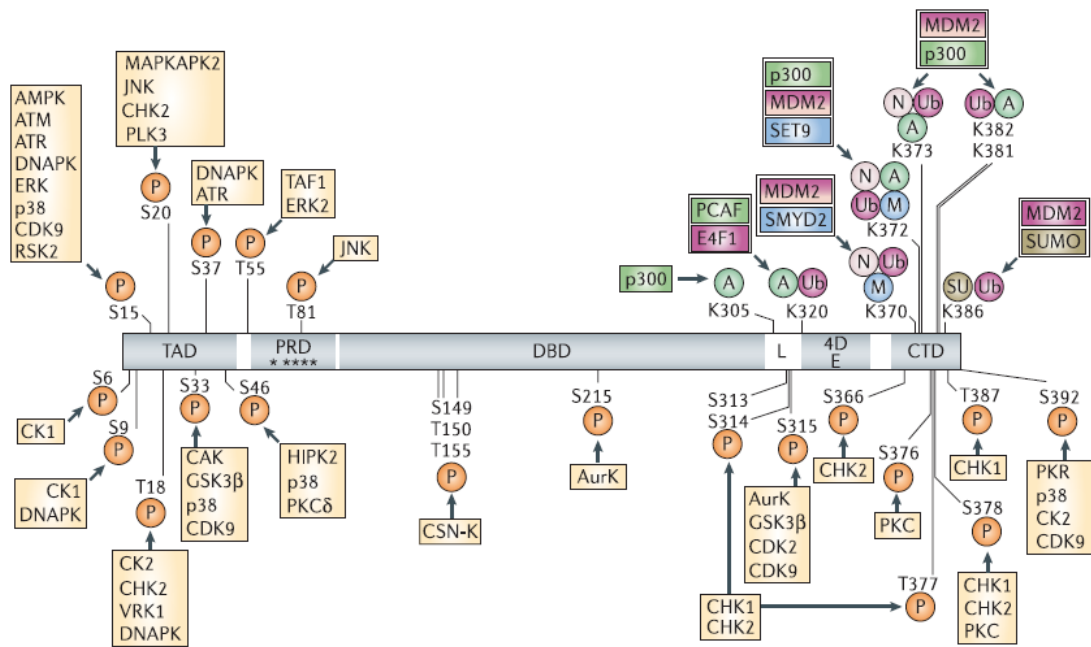


Figure 4. Posttranslational modifications of p53 (Toledo and Wahl, 2006)

Deregulation of p53 in Cancer

A multitude of different tumorigenic mutations of p53 have been characterized. It is estimated that approximately half of all cancers have mutated p53. The other half retains wild type p53 but exhibit deregulated pathways of p53 control which generally leads to the constitutive repression of p53 expression thereby prohibiting its proapoptotic functions. The vast majority of p53 mutations occur in the central DNA-binding domain of p53. These mutations prevent p53 from binding DNA and inhibit the transcriptional activation of its target genes. The deregulation of wild type p53 in cancer can be caused by the overexpression of its negative regulator MDM2. Aberrant MDM2 mediated degradation of p53 can be caused by gene amplification of MDM2 or the loss of ARF, which directly binds to MDM2 in response to cellular stresses and inhibits its function (Momand et al., 1998; Ruas and Peters, 1998). This amazing rate of deregulation across

cancers illustrates the central role of p53 as a tumor suppressor.

Knock-out of *mdm2* results in an embryonic lethal phenotype that can be rescued by complimentary knock-out of *p53*. This is presumably because in the absence of Mdm2, p53 becomes overexpressed and causes aberrant apoptosis during embryogenesis. Mutation of a single allele of p53 is thought to functionally inactivate wild type p53 as it exists in a tetramer for proper transcriptional activity and the presence of a single mutant protein in this complex is inhibitory. Interestingly, *p53*^{+/^{R172H}} / *mdm2*^{-/-} mice fail to be born, however *p53*^{+/^{R172H}} embryos exposed to ionizing radiation show decreased apoptosis in the hypothalamus compared to *p53*^{+/-} mice (Lang et al., 2004). Furthermore, tumor development and metastatic potential in *p53*^{+/^{R172H}} mice have distinct spectrums dependent upon the strain of mice (Lang et al., 2004; Olive et al., 2004). Therefore, the function of mutant p53 varies depending upon the genetic background and cellular context in which it is assayed. Much research remains to be done to determine the multitude of factors that determine the function of both wild type and mutant p53 in different cellular settings and in response to stresses.

Transcription-Dependent p53 Apoptosis

Expression of Mdm2, an E3-ligase responsible for targeting p53 for proteasomal degradation, is transcriptionally activated by p53, which results in a negative feedback loop designed to maintain p53 at appropriate levels. Other E3 ligases including COP1, Pirh2 and ARF-BP1 are also capable of ubiquitinating p53 and repressing its expression (Chen et al., 2005; Dornan et al., 2004; Leng et al., 2003). Cellular stresses cause posttranslational modifications to p53 which negatively affects the ability of Mdm2 to

bind p53. For instance, DNA damage results in phosphorylation of p53 by ATM and Chk2 at serine 15 and serine 20, respectively (Toledo and Wahl, 2006). These two sites are within the Mdm2 binding domain of p53 and prevent the interaction between these two proteins. Phosphorylation of the N-terminus of p53 results in the increased association with histone acetyltransferases p300/CBP which can also cause acetylation of p53 at multiple lysine residues. Interestingly, Mdm2 appears to have several contacts points with p53 as it has recently been discovered that the C-terminus and DNA binding domains of p53 can also bind Mdm2 (Tang et al., 2008; Yu et al., 2006). Recruitment of Mdm2-p53 complexes to p53 responsive promoters results in their transcriptional repression (Ohkubo et al., 2006). Acetylation of p53 can prevent Mdm2 interaction with p53 at the p21 promoter and relieve its repressive effects. These multiple modes of Mdm2 mediated repression of p53 has led to the development of novel small molecule inhibitors that disrupt their interaction such as Nutlin-3 (Vassilev, 2007).

Activated p53 can potentiate cell cycle arrest and apoptosis by inducing transcription of numerous cell cycle regulators and proapoptotic genes such as p21, Bax, DR5 and Puma as well as transcriptional repression of antiapoptotic proteins Bcl-2 and Mcl-1 (el-Deiry, 1998; Fridman and Lowe, 2003; Miyashita et al., 1994; Pietrzak and Puzianowska-Kuznicka, 2008; Yu et al., 2001). Histone acetyltransferases (HATs) such as p300/CBP or PCAF and histone deacetylases (HDACs) such as HDAC1, 2, 3 or SIRT1 regulate the acetylation of p53 at specific lysine residues (Gu and Roeder, 1997; Luo et al., 2000; Sakaguchi et al., 1998; Vaziri et al., 2001). The amount and specificity of p53 acetylation is regulated by diverse mechanisms in response to various cellular stresses (Luo et al., 2004; Sakaguchi et al., 1998). Acetylation of p53 enhances its DNA

binding activity and stability and may be responsible for determining the specific context in which p53 will function (Gu and Roeder, 1997; Luo et al., 2004). HDACi-induced hyperacetylation of p53 results in p53 transactivation-dependent apoptosis (Henderson et al., 2003; Roy et al., 2005; Terui et al., 2003).

Transcription-Independent p53 Apoptosis

In addition to its function as a transcription factor, p53 promotes cell death through transcription-independent mechanisms as it can cause apoptosis in the presence of transcription and translation inhibitors (Caelles et al., 1994). In addition, p53 induces transcription-independent apoptosis through increasing Fas cell surface translocation and activation of caspase-8 (Bennett et al., 1998; Ding et al., 2000). Interestingly, mutant p53 lacking transcriptional activity is fully capable of inducing apoptosis (Bissonnette et al., 1997; Chipuk et al., 2003; Haupt et al., 1995; Kokontis et al., 2001; Yamaguchi et al., 2004).

The best characterized transcription-independent apoptotic function of p53 involves the intrinsic mitochondrial apoptotic pathway. Although normally a cytoplasmic protein, cellular stresses promote wild type p53 accumulation in the cytoplasm and mitochondria where it induces mitochondrial outer membrane permeabilization through displacement of BH3-only proteins from Bcl-2 and Bcl-XL, promoting the oligomerization of Bak and the activation of Bax (Chipuk et al., 2003; Dumont et al., 2003; Erster et al., 2004; Leu et al., 2004; Pietsch et al., 2007). Furthermore, p53 may directly interact with Bax and promote its activation (Chipuk et al., 2004). Conversely, unlike their wild type counterparts, some naturally occurring DNA-binding domain

mutants of p53 lack the capacity to bind Bcl-XL and promote cytochrome c release at the mitochondria (Mihara et al., 2003; Tomita et al., 2006). This leaves open the possibility that mutant p53 may promote apoptosis in a transcription-independent manner other than its previously described role at the mitochondria. It is increasingly apparent that p53 has a dual role in the initiation of apoptosis through both transcription-dependent and independent mechanisms. Transcription-independent binding to Bcl-XL and displacement of Bax can combine with transcription-dependent upregulation of Puma which can directly activate Bax.

In this report, we provide evidence that HDACi induce apoptosis through a p53 acetylation-dependent but transactivation-independent mechanism. Expression of transactivation deficient p53 mutants in p53-null HCT116, H1299, K562, and MEF cells enhances apoptosis initiated by HDACi. Conversely, knockdown of endogenous mutant p53 in HT-29 and SW480 cells abrogates SAHA or LAQ824 induced apoptosis. Purification of p53 binding proteins and mass spectrometry analysis revealed a novel interaction between p53 and Ku70. This interaction is independent of p53 acetylation. However, acetylation at its carboxy-terminus is required for p53 to prevent and/or displace Bax from its inhibitory interaction with Ku70, thus allowing this key proapoptotic member of the Bcl-2 family to target mitochondria, generate ROS, and initiate apoptosis in response to HDACi. Furthermore, knockdown of Ku70 promotes apoptosis in p53-null but not p53 mutant cells treated with HDACi. These results highlight a novel mechanism by which acetylated p53 restrains the Bax/Ku70 interaction to potentiate HDACi-induced apoptosis.

Chapter Two: Src Mediated Suppression of Anoikis

Abstract

Anoikis, a Bax dependent apoptosis triggered by detachment from the extracellular matrix, is often dysfunctional in metastatic cancer cells. Using wild type and c-Src transformed NIH3T3 cells as a model we identified Mcl-1 degradation and Bim upregulation as a critical determinant of anoikis initiation. Detachment rapidly degraded Mcl-1 via a GSK-3 β -dependent proteasomal pathway and transcriptionally upregulated Bim expression. Mcl-1 degradation in the presence of Bim was sufficient to induce anoikis. By analyzing non-metastatic Saos-2 and metastatic derivative LM7 cells, we confirmed that dysregulation of Mcl-1 degradation and Bim induction during detachment contributes to decreased anoikis sensitivity of metastatic cells. Furthermore, knockdown of Mcl-1 or pharmacological inhibition of the PI3-K/Akt and MAPK pathways that suppress Mcl-1 degradation and Bim expression could markedly sensitize metastatic breast cancer cells to anoikis and prevent metastases *in vivo*. Therefore, Mcl-1 degradation primes the cell for Bax activation and anoikis, which can be blocked by oncogenic signaling in metastatic cells.

Results

Src Signaling Ablates the Anoikis Response due to an Inhibition of Bax Activation

The anoikis response is known to be inhibited in cells expressing the Src oncogene (Windham et al., 2002), but the mechanisms involved are poorly understood.

To determine the characteristics of the anoikis response, parental and Src transformed NIH3T3 cells were forcibly detached on polyHEMA coated plates for varying lengths of time. Wild type cells were observed to rapidly loose viability in a time dependent manner while cells with active c-Src 527F and v-Src exhibited limited cell death as determined by a trypan blue exclusion assay (Figure 5A). The loss of viability in wild type cells correlated to the activation of caspase-3 as measured by the DEVDase activity (Figure 5B).

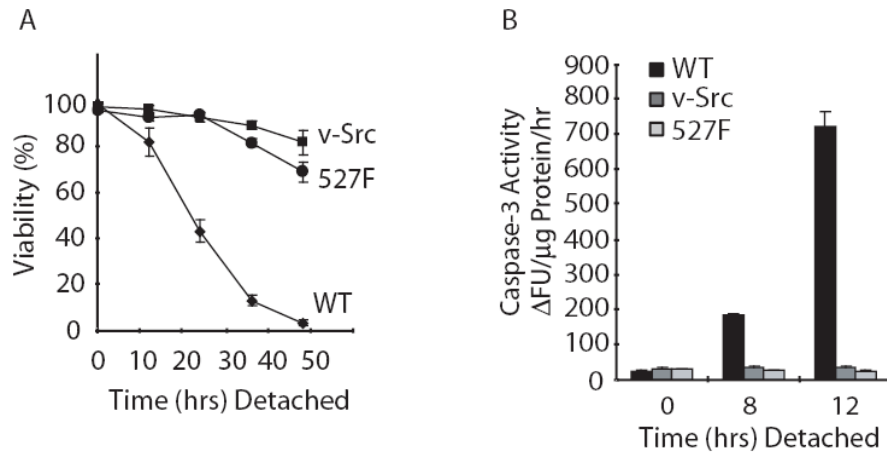


Figure 5. Active Src prevents anoikis. (A) Wild type, c-Src 527F, and v-Src NIH3T3 cells were detached on poly-HEMA coated plates and assayed for viability at the indicated timepoints by trypan blue exclusion assay. (B) Activity of caspase-3 in the aforementioned cell lines after 0, 8, and 12 hours of detachment. Data represented as mean \pm SD, n=3.

The activation of Bax is known to be an initiating event in the activation of the caspase cascade during anoikis (Valentijn et al., 2003). Therefore, we assessed the activation status of Bax by immunoprecipitation (Figure 6) with the Bax 6A7 monoclonal antibody and immunostaining (Figure 7) with the Bax N20 polyclonal antibody. Both of

these antibodies specifically recognize conformationally active Bax. Figure 6 illustrates that Bax activation is completely inhibited in 527F and v-Src cells while wild type cells exhibit a time dependent increase in the conformationally changed Bax protein beginning as early as 8 hours post detachment. Similarly, the immunofluorescence staining in Figure 7 demonstrates that Bax becomes active in wild type cells but not 527F expressing cells in response to cell detachment. However, treatment with the Src family kinase inhibitor dasatinib clearly restored detachment-induced Bax activation in 527F cells (Figure 7A & B).

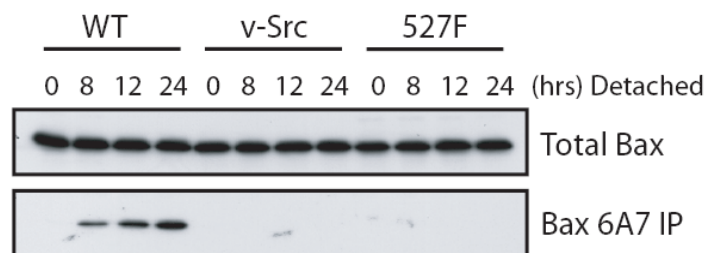


Figure 6. Active Src prevents detachment-induced Bax activation. Wild type, v-Src, and c-Src 527F cells were forcibly detached on poly-HEMA coated plates for 0, 8, 12, and 24 hours. Active Bax was immunoprecipitated using the conformation specific Bax 6A7 antibody.

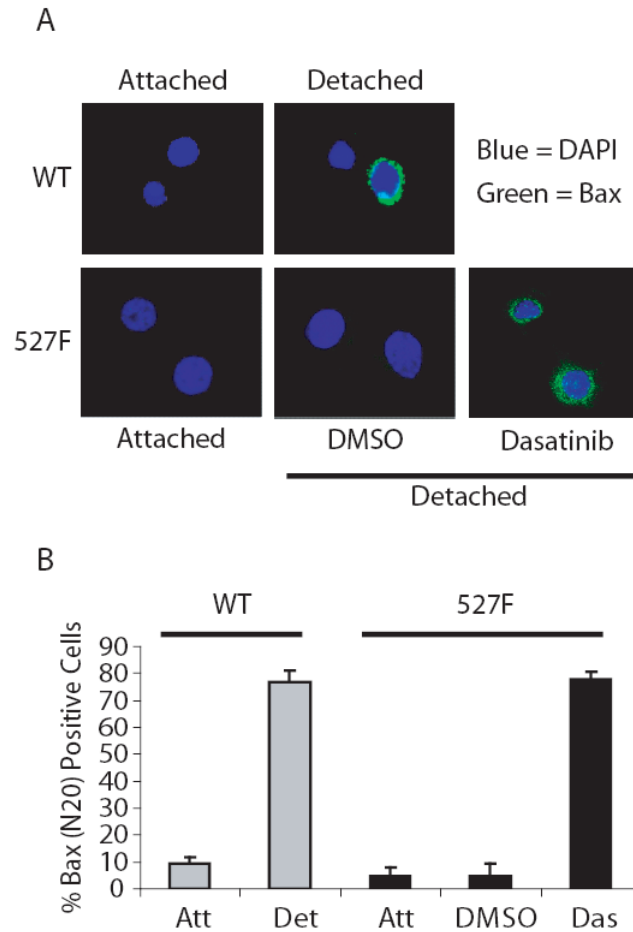


Figure 7. Src inhibition restores Bax activation in response to detachment. (A) NIH3T3 wild type and c-Src 527F cells were immunostained for active Bax in attached and 8 hours detached samples using the conformation specific Bax N20 antibody. c-Src 527F cells were treated with control DMSO or 50 nM dasatinib during detachment. (B) Quantification of the percentage of cells staining positive for active Bax; experiment represented as average \pm SD, n=3. Att, attached. Det, detached. Das, dasatinib.

Mcl-1 and Bim are Critical Regulators of Anoikis

To determine the role of protein neogenesis in the activation of Bax during anoikis, cycloheximide (CHX) was used to block *de novo* protein synthesis in both wild type and 527F cells detached on polyHEMA. CHX markedly reduced detachment-

induced Bax activation and cellular caspase-3 activity in wild type cells as well as 527F cells treated with dasatinib (Figure 8A & B). This prompted us to examine the gene expression profiles associated with anoikis in 527F cells treated with or without dasatinib by microarray (Figure 9). There was a clear increase in the induction of Bim and Puma, both known activators of Bax. There was also a slight decrease in transcription of the antiapoptotic proteins Mcl-1 and Bcl-XL. Members of the caspase family were generally unchanged; however, their involvement in Bax activation was ruled out through the use of the pan caspase inhibitor z-VAD-fmk which was unable to inhibit Bax conformational change during detachment (Figure 10).

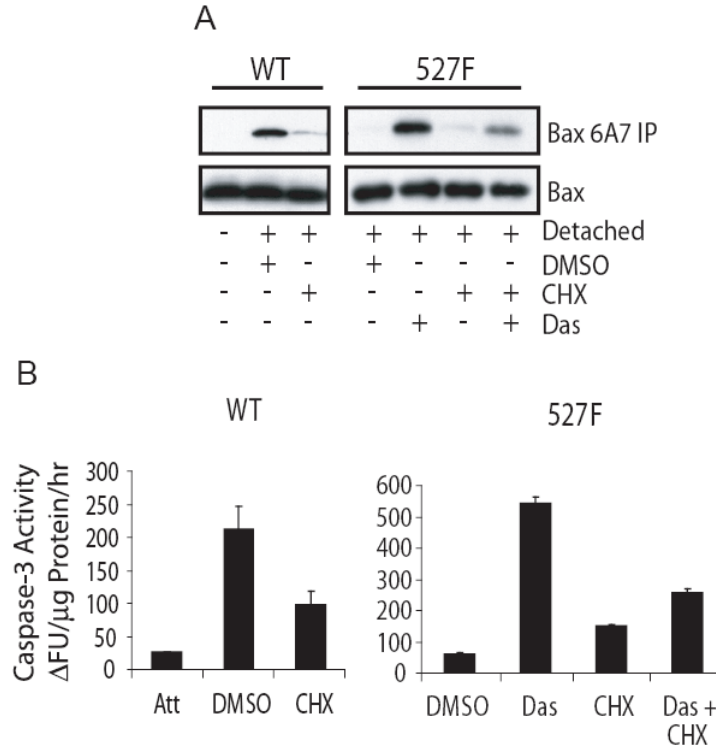


Figure 8. *De novo* protein synthesis promotes anoikis. Wild type and c-Src 527F NIH3T3 cells were cultured on polyHEMA-coated plates containing DMSO control or 50 nM dasatinib, 10 μg/mL CHX or the combination of dasatinib and CHX for 8 hours and subjected to (A) immunoprecipitation with anti-Bax 6A7 antibody and (B) caspase-3 activity assay; mean \pm SD, n=3.

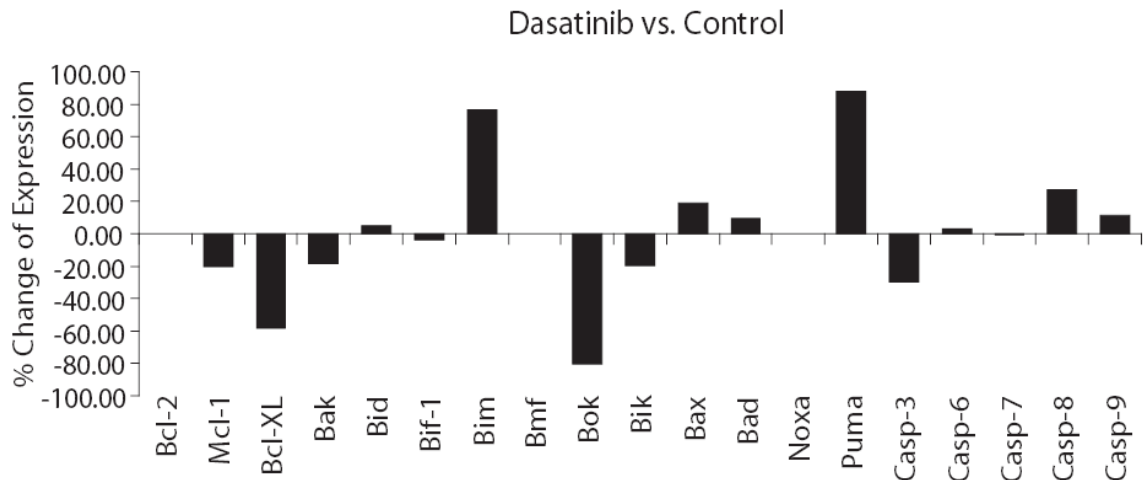


Figure 9. Detachment induced changes in the microarray profile of apoptosis related genes. NIH3T3 cells expressing c-Src 527F were detached on polyHEMA-coated plates in the presence of DMSO or 50 nM dasatinib for 8 hours and subjected to microarray analysis. The bar graph shows the up- and down-regulation of apoptosis genes by dasatinib compared to DMSO control.

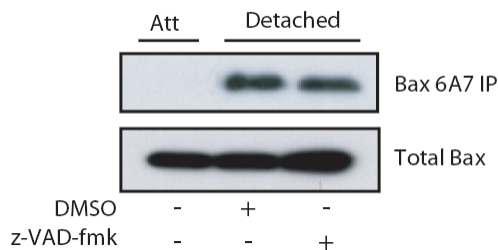


Figure 10. Caspase activity is not required for detachment induced Bax activation. NIH3T3 wild type cells were assayed for conformationally active Bax in control attached samples and 8 hours detached conditions in the presence of either DMSO control or 25 μ M z-VAD-fmk.

The microarray analysis allowed us to form a short list of Bcl-2 family proteins that are differentially transcribed between anoikis responsive and unresponsive cells.

However, the prevalence or absence of transcripts does not always coincide with the

expression of the protein product. To this end, we examined the protein expression profiles of BimEL, Puma, Bcl-XL, Mcl-1 and Bax in 527F cells detached and treated with dasatinib or DMSO and compared them to wild type cells (Figure 11). Several reports have suggested that Bcl-XL is induced as the result of Src signaling and that this provides resistance to anoikis (Coll et al., 2002; Rosen et al., 2001). However, we observed little or no decrease in Bcl-XL at the time of Bax activation in either dasatinib treated 527F or wild type cells. Similarly, there was no significant increase in the protein levels of Bax in either of the two cell types. We did find that Mcl-1 and Bim were the most dynamically regulated proteins analyzed. In particular, Mcl-1 expression was substantially reduced while Bim was increased during anoikis; this response was also found in detached 527F cells treated with dasatinib indicating the relevance to a restored anoikis response by Src inhibition. Puma was moderately induced in dasatinib treated 527F but not in wild type cells, suggesting that Puma may not be a key regulator of anoikis.

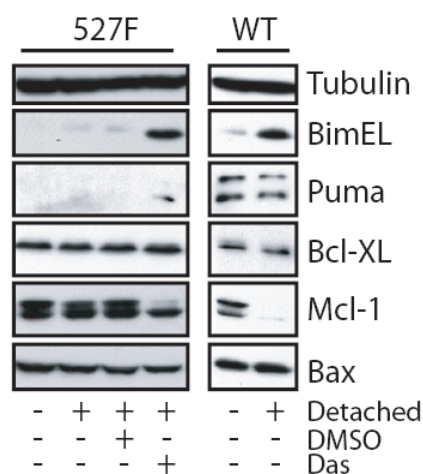


Figure 11. Bcl-2 family protein expression during anoikis. Wild type and c-Src 527F

NIH3T3 cells were cultured in normal conditions or detached on polyHEMA-coated plates with DMSO or 50 nM dasatinib (Das) for 8 hours and subjected to western blot

The results from the microarray in relation to Mcl-1 and Bim were validated using semi-quantitative RT-PCR in samples of wild type and 527F cells (Figure 12).

Transcripts of Mcl-1 were marginally decreased in dasatinib treated 527F cells.

Interestingly, there did not appear to be any decrease in the transcription of Mcl-1 in the wild type samples, indicating that post-transcriptional regulation was responsible for the observed decrease in protein levels. Contrastingly, Bim transcripts were increased dramatically by detachment in wild type and dasatinib treated 527F cells. Bim transcription is known to be positively regulated through the transcription factor Foxo3a which is negatively regulated by Akt (Gilley et al., 2003). Therefore, the ability of Akt signaling in 527F cells to inhibit Bim expression was assessed through the use of LY294002, a PI3-K inhibitor. Indeed, this inhibitor restored Bim induction, indicating that the Src/Akt/Foxo3a pathway is likely involved in the transcriptional suppression of this proapoptotic protein.

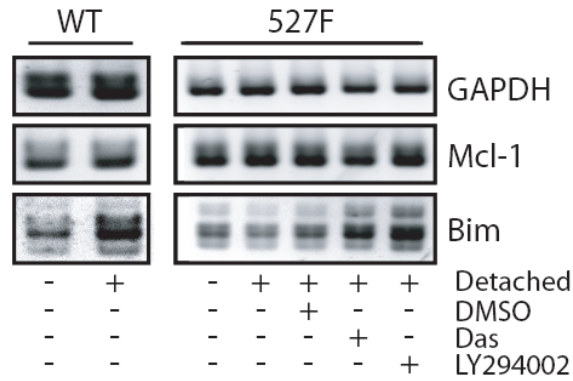


Figure 12. Semi-quantitative RT-PCR analysis of Mcl-1 and Bim in response to anoikis. Wild type and c-Src 527F NIH3T3 cells were cultured in normal conditions or detached on polyHEMA-coated plates with DMSO, 50 nM dasatinib (Das), or 50 μ M LY294002 for 8 hours and subjected to semi-quantitative RT-PCR analysis.

Mcl-1 and Bim Regulate Detachment Induced Bax Activation

The functional importance of increased Bim expression in the anoikis response was assessed by shRNA mediated targeted knockdown. Both wild type and 527F cells were infected with Bim shRNA (shBim) or control retrovirus. Stable pools were detached on polyHEMA plates and their ability to initiate Bax activation and anoikis was assayed. Decreased Bim expression led to a similar decrease in the activation of Bax (Figure 13A) as well as the caspase-3 activity (Figure 13B) in cells cultured on polyHEMA plates. These results suggest that Bim is the major activator of Bax during anoikis, and the Puma induction seen in Figure 7 is of little functional relevance.

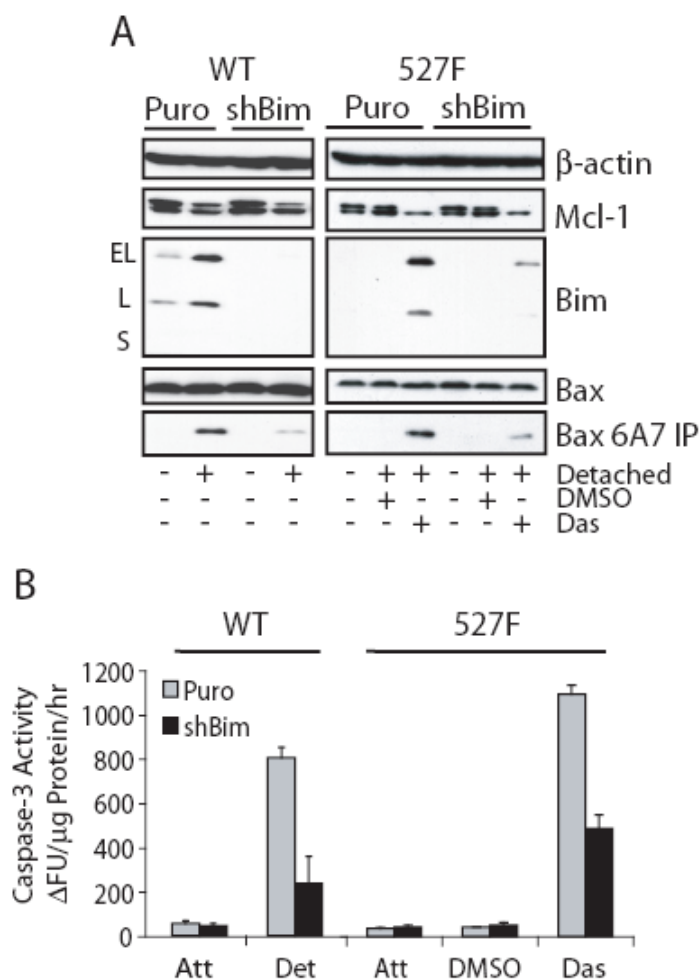


Figure 13. Knockdown of Bim prevents Bax activation in response to detachment. Wild type and c-Src 527F cells were infected with control (Puro) or Bim shRNA (shBim) retroviruses and selected for 14 days on puromycin. The resulting puromycin-resistant transfectants were maintained in normal culture (Att) or detached (Det) on polyHEMA-coated plates with DMSO or 50 nM dasatinib for 8 hours and subjected to (A) immunoprecipitation with anti-Bax 6A7 antibody and (B) assayed for caspase-3 activity; mean \pm SD, n=3.

To test the functional significance of Mcl-1 repression during detachment, increasing amounts of the Mcl-1 construct were transfected into wild type cells along with the pGL3 Luciferase reporter construct. By measuring luciferase activity, we found that overexpression of Mcl-1 led to a dose dependent suppression of cell death in

detached as compared to attached conditions (Figure 14A). Bim alone was able to induce an apoptotic response in 527F cells as transfection of increasing amounts of the Bim construct led to a dose dependent decrease in cell viability (Figure 14B). This demonstrates that decreased Mcl-1 expression is vital to the induction of anoikis and that overexpression of Bim can induce apoptosis in 527F expressing cells, presumably through a mechanism that activates Bax once antiapoptotic Bcl-2 members are saturated by Bim.

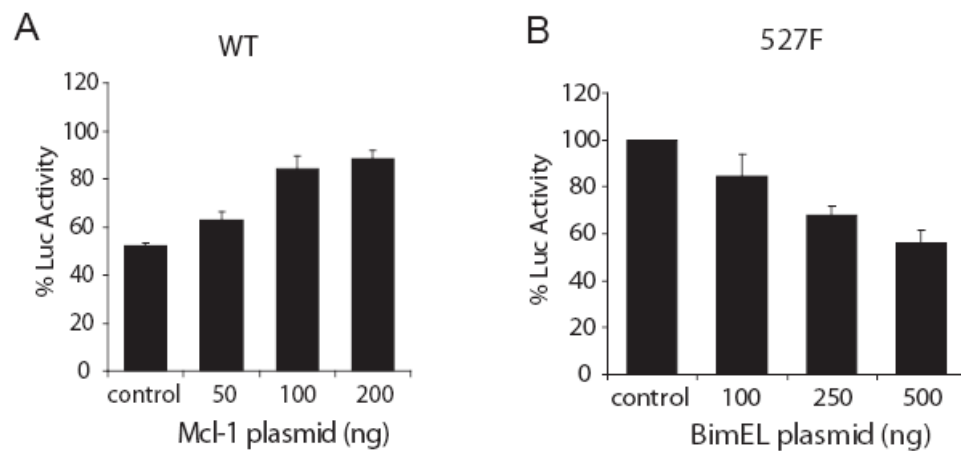


Figure 14. Overexpression of Mcl-1 or Bim alters the anoikis response. (A) Wild type (WT) and (B) Src 527F NIH3T3 cells were co-transfected with increasing amounts of Mcl-1 or Bim expression plasmids, respectively, along with 10 ng pGL3-actin luciferase reporter plasmid. Luciferase expression was assayed as a measure of cell viability; mean \pm SD, n=3.

Anoikis is Regulated by Proteasomal Degradation of Mcl-1

Mcl-1 is an ephemeral protein that is degraded in a proteasome dependent manner. This regulatory mechanism allowed us to study if stabilized endogenous Mcl-1

contributes to inhibition of anoikis. As shown in Figure 15, the proteasome inhibitor MG132 was able to stabilize Mcl-1 in wild type NIH3T3 cells detached on polyHEMA in a dose dependent manner with maximal stabilization occurring at 100 and 1000 nM. This stabilization of Mcl-1 correlated with a similar dose dependent decrease in Bax activation. We also observed the stabilization of Mcl-1 and the complete inhibition of Bax activation in detached 527F cells treated with the combination of dasatinib and MG132 (Figure 15). The addition of MG132 to the lysate of detached wild type cells had no effect on the ability to immunoprecipitate active Bax (data not shown).

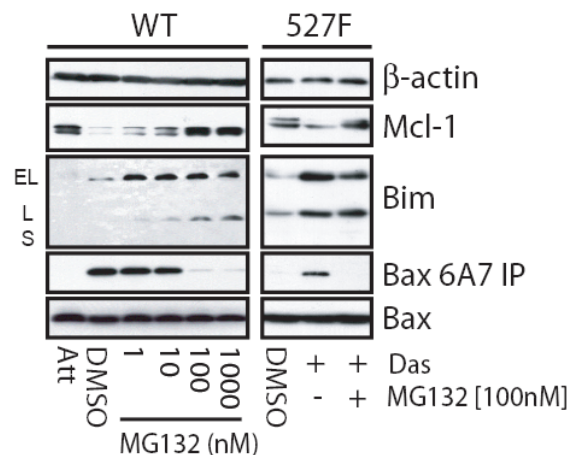


Figure 15. Proteasome inhibition prevents detachment-induced Bax activation. Wild type NIH3T3 cells were maintained as attached on normal plates or detached on polyHEMA-coated plates containing DMSO or increasing amounts of MG132. c-Src 527F NIH3T3 cells were detached and treated with DMSO or 50 nM of dasatinib with or without 100 nM of MG132 for 8 hours. Cells were then subjected to anti-Bax 6A7 immunoprecipitation/immunoblot analysis.

Samples from the wild type cells detached and exposed to the MG132 gradient were also assayed for chymotrypsin-like activity and caspase-3 activity through *in vitro*

protease assays. In concordance with the above results, caspase-3 activity was decreased similar to the chymotrypsin activity inhibited by MG132 (Figure 16). The repression of Bax activation and anoikis in wild type NIH3T3 cells was also observed in a similar manner using Velcade, a proteasome inhibitor currently in clinical trials (Figure 17). This study identifies the ability of proteasome inhibitors to prevent Bax activation and anoikis in response to detachment.

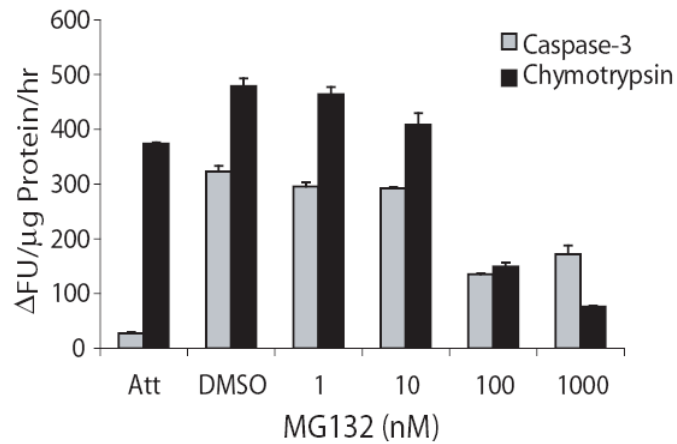


Figure 16. MG132 mediated proteasome inhibition represses detachment-induced caspase-3 activation. Wild type NIH3T3 cells were collected in normal attached conditions (Att) or after 8 hours of forced detachment with treatment of DMSO control or increasing amounts of MG132. Chymotrypsin activity was assessed to determine the inhibition of the proteasome mediated by MG132 and caspase-3 assay was performed to analyze apoptotic induction.

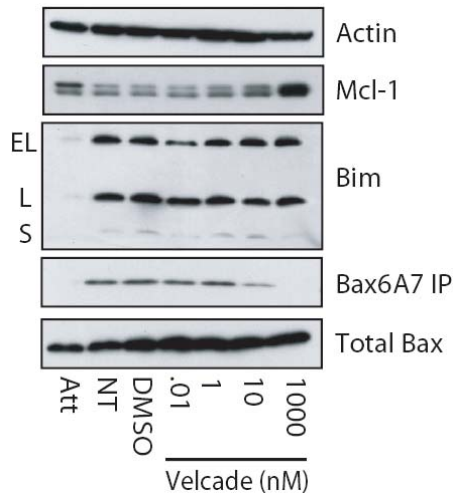


Figure 17. Velcade-mediated proteasome inhibition stabilizes Mcl-1 and prevents detachment-induced Bax activation. Wild type NIH3T3 cells were assayed for Mcl-1, Bim, and Bax expression and the activation of Bax in attached (Att) and 8 hours detached cells that were untreated (NT) or incubated with DMSO or the indicated concentration of Velcade.

To determine whether MG132 mediated inhibition of Bax activation was due to the stabilization of Mcl-1 but not another unidentified protein(s), retroviral shRNA targeting Mcl-1 was infected into wild type cells to decrease its accumulation upon MG132 treatment. Three shRNA constructs were designed, of which constructs 1 and 3 (c-1 and c-3) were the most effective at inhibiting Mcl-1 expression (Figure 18A). Treatment with MG132 led to the nearly complete inhibition of Bax activation in the Puro control line, but was less effective in suppressing the Bax conformational change when there was less Mcl-1 stabilized. An inverse correlation was found between the amount of Mcl-1 accumulated in MG132 treated samples and the activation of Bax. Similarly, there is decreased repression by MG132 on caspase-3 activation in Mcl-1 knockdown cells compared to control (Figure 18B). These observations strongly suggest that anoikis is dependent on the proteasomal depletion of Mcl-1.

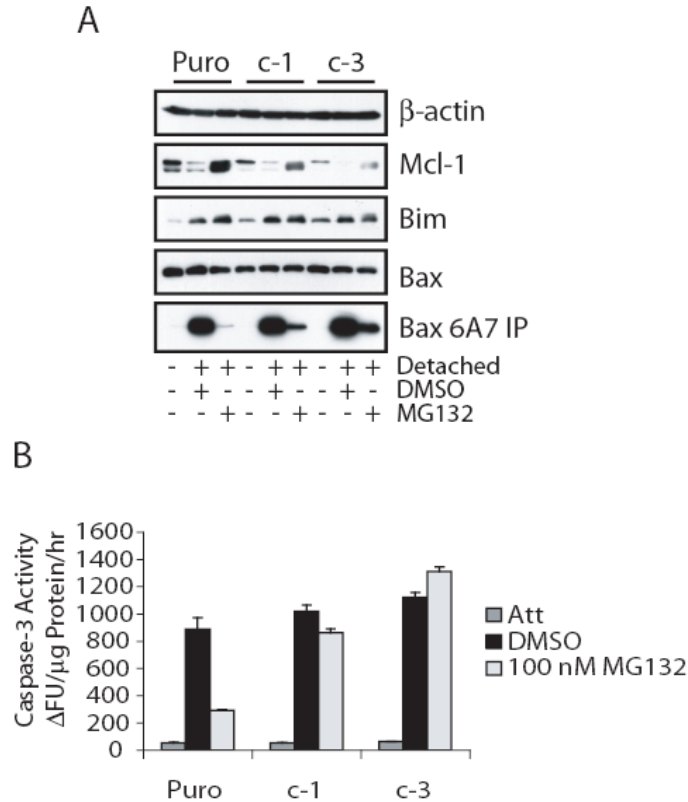


Figure 18. Proteasome mediated suppression of anoikis is mediated by the accumulation of Mcl-1. NIH3T3 cells stably expressing Mcl-1 shRNA (c-1 or c-3) were left in normal culture (control) or detached on polyHEMA-coated plates in the presence of DMSO or 100 nM MG132 for 8 hours and subjected to (A) anti-Bax 6A7 immunoprecipitation/immunoblot analysis and (B) caspase-3 assay.

Mcl-1 Degradation Elicits a Robust Anoikis Response

Thus far it has been evident that both Mcl-1 degradation and Bim induction are key regulators of the normal anoikis response. While overexpression of Mcl-1 can undoubtedly protect cells from anoikis (Figure 18), other groups have shown that overexpression of other antiapoptotic Bcl-2 family proteins can also have the same effect (Rosen et al., 2000). Therefore, the vitality of Mcl-1 degradation for anoikis induction

was evaluated using retrovirally transduced NIH3T3 cells co-expressing Bcl-2, Bcl-XL, or Mcl-1 with the proapoptotic BimEL counterpart via a bicistronic mRNA construct (Figure 19A). Repression of protein translation using CHX illustrates that the instability of Mcl-1 is essential for the induction of anoikis, while the co-expression of Bcl-2:Bim or Bcl-XL:Bim had no effect on cell death due to the maintenance of the anti- and proapoptotic balance (Figure 19A & B). The anoikis response to Mcl-1 degradation could be completely resolved by MG132 mediated proteasome inhibition (Figure 20A & B). We confirmed that releasing Bim from the Bcl-XL:Bim complex by ABT-737, a specific Bcl-2/Bcl-XL inhibitor, was also capable of inducing an apoptotic response (Figure 20B). The uniquely short half-life of Mcl-1, compared to other antiapoptotic proteins such as Bcl-2 and Bcl-XL, makes this an essential element of anoikis initiation.

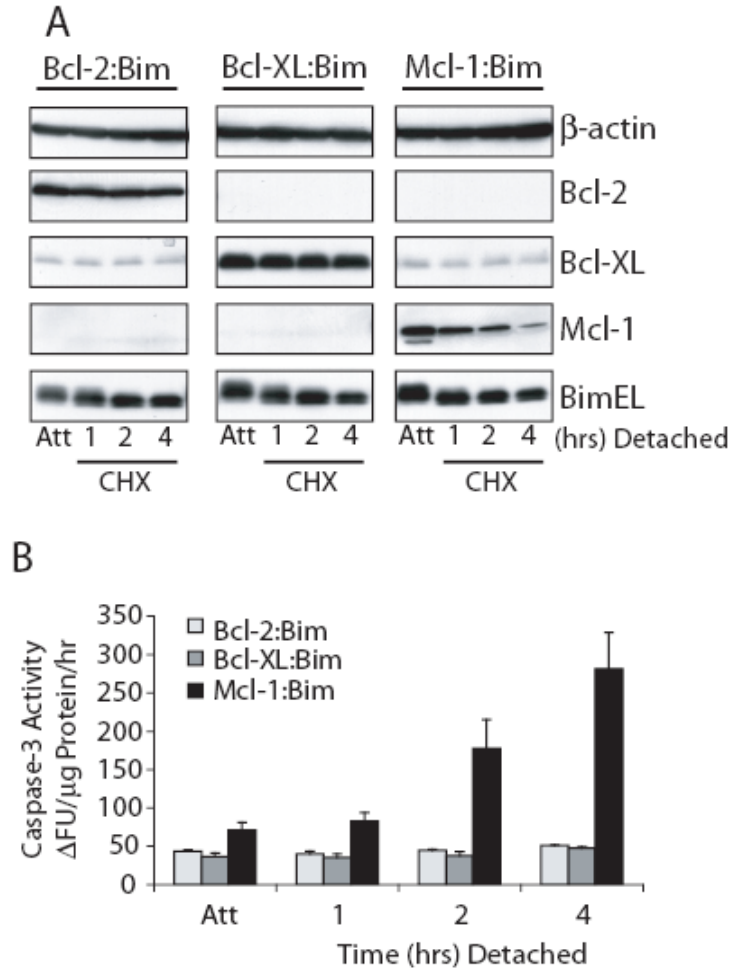


Figure 19. Mcl-1 degradation alone is sufficient to induce anoikis in the presence of Bim. NIH3T3 cells were retrovirally transduced with constructs expressing Bcl-2-IRES-Bim, Bcl-XL-IRES-Bim, or Mcl-1-IRES-Bim. Cells were untreated or pretreated with 10 μ M CHX for 30 minutes then detached for the indicated times in the presence of CHX and analyzed for (A) expression of the proteins by immunoblot and (B) caspase-3 activation; mean \pm SD, n=3.

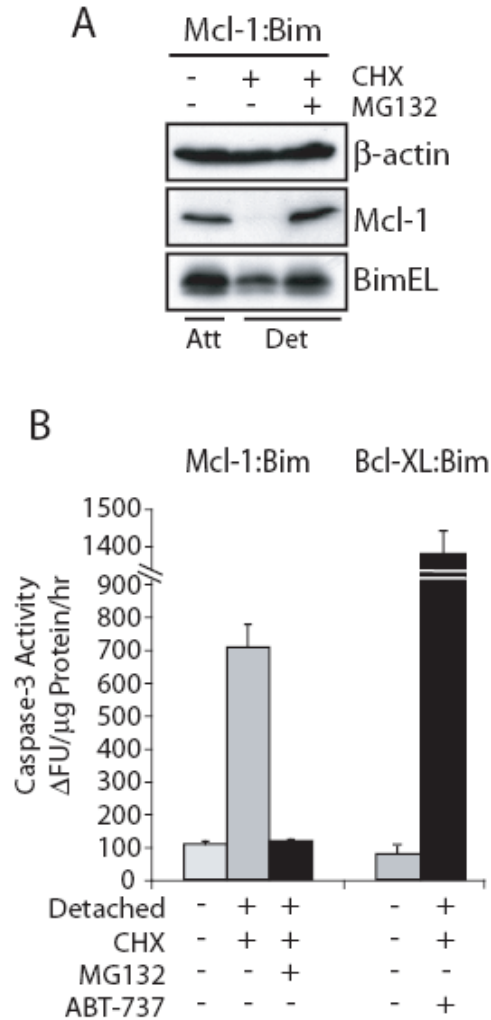


Figure 20. Anoikis in Mcl-1-IRES-Bim cells induced by CHX can be prevented by proteasome inhibition. NIH3T3 cells expressing Mcl-1-IRES-Bim or Bcl-XL-IRES-Bim were untreated or pretreated for 30 minutes with CHX or CHX and MG132 then detached for 5 hours in the presence of their respective inhibitors and analyzed for (A) protein expression and (B) caspase-3 activation; mean \pm SD, n=3.

Mcl-1 Degradation is Mediated by GSK-3 β

The phosphorylation of Mcl-1 by GSK-3 β at Ser159 is known to promote an increased turnover of Mcl-1 (Maurer et al., 2006). To determine if this phosphorylation controls Mcl-1 degradation during anoikis, the GSK-3 β inhibitor TDZD-8, a non-ATP

competitive inhibitor, was used to treat cells beginning immediately with detachment. As shown in Figure 21A, TDZD-8 was able to stabilize Mcl-1 in both wild type and 527F cells when detached or sensitized with dasatinib, respectively. This stabilization was once again associated with a decrease in Bax activation and inhibition of the apoptotic response (Figure 21A & B). To further validate the role of GSK-3 β in the degradation of Mcl-1 induced by detachment, wild type and GSK-3 β knockout MEFs were either attached or detached on polyHEMA plates for 12 hours in the presence of DMSO or LY294002. Detached wild type MEFs showed a decrease in the protein levels of Mcl-1 as did treatment with LY294002 (Figure 22). However, the GSK-3 β null cells did not exhibit a decrease in Mcl-1 protein levels in response to detachment, and treatment with LY294002 resulted in only a slight decrease of Mcl-1; this decrease is likely due to the functional redundancy of GSK-3 α .

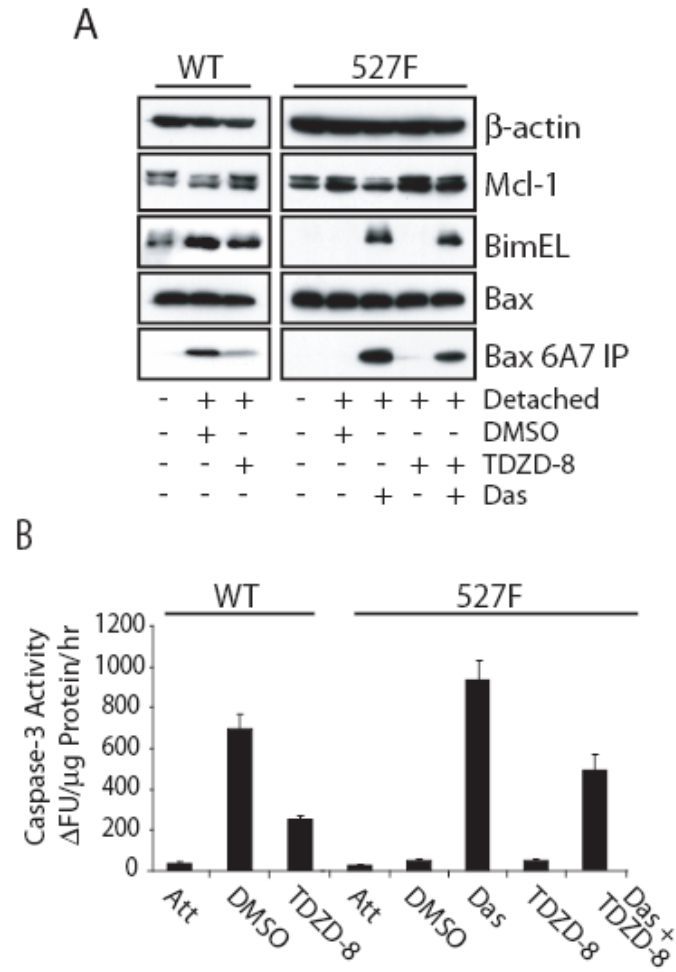


Figure 21. Inhibition of GSK-3 β signaling stabilizes Mcl-1 and prevents Bax activation and anoikis. Wild type and c-Src 527F 3T3 cells were maintained in normal culture or detached on polyHEMA-coated plates containing DMSO, 25 μ M TDZD-8, 50 nM dasatinib, or both inhibitors for 8 hours and subjected to (A) anti-Bax 6A7 immunoprecipitation/immunoblot and (B) caspase-3 activity assays; mean \pm SD, n=3.

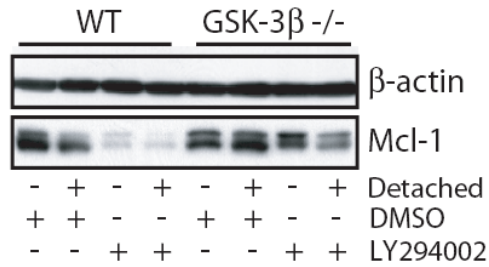


Figure 22. Knockout of GSK-3 β prevents detachment-induced Mcl-1 degradation. Wild type and GSK-3 β null MEFs were treated with DMSO or 25 μ M LY294002 in normal or forced detachments conditions for 12 hours and Mcl-1 expression was determined by western blot.

To determine if phosphorylation of Ser159 is required for Mcl-1 degradation during anoikis, constructs encoding the human Mcl-1 wild type and S159A mutant were transfected into wild type NIH3T3 cells. These cells were then detached and treated with CHX to observe the degradation of Mcl-1. Figure 23 shows that the half-life of wild type Mcl-1 is very short when compared to that of the S159A mutant. This suggests that Mcl-1 is degraded in response to a phosphorylation priming event that in turn targets the protein for ubiquitination and degradation. Moreover, detachment was found to decrease the levels of phospho-Akt in wild type NIH3T3 cells (data not shown), which may explain the increased degradation of Mcl-1 regulated by GSK-3 β .

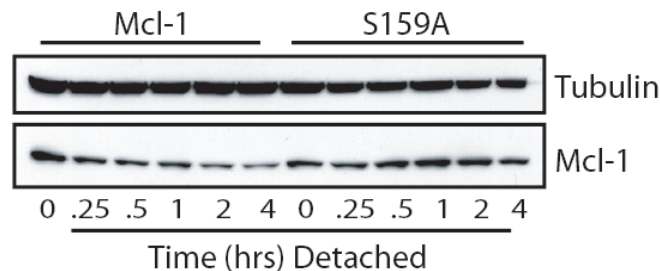


Figure 23. Mutation of the GSK-3 β phosphorylation site on Mcl-1 prevents detachment-induced degradation. NIH3T3 cells transfected with wild type or S159A mutant Mcl-1 were detached on polyHEMA plates for the

indicated times in the presence of CHX to determine the half-life of Mcl-1 during detachment by immunoblot analysis.

Src Regulated Akt and Erk Signaling Control Mcl-1 and Bim Expression

Src is known to promote survival signaling through multiple pathways. Two of the most well defined pathways controlled by Src are Akt and Erk, both of which have been implicated in preventing anoikis but debate remains over their involvement (Fukazawa et al., 2004; Martin et al., 2006). We therefore explored the consequences of Src, Akt, and Erk inhibition on Mcl-1 and Bim protein levels in 527F cells treated with dasatinib, LY294002, U0126, or the combination of LY294002 and U0126 during detachment (Figure 24A). Dasatinib inhibited signaling through both Akt and Erk, while treatment with LY294002 or U0126 specifically inhibited their target's pathways. Inhibition of Akt but not Erk1/2 resulted in a significant decrease in Mcl-1 levels but did not extend to the levels observed in dasatinib treated cells. Also, Akt inhibition led to the appearance of multiple bands of Bim that are presumed to be Erk phosphorylated because U0126 inhibited these slower migrating bands and stabilized the Bim protein. Unlike dasatinib, however, treatment with LY294002, U0126 or the combination did not lead to the activation of Bax and caspase-3 (Figure 24A & B).

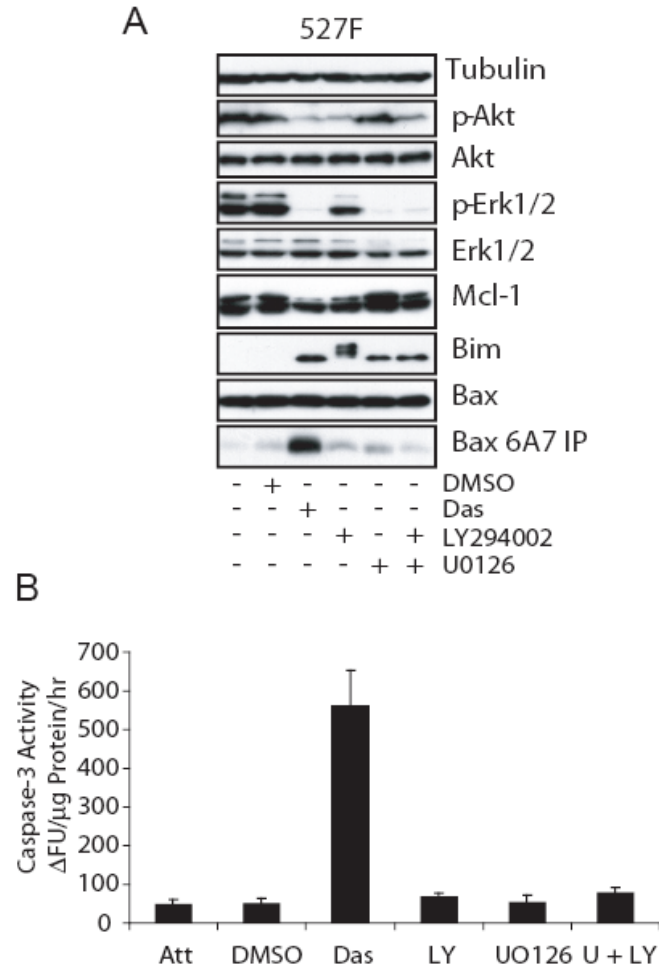


Figure 24. Src signaling through Akt and Erk1/2 controls the expression profiles of Mcl-1 and Bim during detachment. c-Src 527F NIH3T3 cells were cultured in normal conditions (Att) or detached on polyHEMA-coated plates containing DMSO, 50 nM dasatinib, 50 μM LY294002, 10 μM U0126, or the combination of LY294002 and U0126 for 8 hours and subjected to (A) immunoprecipitation/immunoblot analysis with the indicated antibodies and (B) caspase-3 activity assay; mean \pm SD, n=3.

It is possible that decreasing Mcl-1 and increasing Bim expression by LY294002 and/or U0126 could not reach the threshold to shift the anti- and proapoptotic balance of the cell towards anoikis. To test this possibility, we used the c-3 shMcl-1 construct (Figure 18) to knockdown Mcl-1 expression in detached 527F cells treated with

dasatinib, LY294002, U0126, or the combination of LY294002 and U0126. Knockdown of Mcl-1 led to a more anoikis sensitive phenotype in the presence of these kinase inhibitors determined by caspase-3 activity (Figure 25A). Importantly, the activation of Bax was also found to be enhanced in Mcl-1 knockdown cells over that of control (Figure 25B). This not only indicates that the level of Mcl-1 is critical for the initiation of Bax activation in response to Akt and Erk inhibition, but also illustrates the multi-faceted role of Src and implicates other unidentified Src pathways that contribute to anoikis suppression.

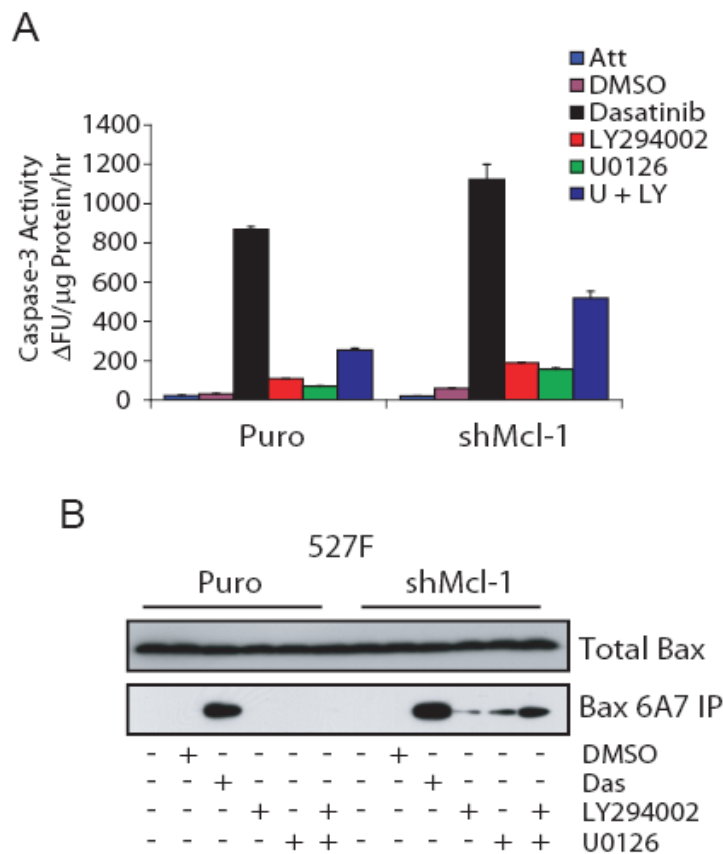


Figure 25. Knockdown of Mcl-1 enhances the anoikis response in c-Src 527F cells treated with kinase inhibitors. c-Src 527F NIH3T3 cells stably expressing Mcl-1 shRNA (shMcl-1) or control vector (Puro) were treated as in Figure 24

and subjected to (A) caspase-3 activity assay and (B) immunoprecipitation with anti-Bax 6A7 antibody; mean \pm SD, n=3.

Metastatic Cancers Exhibit Reduced Mcl-1 Degradation and Bim Induction

Resistance to anoikis is known to enhance the metastatic potential of cancer cells by affording them increased survival potential once detached from the ECM. Using the non-metastatic osteosarcoma cell line Saos-2 and its metastatic derivative LM7 (Jia et al., 1999), we found that parental Saos-2 cells were sensitive to anoikis which correlated with Mcl-1 degradation and Bim induction (Figure 26A & B). However, LM7 cells exhibited increased basal levels of Mcl-1 and maintained this expression during detachment, as well as decreased induction of Bim. Inhibition of Src signaling using dasatinib in both cell lines caused Bim induction and Mcl-1 degradation and increases in caspase-3 activity. The relatively slight increase in dasatinib sensitivity in LM7 cells likely indicates that oncogenic signaling other than Src is responsible for anoikis resistance. To determine if the stabilization of Mcl-1 in LM7 cells was required for their resistance to anoikis, we inhibited Mcl-1 expression using lentiviral delivered shRNA. Knockdown of Mcl-1 was able to restore caspase-3 activation to levels similar to parental Saos-2 cells, while non-targeting scrambled shRNA had no effect on anoikis compared to uninfected LM7 cells (Figure 26C).

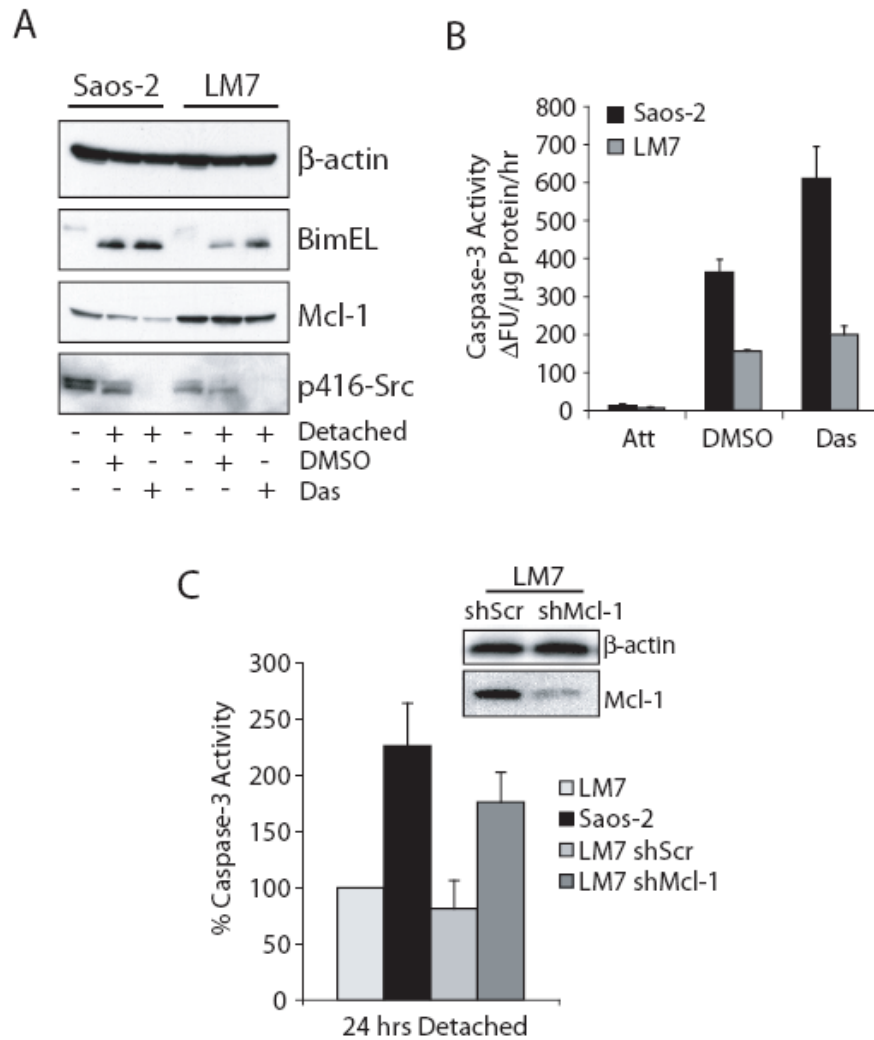


Figure 26. Metastatic progression in osteosarcoma is associated with deregulation of Mcl-1 and Bim. Saos-2 and LM7 cells were untreated or detached on polyHEMA in the presence of DMSO or 50 nM dasatinib for 24 hours. Protein expression profiles were assayed by (A) immunoblot, and (B) apoptotic index was measured by caspase-3 activity. (C) LM7 cells were infected with lentivirus containing control shScr or shMcl-1 constructs. Stably infected cells were analyzed for Mcl-1 expression by immunoblot (insert). The anoikis response of the transfectants was measured by the activation of caspase-3 in response to detachment and compared to the anoikis resistant parental LM7 cells; mean \pm SD, n=3.

Similarly, the highly metastatic breast cancer MDA-MB-231 cells were also

observed to be resistant to anoikis. This was correlated with the maintenance of Mcl-1 expression and repressed Bim induction (Figure 27, lower panel), indicating the dysregulation of the normal response of these proteins to detachment is a contributing factor to metastatic potential. To decipher the signaling pathways involved in the observed expression profiles of Mcl-1 and Bim during detachment, we treated the cells with dasatinib, LY294002, U0126, or the combination of LY294002 and U0126. Inhibition of MEK or PI3-K individually resulted in similar apoptotic indices as measured by caspase-3 activation (Figure 27, upper panel). Similar to results seen in Figure 24A, PI3-K inhibition caused a decrease in Mcl-1 expression, likely through the derepression of GSK-3 β , as well as a limited increase in Bim expression, albeit the Bim protein was highly phosphorylated and likely rapidly turned over (Figure 27, lower panel). Inhibition of MEK with U0126 had no effect on Mcl-1 expression but did confirm an earlier report of increased Bim expression upon Erk1/2 inhibition (Fukazawa et al., 2004). Importantly, the combinational treatment of LY294002 and U0126 enhanced the anoikis response which correlated with Mcl-1 degradation and Bim induction. The failure of dasatinib to sensitize these cells to anoikis is likely the result of independent downstream oncogenic signaling mediated by previously identified mutations in K-RAS and BRAF (Hollestelle et al., 2007) which activate both the PI3-K/Akt and MEK/Erk pathways (Campbell et al., 1998).

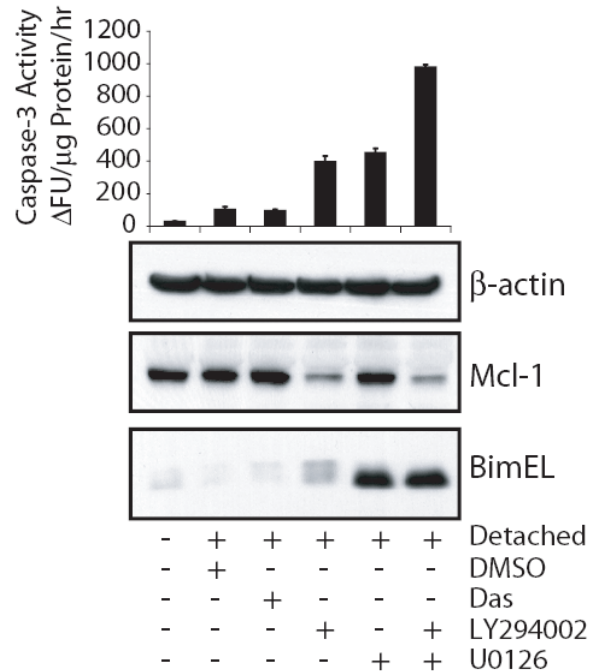


Figure 27. The concerted effects of decreased Mcl-1 and increased BimEL expression correlate with enhanced anoikis response. MDA-MB-231 breast cancer cells were untreated or detached on polyHEMA-coated plates containing DMSO, 50 nM dasatinib, 25 μM LY294002, 10 μM U0126, or the combination of LY294002 and U0126 for 24 hours and subjected to immunoblot analysis with the indicated antibodies (lower panel) and caspase-3 activity assay (upper panel).

To determine if Mcl-1 degradation alone was able to promote anoikis in the MDA-MB-231 cells similar to PI3-K inhibition, we again knocked down Mcl-1 (Figure 28A, insert) and assayed the response to detachment. After only 24 hours of detachment, cells with depleted Mcl-1 displayed markedly increased caspase-3 activity and by 48 hours contained a much higher fraction of dead cells (Figure 28A & B). To further confirm that the loss of Mcl-1 function but not other antiapoptotic Bcl-2 members is essential for initiation of anoikis in metastatic cancer cells, we tested the ability of ABT-737, which targets Bcl-2, Bcl-XL and Bcl-w but not Mcl-1 (Oltersdorf et al., 2005), to

induce apoptosis of detached MDA-MB-231 cells. As expected, treatment with ABT-737 was unable to trigger anoikis in control cells, although it significantly enhanced detachment induced apoptosis of Mcl-1 knockdown cells (Figure 28A & B). Furthermore, *in vivo* experiments demonstrated that knockdown of Mcl-1 alone can significantly reduce tumor establishment of metastatic MDA-MB-231 cells in lungs of nude mice (Figure 29A & B). These results strongly imply that inhibition of Mcl-1 degradation during detachment is vital to the establishment of metastatic cells.

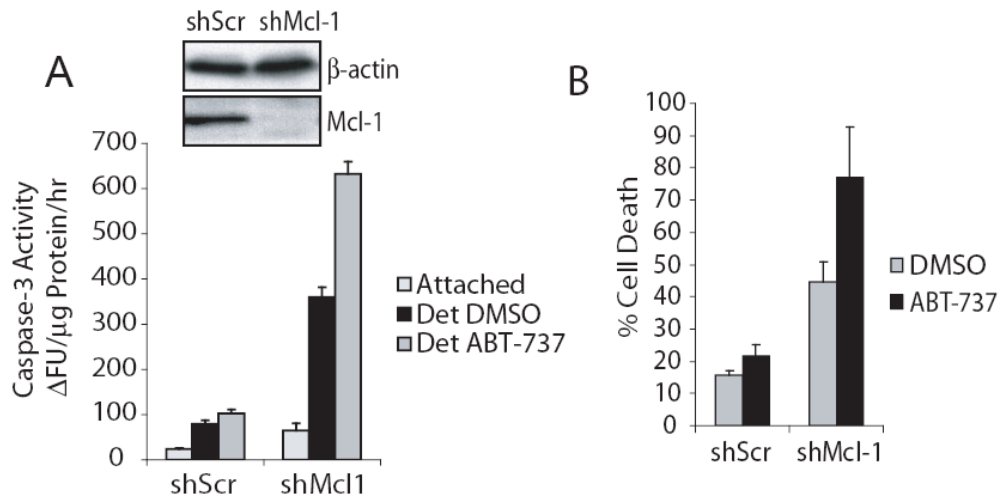


Figure 28. Mcl-1 depletion sensitizes human breast cancer cells to anoikis. MDA-MB-231 cells were infected with lentivirus encoding control shScr or shMcl-1 constructs and selected by 0.5 μ g/mL puromycin for 14 days. The knockdown of Mcl-1 was confirmed by immunoblot (insert). The anoikis response of shScr and shMcl-1 cells cultured in the presence of DMSO or 100 nM ABT-737 was measured by (A) caspase-3 activation and (B) LDH release after 24 or 48 hours detachment respectively; mean \pm SD, n=3.

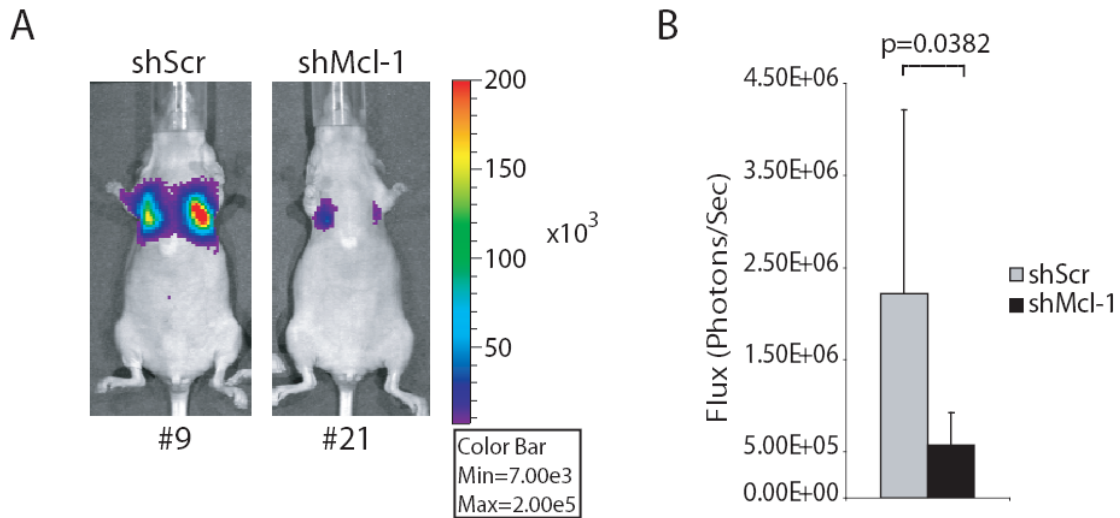


Figure 29. Mcl-1 depletion reduces human breast cancer metastasis *in vivo*. MDA-MB-231 cells constitutively expressing luciferase were infected with shScr or shMcl-1, injected into nude mice and tumor establishment was measured three weeks post-injection. (A) Representative mice and (B) photon emissions were quantified; mean \pm SD, n=9.

Discussion

Despite recent advances in early detection and new therapeutic options for cancer patients, metastatic progression is attributed to 90% of human cancer fatalities (Weigelt et al., 2005). Anoikis is a vital regulatory mechanism that can prevent metastases in a process that requires Bax translocation to mitochondria, which is inhibited by survival kinase signaling found in many human cancers (Gilmore et al., 2000). In spite of our current knowledge of apoptosis, how metastatic cancers escape anoikis remains poorly defined. In this report, we are the first to provide evidence of the unique importance of Mcl-1 degradation in the anoikis response. The Mcl-1 protein rapidly undergoes proteasome-dependent degradation in normal NIH3T3 cells at very early time points after detachment, which allows the subsequently induced Bim to activate Bax. Indeed,

depletion of Mcl-1 alone is not enough to activate Bax under conditions in which the induction of Bim is blocked. Conversely, stabilization of Mcl-1 by proteasome inhibition suppresses Bax activation during anoikis, whereas overexpression of Bim causes dose dependent apoptosis. Together, these results suggest a model for anoikis where Mcl-1 degradation is required as a priming event to sensitize the cell towards Bax activation, which occurs in a manner that is dependent on the BH3-only protein Bim induction. These events are suppressed by active Src, Akt, and Erk1/2 signaling thereby conferring cancer cells with activating mutations in these pathways a survival advantage upon detachment from the ECM.

The ability of cancers to inhibit anoikis is propagated by the inability of these cells to activate Bax after the loss of adhesion. Bim, a known activator of Bax, is suppressed in active Src expressing cells through a mechanism controlled by Akt and Erk1/2 signaling pathways. The transcriptional repression of Bim in 527F cells is likely through the Akt pathway, which is known to phosphorylate the Bim transcription factor Foxo3a and promote its retention in the cytoplasm by interacting with 14-3-3 (Brunet et al., 1999). BimEL is also regulated posttranslationally by phosphorylation of Ser69 by Erk1/2, which promotes its targeting to the proteasome and degradation (Luciano et al., 2003). JNK is also known to control transcription and sequestration of Bim (Lei and Davis, 2003; Whitfield et al., 2001), but upon treatment of 527F cells with JNK inhibitors there are no significant changes in the Bim expression or apoptosis (data not shown).

Although Src can directly phosphorylate STAT3 thereby promoting the expression of Mcl-1 (Bowman et al., 2001), our microarray and semi-quantitative RT-PCR analyses indicate that the Mcl-1 transcripts decrease marginally during anoikis in

dasatinib sensitized 527F but not wild type cells. At posttranslational levels, however, we found that GSK-3 β mediated phosphorylation at Ser159 of Mcl-1 in response to detachment promotes its rapid proteasomal degradation. Akt negatively regulates GSK-3 β by phosphorylation at Ser9 (Cross et al., 1995). Consistently, our studies in 527F and human metastatic cancer cells indicate that Mcl-1 expression is mainly regulated posttranslationally through inhibition of GSK-3 β activity by Akt. However, in 527F cells the lower expression of Mcl-1 and higher sensitivity to anoikis observed when treated with dasatinib compared to LY294002 suggest that other regulatory mechanisms mediated by Src, such as STAT3-dependent transcription, are also involved.

Mcl-1 degradation is required but by itself is insufficient to induce anoikis which requires the subsequent induction of Bim. Once there is an excess of Bim in relation to Mcl-1 the activation of Bax is initiated. These coordinated events determine the anoikis response, which are deregulated in cells with active oncogenic signaling. The comparison of human osteosarcoma cell line Saos-2 and its metastatic derivative LM7 illustrates that stabilization of Mcl-1 during detachment can afford metastatic cells considerable survival advantage. Consistent with this finding, knockdown of Mcl-1 enhances the sensitivity of metastatic LM7 and MDA-MB-231 cells to anoikis and can prevent establishment of metastases. Likewise, repression of the PI3-K/Akt and MAPK signaling pathways that control Mcl-1 stability and Bim expression can resensitize metastatic breast cancer cells to anoikis.

The unique role of Mcl-1 in the anoikis response is likely attributed to its short half-life as its depletion coincides with the commitment of the cell to anoikis. The mechanism of Mcl-1 mediated anoikis inhibition appears to be non-redundant with that of

Bcl-2 or Bcl-XL as ABT-737 is unable to initiate anoikis whereas loss of Mcl-1 does. However, the failure of initiation of anoikis by inhibition of Bcl-2/Bcl-XL does not exclude the role that these antiapoptotic Bcl-2 members play in suppression of anoikis. Indeed, treatment with ABT-737 enhances anoikis initiated by knockdown of Mcl-1 in MDA-MB-231 cells. This finding is consistent with others that show an increased apoptotic response when Mcl-1 inhibition is combined with ABT-737 (Chen et al., 2007). Moreover, there is no significant decrease in the protein levels of Bcl-XL at early timepoints of anoikis where Bax conformational change and caspase activation are observed. Therefore, Bcl-XL overexpression in cancer cells may promote viability by repressing the velocity of the apoptotic insult initiated by Mcl-1 degradation when detached from the ECM. The prolonged viability may give the cells more time to re-attach to the ECM at a distal site or for the accumulation of additive mutations that can disrupt anoikis execution. Due to the unique characteristics of Mcl-1, such as a short half-life, higher affinity to Bim, and verified intricate regulation, we propose that Mcl-1 serves at the convergence point of many resultant signals downstream of detachment from the ECM that mediates the initiation of an anoikis response and the prevention of metastasis.

Materials and Methods

Reagents

Poly(2-hydroxyethyl methacrylate) (polyHEMA), caspase-3 assay kit, cycloheximide, MG132, oligonucleotides for shRNA constructs, and monoclonal antibodies specific for Bax (clone 6A7), α -tubulin and β -actin were purchased from Sigma. 3-[3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) was

purchased from MP Biomedical. Anti-mouse Mcl-1 was purchased from Rockland. Monoclonal anti-Akt and polyclonal anti-p473 Akt antibodies were purchased from R&D Systems. Anti-p202/204 Erk1/2 and Anti-Erk1/2 polyclonal antibodies and U0126 were purchased from Cell Signaling. Polyclonal Bax (N20) and Goat anti-rabbit IgG-HRP were purchased from Santa Cruz. Polyclonal anti-Bim antibody, TDZD-8, and the fluorogenic chymotrypsin substrate III were purchased from Calbiochem. The anti-human Mcl-1 antibody was purchased from BD Biosciences. LY294002 was purchased from Alexis.

Plasmids

The pcDNA3-BimEL vector was described previously (Yamaguchi and Wang, 2002). The pcDNA3.1/V5-His-TOPO plasmids encoding wild type and mutant Mcl-1 were described previously (Maurer et al., 2006). Oligos for shRNAs targeting Bim 5'-GTTCTGAGTGTGACAGAGA-3' and Mcl-1 5'-GAGGACGACCTATACCGCC-3' (c-1) and 5'-GCCCTAATTAACAACGTTG-3' (c-3) were synthesized and cloned into the Bgl II and Sal I restriction sites of LTRH1-puro (Ken Watanabe, National Center for Geriatrics & Gerontology, Aichi, Japan). The retroviral constructs expressing Bcl-2-IRES-Bim, Bcl-XL-IRES-Bim, and Mcl-1-IRES-Bim were described previously (Kim et al., 2006). The pLKO.1-based lentiviral shRNA targeting human Mcl-1, TRCN0000005517 was purchased from Open Biosystems. The pLKO.1-based scrambled control shRNA vector was purchased from Sigma.

Cell Culture and Transfection

Wild type, v-Src, and c-Src 527F NIH3T3 cells were previously described (Yu et al., 1995). GSK-3 β ^{-/-} MEF cells were kindly provided by James Woodgett (Hoeftlich et al., 2000). NIH3T3 and MEF cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Saos-2, LM7, and MDA-MB-231 cell lines were maintained in MEM medium supplemented with 10% FBS, 1% penicillin/streptomycin, 1 mM NaPyruvate, and 1x MEM non-essential amino acids. To induce anoikis, cells were maintained in the same media supplemented with 1% (for mouse cell lines) or 10% (for human cell lines) FBS in polyHEMA coated plates. Transfection was completed with Lipofectamine2000 (Invitrogen) according to the manufacturer's recommendations. Recombinant retrovirus and lentivirus were produced in Amphotropic 293T packaging cells and 293FT cells with ViraPowerTM Packaging Mix (Invitrogen), respectively.

Caspase-3, Chymotrypsin and LDH release

Caspase-3 activation was assayed as DEVDase activity with the Caspase-3 fluorescence assay kit (Sigma). Briefly, cells were lysed in 1% Chaps lysis buffer and cleared by centrifugation at 13,000g for 5 minutes. The supernatant was collected and protein concentration was determined by BCA method. 1X reaction mixture was aliquoted into a 96-well plate and 50 μ g total protein lysate was added. The reaction was incubated at room temperature in the dark for 1 hour and the fluorescence was measured by an automated plate reader. Values are represented as the change in fluorescent units (Δ FU) per μ g protein per hour. The fluorogenic chymotrypsin substrate III (Calbiochem)

was used to measure chymotrypsin-like activity using the same protocol as that used for the caspase-3 assay. Cell death was measured using the LDH cytotoxicity assay (Biovision) according to the manufacturer's recommendations.

Semi-Quantitative RT-PCR

Semi-quantitative RT-PCR was completed using the Qiagen OneStep RT-PCR system according to the manufacturer's recommendations. Primers used for the reactions are: 5'-TGCAGGGTTATGGAATCCTC-3' and 5'-GCCCCTACCTCCCTACAGAC-3' for Bim; 5'-GCAGCTTCAAGTCCACCTTC-3' and 5'-AGATGGCGTAACAACTGGG-3' for Mcl-1; 5'-AATGTGTCCGTCGTGGATCT-3' and 5'-CCCTGTTGCTGTAGCCGTAT-3' for GAPDH.

In Vivo Metastasis Model

MDA-MB-231-luc-D3H2LN cells were obtained from Xenogen and infected with lentiviral Mcl-1 shRNA (shMcl-1) or control scrambled shRNA (shScr). After 10 days selection with 0.5 µg/mL puromycin, 1×10^6 cells were injected into 10-12 week-old Harlan nude mice via the tail vein. Three weeks post-injection mice were imaged using the IVIS200 system (Xenogen) as per the manufacturer's recommendations. Statistical significance was determined using Student's t-test.

Chapter Three:

Src Phosphorylation of Bif-1 Disrupts the Interaction with Bax and Inhibits Anoikis

Abstract

Bif-1 interacts with Bax and enhances its conformational rearrangement resulting in apoptosis. However, the molecular mechanism governing the interaction between Bif-1 and Bax is poorly defined. Here we provide evidence that Bif-1 is phosphorylated, an event which can be repressed by apoptotic stimuli. The protein kinase c-Src binds to and directly phosphorylates Bif-1 on tyrosine 80. Moreover, Src phosphorylation of Bif-1 suppresses the interaction between Bif-1 and Bax resulting in the inhibition of Bax activation during anoikis. Together, these results suggest that phosphorylation of Bif-1 impairs its binding to Bax and represses apoptosis, providing another mechanism by which Src oncogenic signaling can prevent cell death.

Results

Bif-1 Phosphorylation In Vivo is Repressed upon Apoptotic Stimuli

Because Bif-1 binding to Bax is induced by apoptotic stimuli with no apparent changes in either protein's expression profile (Cuddeback et al., 2001; Takahashi et al., 2005; Yamaguchi et al., 2002), we reasoned that the ability of Bif-1 to activate Bax might be regulated by posttranslational modifications. To determine if Bif-1 could be phosphorylated in intact cells, 293T cells were metabolically labeled with $^{32}\text{PO}_4$ and subjected to immunoprecipitation with anti-Bif-1 or control anti-Myc antibodies (Figure

30). A specific ^{32}P -labeled band corresponding to Bif-1 was pulled down with anti-Bif-1 but not control anti-Myc antibody.

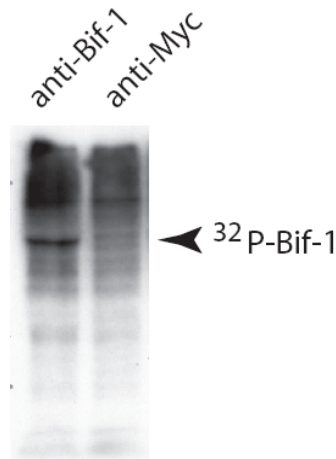


Figure 30. Bif-1 phosphorylation. 293T cells were metabolically labeled with $^{32}\text{PO}_4$ for 6 hours; lysates were collected and subjected to immunoprecipitation with anti-Bif-1 or anti-Myc antibodies followed by SDS-PAGE and autoradiography.

To determine the phosphorylation status of Bif-1 in stressed versus unstressed cells, 293T cells expressing Myc-tagged Bif-1 were metabolically labeled with $^{32}\text{PO}_4$ and treated with DMSO, thapsigargin (THG), or serum starvation (Figure 31). The levels of phosphorylated Bif-1 were found to be highest in unstressed cells. Similarly, the level of tyrosine phosphorylation of Bif-1 was repressed in a time dependent manner upon treatment with staurosporine (STS) (Figure 32). The kinetics of Bif-1 dephosphorylation correlated with the activation of Bax as determined by immunoprecipitation of conformationally active Bax with the 6A7 monoclonal antibody (Figure 32). Together, these results clearly indicate that Bif-1 is phosphorylated in whole cells and apoptotic

stresses promote the dephosphorylation of Bif-1.

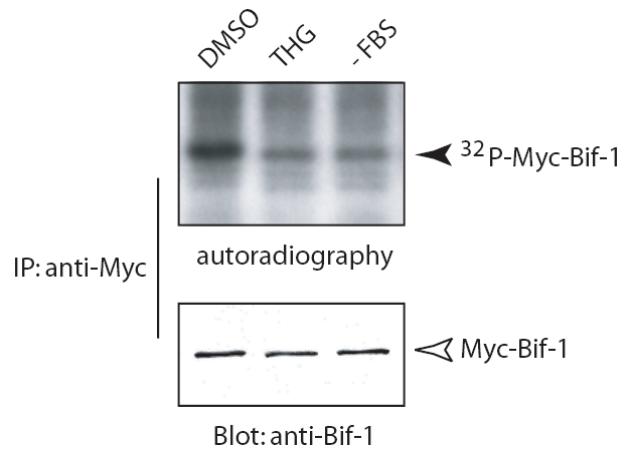


Figure 31. Phosphorylation of Bif-1 is inhibited by cellular stress. 293T cells were transfected with Myc-Bif-1 and labeled with $^{32}\text{PO}_4$ for 6 hours after serum deprivation (- FBS) for 24 hours or exposure to 2 μM thapsigargin (THG) or DMSO for 8 hours. Myc-Bif-1 protein was immunoprecipitated with anti-Myc antibody and subjected to SDS-PAGE/immunoblot and autoradiography.

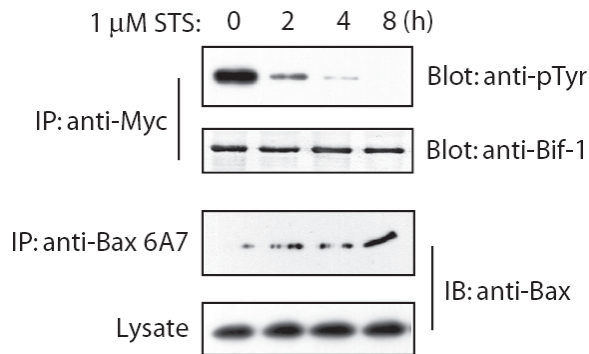


Figure 32. Bif-1 is dephosphorylated in a time-dependent manner upon treatment with staurosporine. 293T cells were transfected with Myc-Bif1 and treated with 1 μM staurosporine (STS) for various times prior to immunoprecipitation/immunoblot analysis with the indicated antibodies.

Src Interacts with Bif-1 in Both Yeast and Mammalian Cells

A search of Bif-1 interacting proteins through the use of a yeast two-hybrid assay resulted in the identification of c-Src kinase. The LexA-c-Src fusion protein specifically interacted with the wild type Bif-1 fused to the B42 transactivation domain in yeast (Figure 33). The region of Bif-1 required for the interaction with c-Src was determined using a series of Bif-1 deletion mutants. It was found that full length Bif-1 was the most effective at binding c-Src but appeared to require the presence of the region between amino acid residues 238-285 (Figure 34).

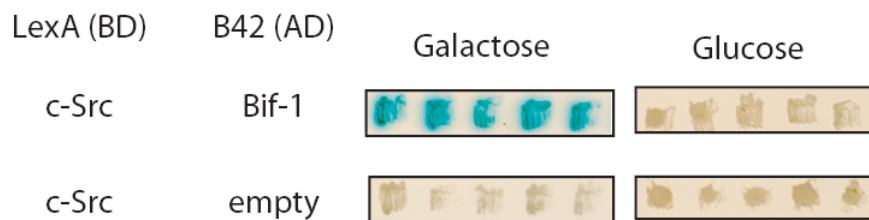


Figure 33. Bif-1 interacts with c-Src in the yeast two-hybrid assay. LexA-c-Src and B42-Bif-1 fusion proteins interact in yeast.

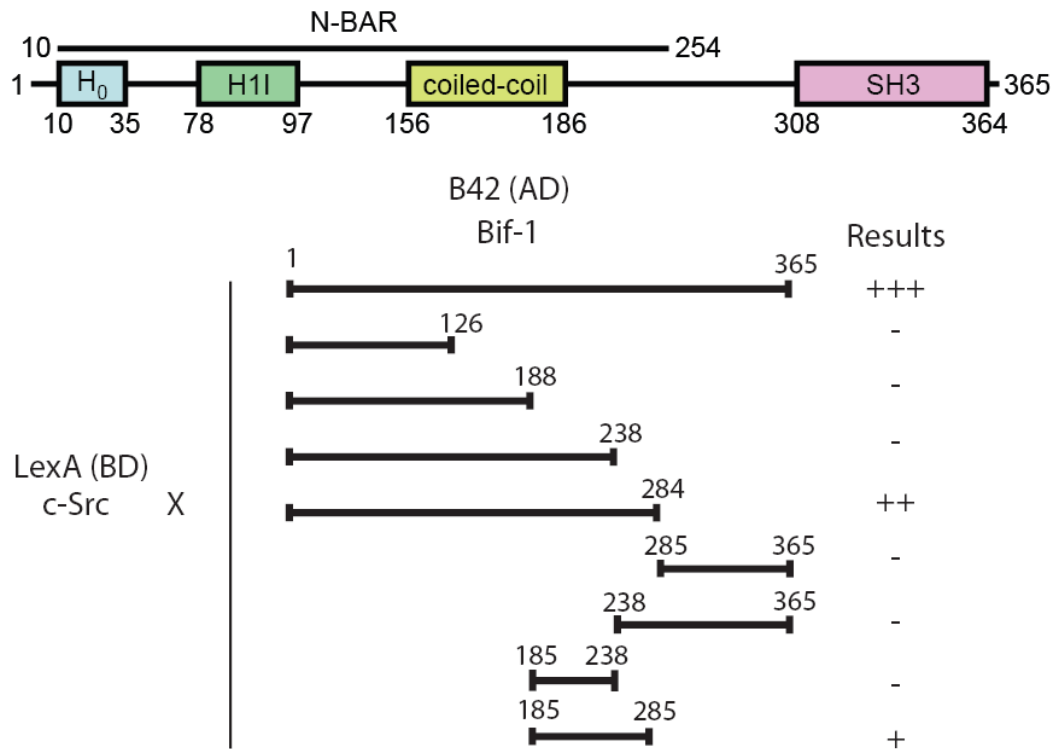


Figure 34. Schematic representation of the domain structure of Bif-1 and mapping the Src binding domain of Bif-1. LexA-c-Src was transfected into yeast with the indicated deletion mutants of Bif-1 fused to B42. Interactions of pairs of fusion proteins were determined by visual examination of β -galactosidase activity and represented graphically.

To determine if Bif-1 was a binding partner for c-Src in mammalian cells, 293T cells were transfected with empty vector, Flag-tagged c-Src or the naturally occurring Src mutant, Src531, which results in a truncated protein that has been shown to be activating, transforming, and tumorigenic (Irby et al., 1999). Immunoprecipitation with anti-Flag antibody revealed that endogenous Bif-1 could indeed interact with both c-Src and mutant Src531 (Figure 35A). Similarly, Bif-1 was found to interact with wild type c-Src and mutant c-Src (Y527F) in 3Y1 rat fibroblasts and NIH3T3 cells, respectively (Figure

35B). Src binding to Bif-1 is therefore a common event with implications in Bif-1 regulation.

Since both STS and THG prevented the phosphorylation of Bif-1 (Figures 27 & 28), we sought to determine whether apoptotic stimulation affects the binding of Src to Bif-1. As shown in Figure 36, H1299 cells treated with STS did not prevent the association of Bif-1 with Src at endogenous levels as compared to DMSO control, but instead prevented the kinase activity of Src as determined by the activation associated pTyr-416 western blot. Alternatively, THG treatment could effectively prevent the association between Bif-1 and Src. Treating cells with THG did not suppress but rather enhanced the activation of Src, which is consistent with previous observations (Chung et al., 2001). Together, these results suggest that apoptotic stresses can prevent the phosphorylation of Bif-1 through either inhibition of Src interacting with Bif-1 or by preventing Src activity.

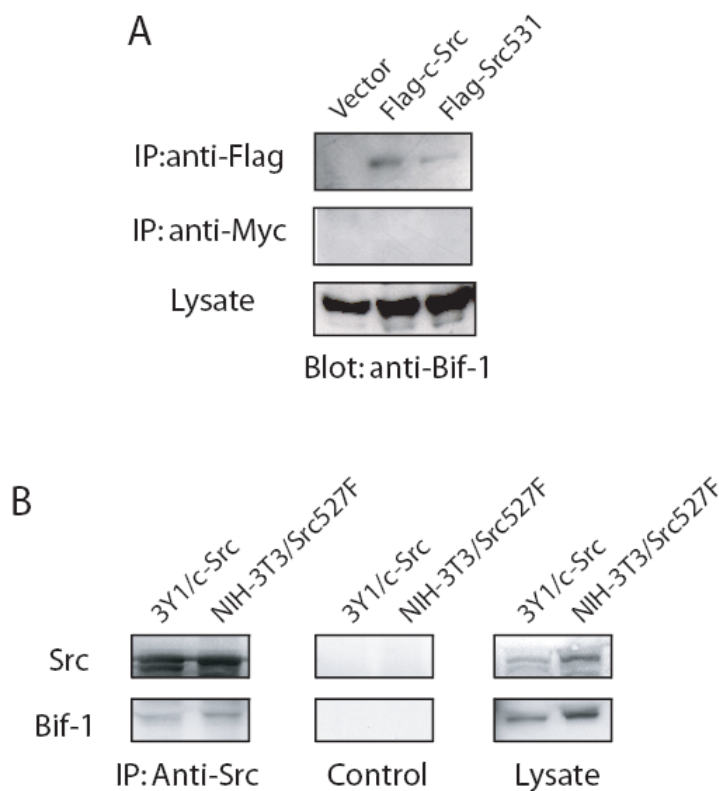


Figure 35. Bif-1 interacts with Src in mammalian cells. (A) 293T cells were transiently transfected with pFlag-c-Src or c-Src531 or parental pFlag-CMV2 vector. After 2 days, immunoprecipitation was performed using anti-Flag antibody or anti-Myc (control) followed by SDS-PAGE/immunoblot analysis with anti-Bif-1 polyclonal antiserum. (B) 3Y1 rat fibroblasts stably expressing c-Src or NIH3T3 cells stably expressing a constitutively active c-Src (Y527F) were subjected to immunoprecipitation with anti-c-Src or control antibody followed by SDS-PAGE/immunoblot analysis.

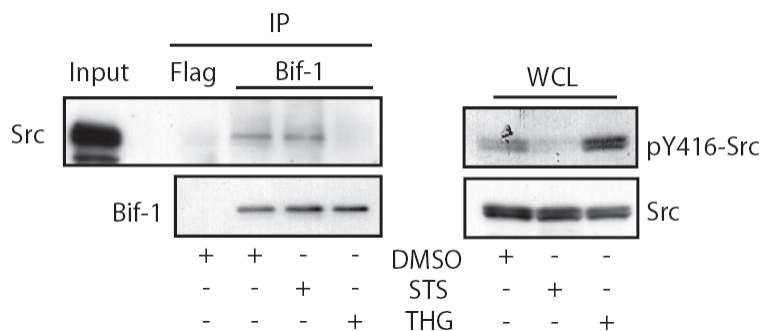


Figure 36. Dephosphorylation of Bif-1 can be achieved via two independent mechanisms in response to cellular stresses. H1299 cells were treated with

1 μ M STS, 2 μ M THG, or DMSO for 8 hours. Left panel, lysates were immunoprecipitated with anti-Bif-1 antibody or control Flag antibody and then probed with anti-Src and anti-Bif-1 antibodies. Right panel, whole cell lysates were probed for Src activity with anti-pY416 antibody.

Src phosphorylation of Bif-1 at Y80

We next sought to determine whether Src could directly phosphorylate Bif-1 using an *in vitro* kinase assay. Incubation of recombinant c-Src with GST-Bif-1 fusion protein but not GST alone resulted in detectable phosphorylation determined by autoradiography (Figure 37A). The subsequent Src phosphorylation site on Bif-1 was narrowed down through the use of deletion mutants (Figure 37B).

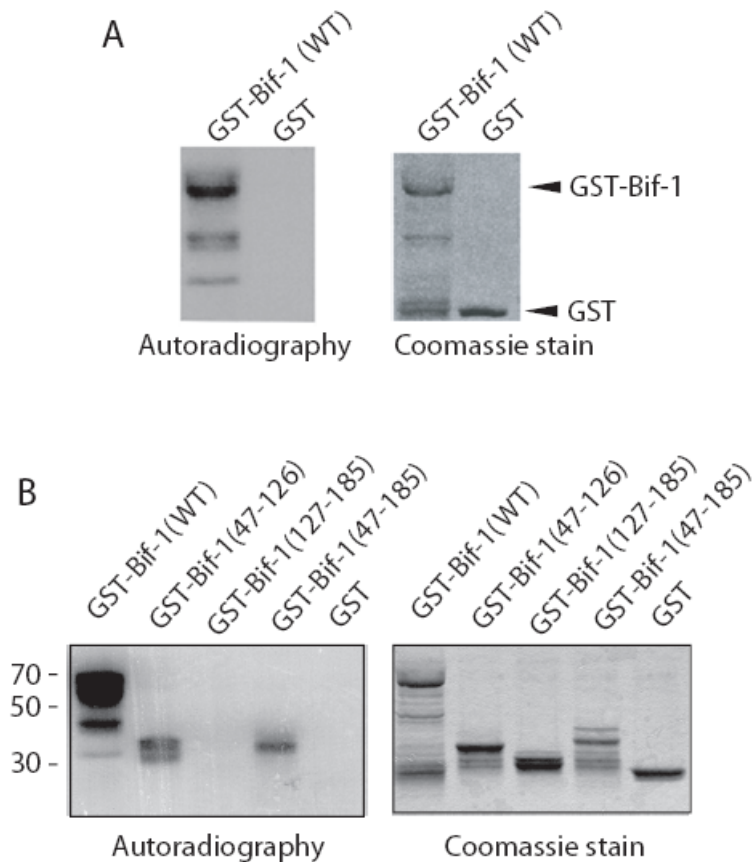


Figure 37. Minimal Bif-1 sequence required for Src phosphorylation. Recombinant c-Src kinase was incubated with GST, GST-Bif-1, or various GST-Bif-1 deletion mutants and [γ - 32 P]-ATP at 30°C for 15 minutes before analysis by SDS-PAGE and autoradiography.

Src mediated phosphorylation of Bif-1 occurred in the N-terminal region of Bif-1 encompassing amino acids 47-126. A closer evaluation of this region revealed a potential Src phosphorylation consensus motif (EEFVY⁸⁰EKLD), and substitution of Y80 to F abrogated Src mediated Bif-1 phosphorylation (Figure 38).

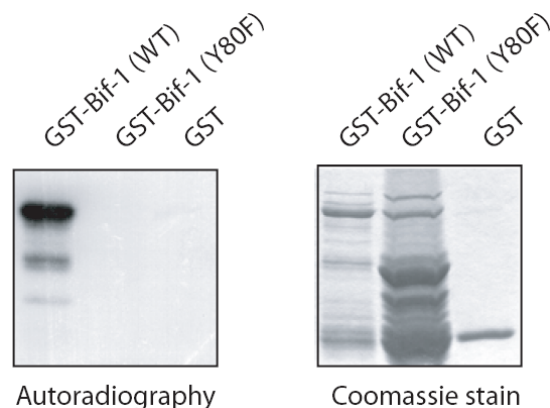


Figure 38. Src phosphorylates Bif-1 at Y80 *in vitro*. Recombinant c-Src kinase was incubated with GST, GST-Bif-1 wild type (WT), or GST-Bif-1 Y80F mutant and [γ - 32 P]-ATP at 30°C for 15 minutes before analysis by SDS-PAGE and autoradiography. Coomassie staining shows the input of GST and GST-Bif-1 proteins.

Src Phosphorylates Bif-1 at Y80 in Intact Cells

Although the *in vitro* kinase assay illustrates that Src directly targets Y80 in Bif-1, it remained to be determined whether this event could be recapitulated in whole cells. Therefore, 293T cells were co-transfected with Flag-Bif-1 along with empty, c-Src, or Src531 constructs. Bif-1 was then immunoprecipitated from lysates using anti-Flag antibody and subjected to immunoblotting with antibodies specific for phosphotyrosine or Bif-1 (Figure 39A). Co-expression of Src with Bif-1 led to a marked increase in tyrosine phosphorylation of Bif-1. It was also found that expression of Src531 could induce the phosphorylation of both endogenous and ectopic Bif-1 regardless of tag presence in 293T cells (Figure 39B).

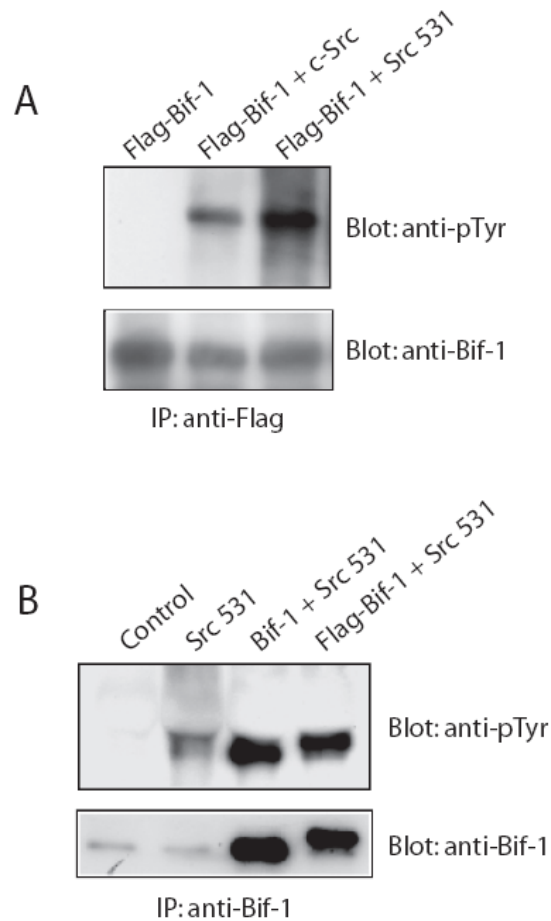


Figure 39. Src phosphorylates Bif-1 in intact cells. (A) 293T cells were transiently co-transfected with Flag-Bif-1 and either c-Src, c-Src531 mutant or empty vector. After two days, Flag-Bif-1 was immunoprecipitated with anti-Flag antibody, followed by SDS-PAGE/immunoblot analysis with anti-Bif-1 rabbit antiserum or anti-phosphotyrosine PY20 antibody. (B) 293T cells were transiently co-transfected with Src531 and plasmids encoding Bif-1, Flag-Bif-1 or parental vector. Cells were lysed 2 days later and Bif-1 was immunoprecipitated with anti-Bif-1 monoclonal antibody and subjected to SDS-PAGE/immunoblot analysis with antibodies specific for Bif-1 or phosphotyrosine.

Performing the same co-transfection experiments confirmed that Y80 was the major phosphorylation target of Src as the Bif-1^{Y80F} mutant showed a significant reduction in the level of tyrosine phosphorylation of Bif-1 (Figure 40). However, it

appears as if there is some level of phosphorylation still present in the Bif-1^{Y80F} mutant. This is likely due to phosphorylation at other tyrosine residues regulated by Src or the immunoprecipitation of endogenous Bif-1.

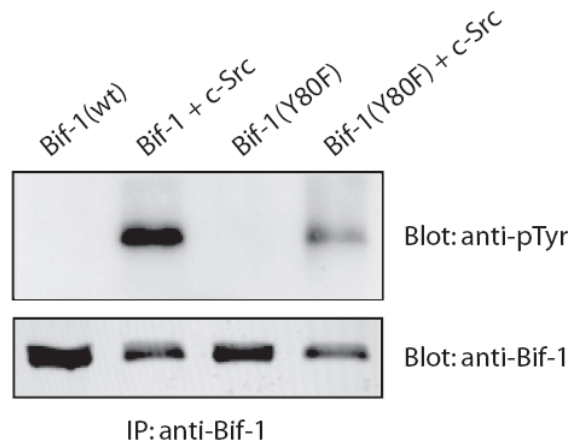


Figure 40. Src phosphorylates Y80 of Bif-1 in whole cells. 293T cells were transfected with Bif-1 or Bif-1(Y80F) alone or together with c-Src expression plasmids. Bif-1 proteins were immunoprecipitated and analyzed 2 days post transfection.

Similarly, A431, 293T, and NIH3T3 cells were assayed for the ability of EGF to stimulate endogenous Bif-1 phosphorylation (Figure 41A). EGF was found to promote an increase in Bif-1 phosphorylation, which could be inhibited by co-treatment with the Src specific inhibitors PD180970 in NIH3T3 cells as well as dasatinib in A431 and 293T cells, indicating that this phosphorylation is associated with intracellular signals that are associated with oncogenic suppression of apoptosis. Pretreatment with pervanadate, a broad spectrum tyrosine phosphatase inhibitor, was found to enhance the amount of Bif-1 phosphorylation on tyrosine by Src (Figure 41B). This indicates that the phosphorylation

of Bif-1 is a tightly regulated process.

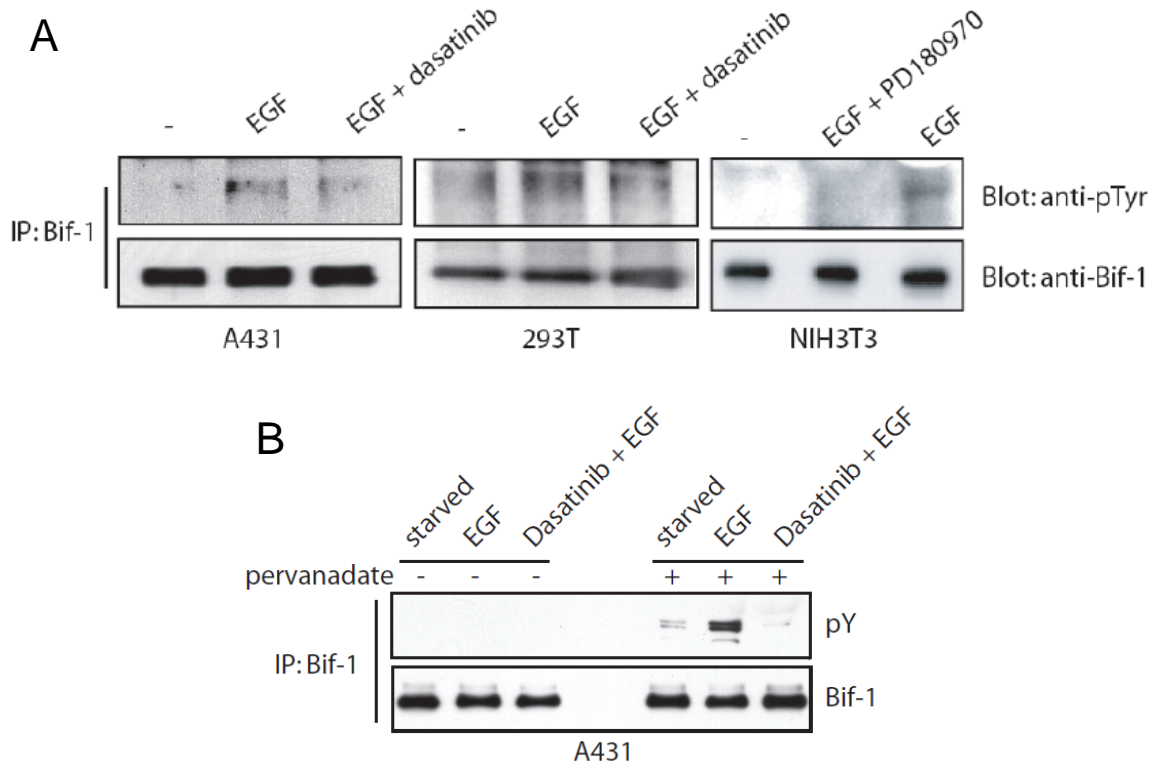


Figure 41. Endogenous phosphorylation of Bif-1 by c-Src. (A) A431, 293T, and NIH3T3 cells were serum starved for 16 hours then treated with or without 500 nM PD180970 or 50 nM dasatinib for 2 hours, and then stimulated with 50 ng/mL recombinant EGF for 5 minutes. Cell lysates were prepared and subjected to immunoprecipitation with anti-Bif-1 antibody followed by SDS-PAGE/immunoblot with anti-phosphotyrosine or anti-Bif-1 antibody. (B) A431 cells were treated as in A with and without pervanadate, which was added 10 minutes prior to the addition of EGF and maintained during the 5 minute incubation with EGF.

Bif-1^{Y80} Phosphorylation by Src Prevents Bif-1 Mediated Bax Activation during Anoikis

Based on the previous knowledge of Bif-1 activation of Bax upon apoptotic stimuli and our current findings of Src mediated phosphorylation of Bif-1, we sought to

determine the role of Src mediated Bif-1 phosphorylation in the context of anoikis initiation. Bif-1^{-/-} MEFs expressing empty control vector (pKI) or re-established wild type or Y80F mutant Bif-1 in the presence of Src531 or control GFP expression were created (Figure 42A) and detached on poly-HEMA coated plates for 24 hours or left attached on normal culture dishes. Lysates were then collected and the interaction between Bif-1 and Bax was determined (Figure 42B). Detachment caused an increase in Bif-1/Bax binding, consistent with our previous findings that apoptotic stimuli increase this interaction (Cuddeback et al., 2001; Takahashi et al., 2005). The Bif-1^{Y80F} mutant was able to bind Bax with the same affinity as wild type. Importantly, co-expression of Src531 with wild type Bif-1 repressed the ability of Bif-1 to interact with Bax in response to detachment. However, the non-phosphorylatable Bif-1^{Y80F} mutant retained Bax binding potential even in the presence of Src expression (Figure 42B). This interaction was specific as immunoprecipitation with normal rabbit serum (NRS) was unable to pull down either Bax or Bif-1 (Figure 42C).

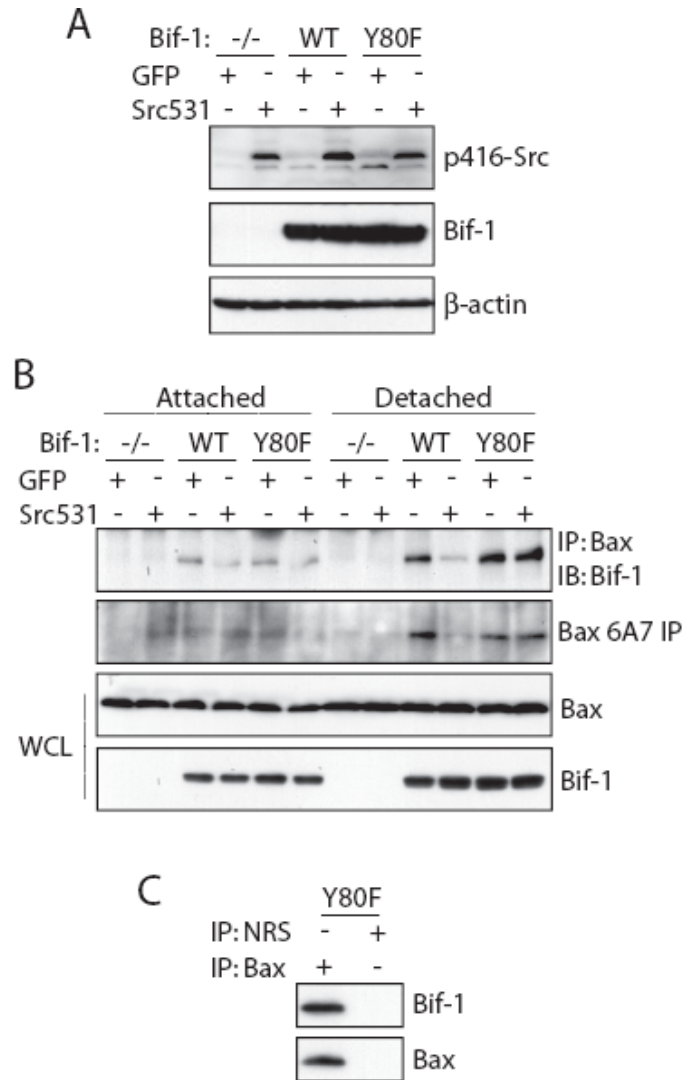


Figure 42. Src phosphorylation of Bif-1 inhibits Bax conformational activation during anoikis. (A) Bif-1 ^{-/-} MEFs were infected with retrovirus encoding empty control, Bif-1 wild type (WT) or Bif-1 mutant (Y80F) and selected on 1.0 µg/mL puromycin for 10 days. These cells were then re-infected with retrovirus encoding GFP or Src531 proteins. Expression of transgenes was confirmed by immunoblot. (B) The cells created in A were left attached or detached on poly-HEMA coated plates for 24 hours. Lysates were prepared and subjected to immunoprecipitation with anti-Bax polyclonal antiserum or monoclonal 6A7 antibody. The resulting immune complexes and whole cell lysates were analyzed by immunoblotting with anti-Bax or anti-Bif-1 antibody. (C) Lysates prepared from Bif-1(Y80F) cells detached for 24 hours were subjected to immunoprecipitation with anti-Bax polyclonal antibodies or normal rabbit serum.

Detachment induced Bax activation was increased in cells expressing Bif-1. Importantly, the enhanced Bax conformational change was inhibited by co-expressing Src531 in wild type Bif-1 but not Bif-1^{Y80F} cells (Figure 42B). Similarly, co-expression of Src531 could partially rescue cells expressing empty or wild type Bif-1 from caspase-3 activation, but overexpression of Src531 had no inhibitory effect on cells expressing the non-phosphorylatable Bif-1^{Y80F} mutant (Figure 43A). These results were confirmed by a cell death assay measuring LDH release (Figure 43B). These findings clearly indicate that Src phosphorylation of Bif-1 has direct biological impacts that affect the ability of a cell to undergo apoptosis.

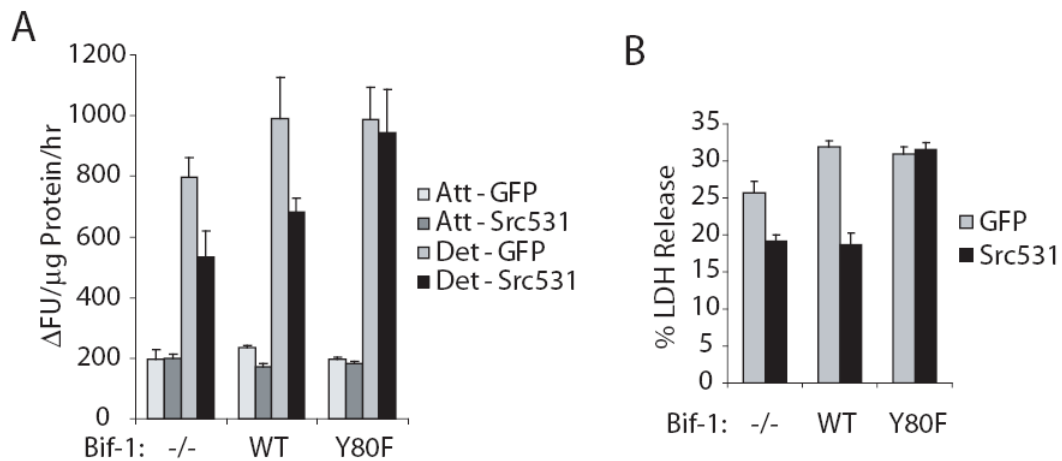


Figure 43. Src phosphorylation of Bif-1 inhibits caspase-3 activation and cell death in response to cell detachment. Cells were left attached or detached on poly-HEMA coated plates for 24 hours. Lysates were prepared and subjected to (A) caspase-3 assays or (B) LDH release. LDH release representation is normalized to their attached conditions; mean \pm SD, n=3.

Discussion

Bax activation is known to control the initiation of apoptosis. However, the exact

mechanism by which the conformational rearrangement and insertion of Bax into the OMM is controlled remains elusive. Although it is generally believed that activating BH3-only molecules such as tBid, Puma, and Bim can directly facilitate the activation of Bax, there remains debate as to whether Bax can directly interact with these molecules (Kim et al., 2006; Willis et al., 2007). Furthermore, it appears that activating molecules, other than BH3-only proteins, are crucial to the ability of Bax to form pores in membranes (Lutter et al., 2000; Ott et al., 2007b; Roucou et al., 2002). These observations have led to the pursuit and discovery of novel Bax interacting proteins, such as Bif-1.

Identification of Bif-1 as a novel Bax interacting factor has led to the finding that Bif-1 is able to potentiate the activation of Bax as well as Bak (Cuddeback et al., 2001; Takahashi et al., 2005). Reasons as to why Bif-1 is able to enhance the activation of Bax are not entirely understood. One possible explanation is that Bif-1 can alter the mitochondrial membrane structure such that it becomes suitable for conformational rearrangement and/or insertion of Bax into the mitochondria. As a member of the endophilin family of proteins, Bif-1 contains the N-BAR domain which is known to promote membrane curvature (Gallop et al., 2006; Peter et al., 2004). Given that Bax accumulates at fission and fusion sites on the mitochondrial membrane and that Bif-1 is known to regulate mitochondrial morphology (Karbowksi et al., 2004; Karbowksi et al., 2002), it is likely that the Bif-1/Bax interaction observed is the consequence of changes in membrane structure mediated by Bif-1. Interestingly, the use of deletion mutants has identified the first eleven amino acids in Bif-1 as essential for its ability to bind Bax (Pierrat et al., 2001). This region of Bif-1 is a part of the amino terminal amphipathic

helix known as helix zero, which is essential for binding and tubulating membranes (Farsad et al., 2001; Gallop et al., 2006). The overlapping Bax and membrane binding functions of this region in Bif-1 may indicate that the interaction between Bif-1 and Bax is dependent on membrane dynamics.

Given the aforementioned role of Bif-1 in the activation of Bax and apoptosis (Cuddeback et al., 2001; Takahashi et al., 2005) as well as its newly defined role in autophagy (Takahashi et al., 2007), the mechanisms that regulate Bif-1 function are of increasing importance. Yeast two-hybrid analysis has identified Src as a Bif-1 interacting protein, an association that is also evident in both exogenous and endogenous expression systems. Furthermore, we have shown that Src can directly phosphorylate Bif-1 at Y80. This residue is part of the internal amphipathic helix common to members of the endophilin family but weakly conserved therein. Previous deletion mutation analysis revealed that this domain is required for membrane binding and tubulating activities of Endophilin A1 (Gallop et al., 2006). Similarly, mutating positively charged residues to negative ones in this region of Endophilin A1 also inhibits membrane binding and tubulation (Gallop et al., 2006). Phosphorylation of Y80 on Bif-1 is likely to alter membrane binding by changing the charge distribution in the internal amphipathic helix, as well as altering the helix organization with the addition of a bulky phosphate group. Mutant Bif-1^{Y80F} would lack the ability of Src to alter the charge structure of the helix and allow binding to membranes, which could explain why this mutant maintained Bax binding potential in cells with active Src. Future studies will be needed to determine specifically how Src mediated phosphorylation at Y80 of Bif-1 affects helix organization, membrane binding, and tubulation potential of the protein.

Materials and Methods

Metabolic Labeling and In Vitro Kinase Assays

For detection of Bif-1 phosphorylation, 293T cells were incubated for overnight in phosphate-free Dulbecco's modified Eagle's medium (DMEM) containing 5% dialyzed fetal bovine serum (FBS), and then labeled with 0.5-1.0 mCi of [32 P]-orthophosphate for 6 hours in the presence of 10% dialyzed FBS. Plates were washed with ice-cold phosphate-buffered saline, and lysed with radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors. Lysates were pre-cleared with Protein G agarose prior to immunoprecipitation with anti-Bif-1 or anti-Myc monoclonal antibodies. For *in vitro* kinase assays, a recombinant c-Src was obtained from Upstate Biotechnology. The phosphorylation reaction was carried out at 30°C for 15 min in 40 μ l reaction mixture (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 0.015% Brij 35, 0.1 mg/ml BSA, 10 μ M ATP, 5 μ Ci [γ - 32 P]ATP (3000 Ci/mmol), 5 units Src kinase) containing 1 μ g of indicated GST fusion protein. The reaction was stopped by addition of 14 μ l of 4 x SDS loading buffer and heat denaturation, resolved by SDS-PAGE and analyzed by autoradiography.

Yeast Two-Hybrid Assays

Two-hybrid assays were performed as previously described (Cuddeback et al., 2001). Briefly, c-Src fused to the LexA DNA binding domain was transfected into *S. cerevisiae* EGY48 cells with the full length or deletion mutants of Bif-1 fused to the B42 transactivation domain. Five independent transformants were grown on either galactose

or glucose containing agar plates for inducing or repressing β -galactosidase activity, respectively.

Expression and Purification of GST-Bif-1

The GST-Bif-1 wild type and deletion mutants proteins were cloned into pGEX-4T-1 plasmid and expressed in the DH5 α strain of *E. coli*. Briefly, transformed cells were grown in LB medium containing ampicillin (100 μ g/ml) at 37°C to an OD_{600nm} of 0.8, then 1 mM IPTG was added to induce protein expression at 37°C for 3 hours. Cells were lysed in PBS (pH=7.4) containing protease inhibitors by sonication and centrifuged at 14,000 x g for 30 min. The resulting supernatant was incubated with glutathione-sepharose 4B (Amersham Bioscience) at 4° for 1 hour then washed three times with PBS, the protein was eluted with 10 mM of reduced glutathione in 50 mM Tris-HCl (pH=8.0) and dialyzed against 50 mM Tris-HCl buffer (pH=7.5).

Immunoprecipitation and Immunoblot Assays

Cells were lysed using 1% Chaps lysis buffer (150 mM NaCl, 10 mM HEPES, pH 7.4, 1% Chaps) containing protease and phosphatase inhibitors. Immunocomplexes were pulled down with the indicated antibodies and washed 3 times in lysis buffer.

Immunoprecipitates were resolved using SDS-PAGE and immunoblotted with the indicated antibodies. For detection of active Bax, anti-Bax 6A7 monoclonal antibody (Sigma) was used for immunoprecipitation and anti-Bax N20 polyclonal antibody (Santa Cruz) was used for subsequent immunoblot analysis. The Bax/Bif-1 interaction was determined by immunoprecipitation of Bax using polyclonal anti-mouse/rat Bax 1696

antiserum (Krajewski et al., 1994) followed by immunoblotting with anti-Bif-1 monoclonal antibody (Imgenex). The Src/Bif-1 interaction was determined in H1299 cell lysate in 1% Chaps lysis buffer by immunoprecipitation of Bif-1 with monoclonal antibody (Imgenex) and subsequent immunoblotting for Src (Cell signaling #2108). For detection of tyrosine phosphorylation of Bif-1, A431, 293T, and NIH3T3 cells were deprived of serum for 18 hours in DMEM containing 0.1% BSA. Cells were then incubated with Src inhibitors PD180970 or dasatinib for 2 hours. Cells were then stimulated by the addition of EGF to 50 ng/mL for 5 minutes, washed once with 5 mL ice-cold PBS, and collected in 900 μ L Buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 5 mM sodium pyrophosphate, 1 mM Na₃VO₄, 2 μ g/ml aprotinin, 2 μ g/mL leupeptin, 100 μ g/ml phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 20 mM *p*-nitrophenyl phosphate, 1% Triton X-100) as previously described (Ren et al., 2004). One mg of pre-cleared cell lysate was incubated with Bif-1 rabbit polyclonal antibody for one hour on ice and then 20 μ L Protein A agarose was added and rocked overnight at 4°C. Beads were washed three times with Buffer A and then boiled in 20 μ L Laemmli sample buffer and resolved by SDS-PAGE. Proteins were transferred onto nitrocellulose membrane (Bio-Rad) and blocked in 3% chicken egg white albumin in TBS-T for 2 hours. The phosphorylated Bif-1 protein was detected with HRP-conjugated anti-phospho-tyrosine (PY20) mouse monoclonal antibody (BD Biosciences).

Retrovirus Production

Retroviral plasmids encoding Bif-1 wild type and Y80F mutant were cloned into

the BglII-XhoI sites of pKI vector. For Src expression, active c-Src531 mutant was cloned into the EcoRI-XhoI sites of pBMN-IRES-GFP vector. Retrovirus production was carried out in Amphotropic 293T cells. Briefly, cells were transfected with the indicated retroviral vector using the calcium phosphate method overnight in the presence of 25mM Chloroquine. Media were replaced the next morning and 36-48 hours later the viral containing media were collected and used to infect the target Bif-1^{-/-} MEFs in the presence of 8 µg/mL polybrene.

Cell Culture and Transfection

293T, NIH3T3, 3Y1, and Bif-1^{-/-} MEFs were maintained in DMEM containing 10% FBS supplemented with 100 µg/mL streptomycin and 100 U/mL penicillin. Transfection was performed using the calcium phosphate precipitation method. For anoikis assays, the indicated MEFs were grown to approximately 80-90% confluency and then forcibly detached on poly(2-hydroxyethyl methacrylate; poly-HEMA) coated culture plates for 24 hours in 1% FBS as previously described (Woods et al., 2007). Control attached cells were also cultured for 24 hours in 1% FBS before collection.

Chapter Four:

p53 Acetylation Is Crucial for Its Transcription-Independent Proapoptotic Functions

Abstract

Acetylation of p53 at carboxy-terminal lysine residues enhances its transcriptional activity associated with cell cycle arrest and apoptosis. Here, we demonstrate that p53 acetylation at K320/K373/K382 is also required for its transcription-independent functions in Bax activation, ROS production, and apoptosis in response to the histone deacetylase inhibitors (HDACi) SAHA and LAQ824. Knockout of p53 in HCT116 cells markedly reduced HDACi-induced apoptosis. Unexpectedly, expression of transactivation-deficient p53 variants sensitized p53-null cancer cells to HDACi-mediated Bax-dependent apoptosis, whereas knockdown of endogenous mutant p53 inhibited HDACi-induced apoptosis. Evaluation of the mechanisms controlling this response led to the discovery of a novel interaction between p53 and Ku70. The association between these two proteins was acetylation independent, but acetylation of p53 could prevent and disrupt the Ku70/Bax complex and enhance apoptosis. These results suggest a new mechanism of acetylated p53 transcription-independent regulation of apoptosis.

Results

Transactivation Activity of p53 Is Dispensable for Apoptosis Induced by SAHA or LAQ824

To investigate the transcription-independent function of p53 in HDACi-mediated cell death, we utilized the previously established p53^{-/-} HCT116 cell line expressing the p53^{QS} transactivation deficient mutant that retains the proapoptotic function of p53 in which two key amino acids (Leu22 and Trp23) in the transactivation domain were replaced with Gln and Ser, respectively (Yamaguchi et al., 2004). The inability of this mutant to transcriptionally activate p53 target genes was confirmed by using p53 responsive luciferase reporter plasmids containing the p21 or Mdm2 promoter (Figure 44).

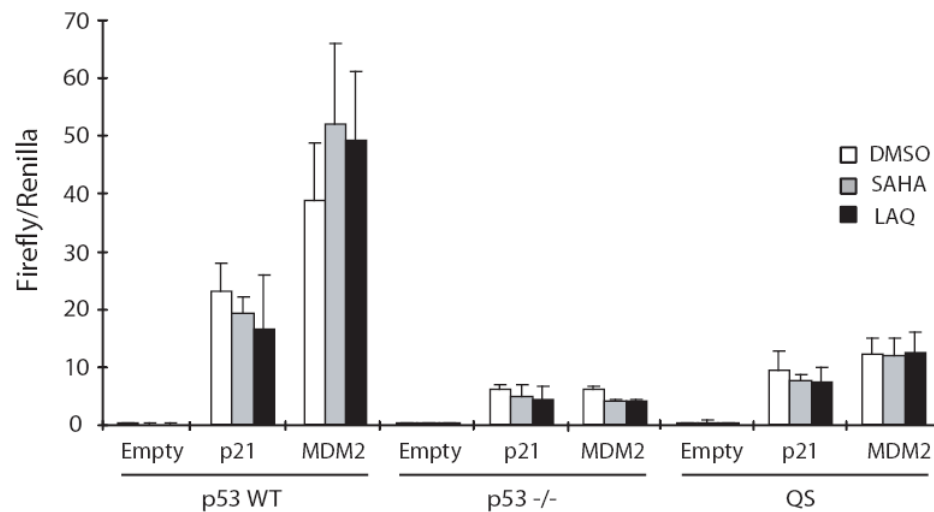


Figure 44. Treatment with HDACi does not robustly induce p53^{QS} transactivation. HCT116 p53^{-/-} cells stably expressing control empty Puro, p53 wild type (WT) or p53^{QS} mutant were transiently co-transfected with firefly luciferase constructs driven by either p21 or mdm2 promoter together with pRL-SV40 renilla luciferase vector. The cells were then treated with DMSO, 5 μ M SAHA or 200 nM LAQ824 and subjected to Dual-Luciferase assay according to manufacturer's instructions (Promega). The results are represented as the mean ratio of firefly/renilla luciferase activities \pm SD, n=3.

When compared to p53^{+/+} HCT116 cells, p53^{-/-} HCT116 cells displayed a drastically reduced cell death (Figure 45) and caspase-3 activity (Figure 46) following

SAHA or LAQ824 treatment. Surprisingly, transfection of not only wild type p53 but also the p53^{QS} mutant with Puromycin (Puro) selection marker completely resensitized p53^{-/-} HCT116 cells to SAHA and LAQ824 (Figures 45-48), suggesting that HDACi require the presence of p53 but not its transcriptional activity to induce apoptosis. Similar results were obtained in three additional independent transfection clones harboring p53^{QS} with Blasticidin (Bsd) selection marker (Figures 45-48).

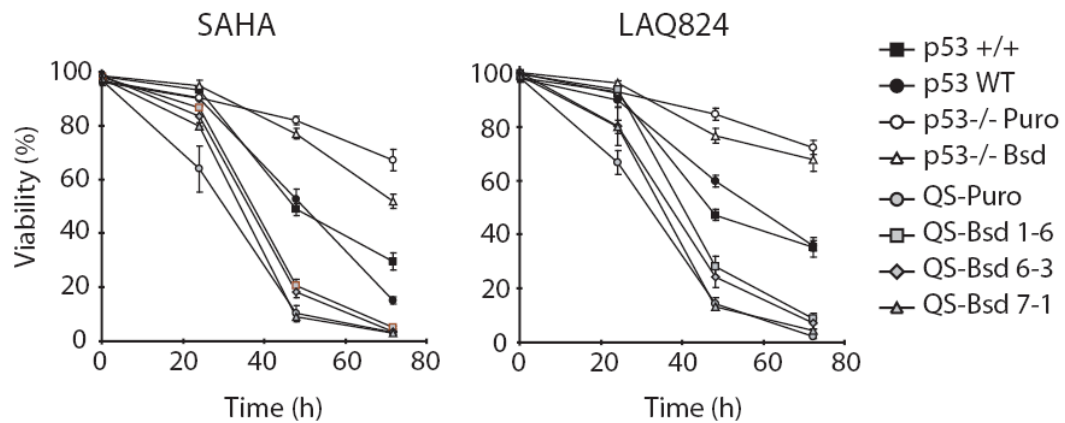


Figure 45. p53 status affects HDACi-induced cell death. Wild type HCT116 (p53+/+) and HCT116 p53^{-/-} cells stably transfected with empty vector (Puro or Bsd), p53 WT or p53^{QS} were treated with either 5 μ M SAHA or 200 nM LAQ824 for the indicated periods of time. The percentage of cell viability was determined by trypan blue dye exclusion assay; mean \pm SD, n=3.

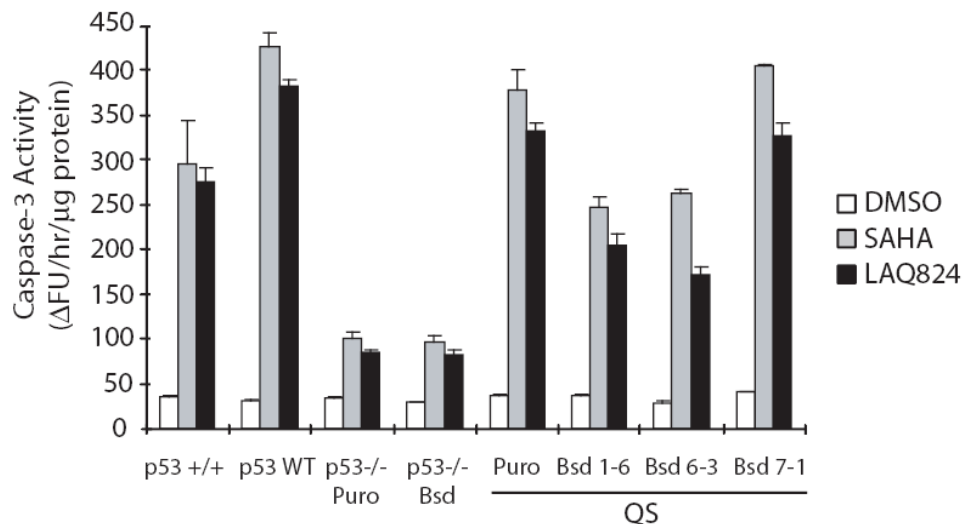


Figure 46. The transactivation-deficient p53^{QS} mutant sensitizes HCT116 p53^{-/-} cells to HDACi-induced caspase-3 activation. HCT116 cell lines expressing p53 as outlined in Figure 45 were treated with DMSO, 5 μ M SAHA or 200 nM LAQ824 for 28 hours and subjected to caspase-3 assay; mean \pm SD, n=3.

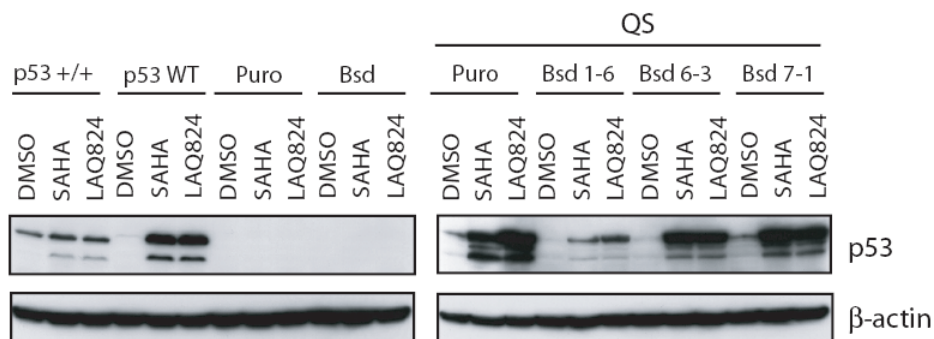


Figure 47. HDACi promotes stabilization of exogenously expressed p53. The same cell lines used in Figures 45 & 46 were treated with DMSO, 5 μ M SAHA or 200 nM LAQ824 for 18 hours and subjected to immunoblot analysis.

Mutations in p53 have been documented in more than half of all human cancers, of which most arise in the DNA binding domain. Since there are no known naturally

occurring mutation at both Leu22 and Trp23 residues of p53 and the transcriptional activity of p53^{QS} is not equal to other common mutations (Johnson et al., 2005), the ability of tumor-associated p53 mutants, R175H, R273H, and D281G (Hinds et al., 1990), to promote apoptosis in response to HDACi treatment in cancer cells was assessed. Consistently, all of these transactivation deficient p53 mutants potentiated HDACi-induced apoptosis in p53^{-/-} HCT116 cells, with p53^{QS} remaining the most potent inducer of apoptosis (Figures 48 & 49).

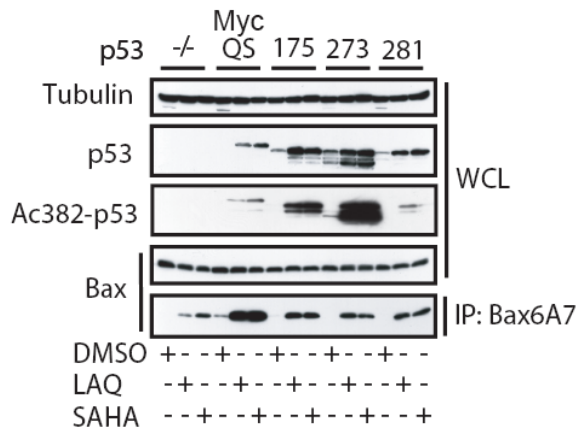


Figure 48. Expression of p53 mutants in HCT116 p53^{-/-} background enhances Bax activation induced by HDACi. HCT116 p53^{-/-} cells were stably transfected with empty, Myc-p53^{QS}, p53^{R175H}, p53^{R273H} or p53^{D281G}, treated with DMSO, 5 μ M SAHA or 200 nM LAQ824 for 24 hours and subjected to anti-Bax 6A7 immunoprecipitation and western blot analysis.

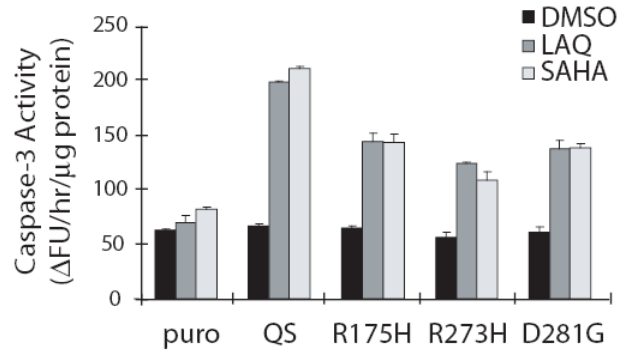


Figure 49. Expression of p53 mutants in HCT116 p53^{-/-} background enhances caspase-3 activation induced by HDACi. HCT116 p53^{-/-} cells were stably transfected with empty, Myc-p53^{QS}, p53^{R175H}, p53^{R273H} or p53^{D281G}, treated with DMSO, 5 μ M SAHA or 200 nM LAQ824 for 24 hours and subjected to caspase-3 activity assay.

Additionally, K562 and H1299 cells (both p53-null) stably transfected with p53 constructs containing R175H or D281G mutations, respectively, showed a marked increase in apoptotic response to LAQ824 treatment, as demonstrated by the ability to activate Bax and caspase-3 (Figures 50 & 51). Bax activation was determined by immunoprecipitation with anti-Bax 6A7 monoclonal antibody that only recognizes the active conformer of Bax (Hsu and Youle, 1997).

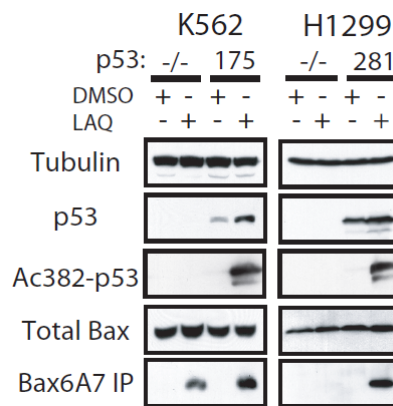


Figure 50. Naturally occurring p53 mutations promote Bax activation after HDACi

treatment. K562 and H1299 cells were stably transfected with p53^{R175H} and p53^{D281G}, respectively, treated with DMSO or LAQ824 (100 nM for K562 and 200 nM for H1299) for 24 hours and subjected to western blot analysis and anti-Bax 6A7 immunoprecipitation.

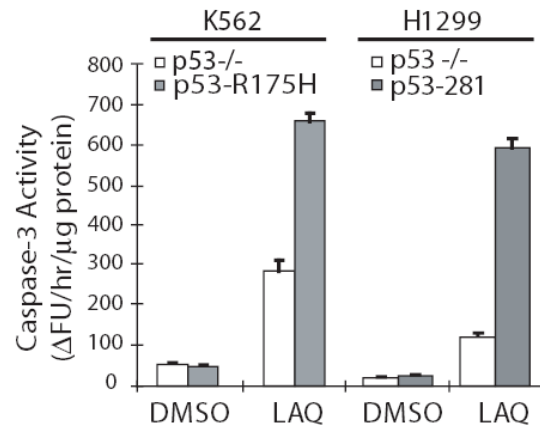


Figure 51. Expression of naturally occurring p53 mutants promotes caspase-3 activity in response to HDACi. K562 and H1299 cells were stably transfected with p53^{R175H} and p53^{D281G}, respectively, treated with DMSO or LAQ824 (100 nM for K562 and 200 nM for H1299) for 24 hours and subjected to caspase-3 assay.

Notably, after exposure to SAHA or LAQ824, the exogenously expressed p53 proteins were robustly increased (Figures 47, 48, & 50). Proteasome-directed degradation of p53 is tightly regulated through specific ubiquitin ligases such as Mdm2 (Honda and Yasuda, 1999). The increase in p53 protein levels by HDACi coincides with acetylation of p53 (Figures 48 and 50) that contributes to the inhibition of Mdm2-mediated p53 ubiquitination (Li et al., 2002). In addition, microarray analysis indicated that the mRNA levels of exogenous p53 were increased two to five fold by HDACi treatment (data not shown). We speculate that the exogenously integrated p53 genes are normally silenced by HDACs and reactivated by HDACi. This may explain at least in part why the

upregulation of endogenous p53 by HDACi was less than that of exogenous p53 (Figure 47). To evaluate the consequences of p53 stabilization after HDAC inhibition, H1299 p53^{-/-} and p53^{D281G} cells were treated with DMSO (vehicle), MG132 (proteasome inhibitor), or LAQ824. Inhibition of the proteasome has been shown to induce p53 accumulation without specific modifications and activation (Sakaguchi et al., 1998). Both MG132 and LAQ824 promoted a similar amount of p53 stabilization, but only LAQ824 treatment resulted in an increase in p53 acetylation (Figure 52). While MG132 caused a similar loss of viability in both p53^{-/-} and p53^{D281G} cell lines, LAQ824 specifically promoted apoptosis in p53^{D281G} expressing cells (Figure 53). Therefore, the presence of mutant p53 does not predispose cells to apoptosis under certain stresses, but HDACi preferentially promotes apoptosis in p53 expressing cells. This unique ability of HDACi correlates with the acetylation of p53 but not just its accumulation.

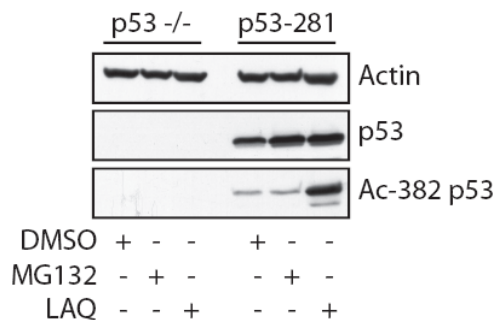


Figure 52. p53 expression and acetylation in response to HDACi and proteasome inhibition in H1299 cells. H1299 p53^{-/-} and p53^{D281G} cells were treated with DMSO, 200 nM LAQ824, or 1 μ M MG132 for 24 hours and subjected to western blot analysis.

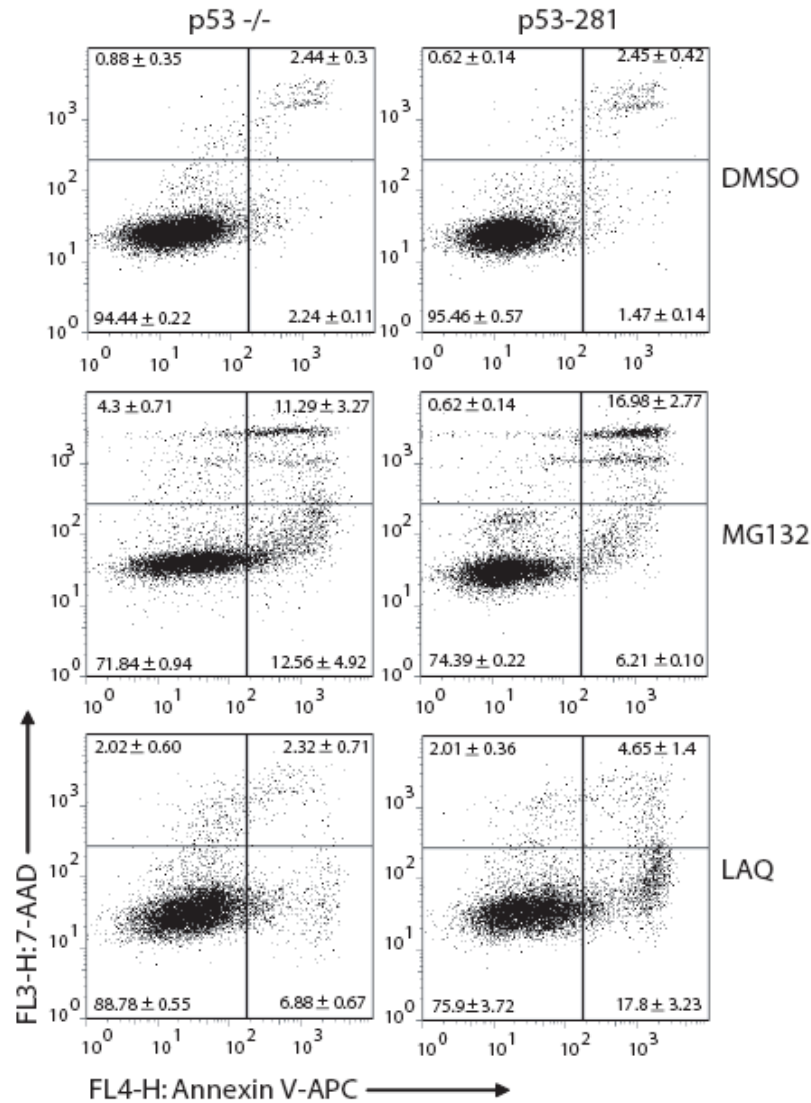


Figure 53. HDACi but not proteasome inhibitor induced cell death is enhanced by mutant p53 expression. H1299 p53^{-/-} and p53^{D281G} cells were treated with DMSO, 200 nM LAQ824, or 1 μ M MG132 for 24 hours and subjected to Annexin V-APC/7AAD staining and analysis by flow cytometry. Data are represented as mean \pm SD, n=3.

To further evaluate the potential of p53 to initiate HDACi-induced apoptosis, two colon carcinoma cell lines SW480 and HT-29 harboring R273H/P309S and R273H p53 mutations, respectively, were infected with lentiviral constructs expressing p53 shRNA (shp53), scrambled (shScr) shRNA or empty vector. Infection with the shp53 lentivirus

effectively knocked down p53 expression in both cell lines (Figure 54). Loss of mutant p53 conferred significant resistance to HDACi-induced caspase-3 activation and cell death (Figure 55A & B). The viability and caspase-3 results were also confirmed by Annexin V-APC/7AAD staining of shScr and shp53 SW480 clones (Figure 56). Furthermore, knock-in of murine p53-R172H, corresponding to human R175H mutation, results in enhanced cell death response of MEFs over control p53 null cells when treated with LAQ824 (Figure 57). These results highlight the functional significance of endogenous p53 status in determination of apoptotic index in response to HDACi regardless of its transactivational ability.

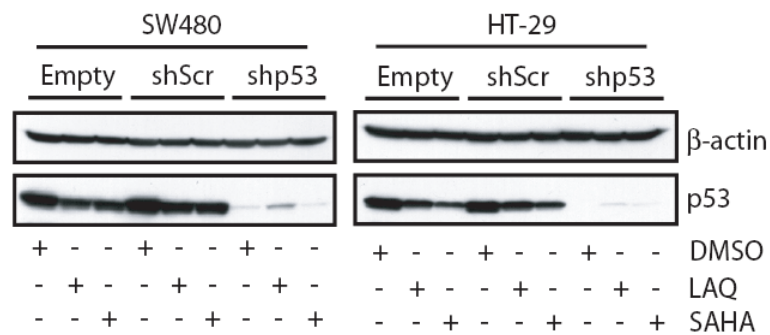


Figure 54. Knockdown of mutant p53 in SW480 and HT-29 cell lines. SW480 and HT-29 colon carcinoma cell lines were infected with lentiviral empty, scrambled shRNA (shScr) or p53 shRNA (shp53) constructs and selected on puromycin for 10 days. Cells were treated with DMSO, 5 μ M SAHA or 200 nM LAQ824 for 36 hours and subjected to immunoblot analysis.

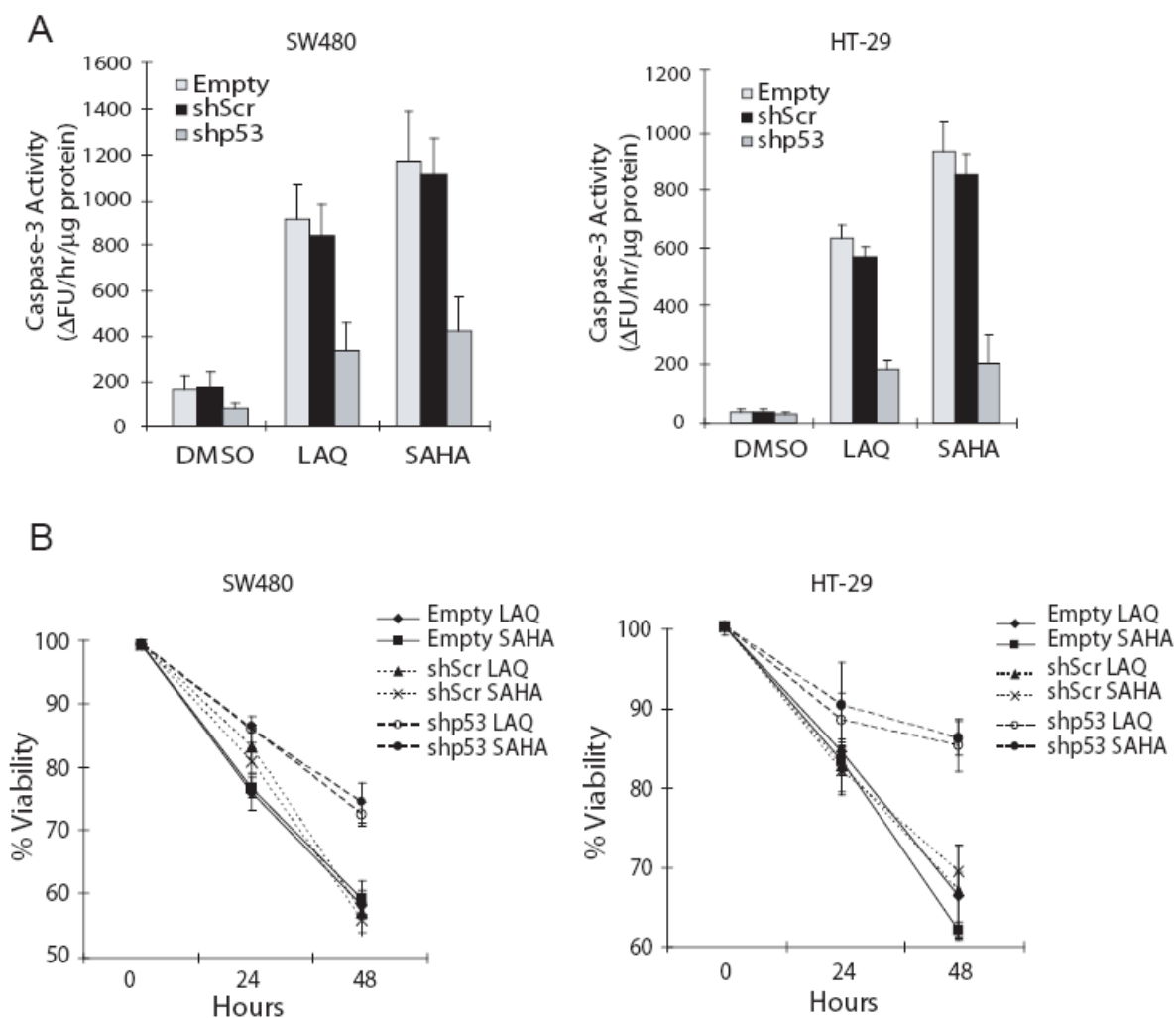


Figure 55. Knockdown of mutant p53 in SW480 and HT-29 cell lines prevents caspase-3 activation and cell death. (A) Cells described in Figure 54 were treated with DMSO, 5 μ M SAHA or 200 nM LAQ824 for 36 hours and subjected to caspase-3 assay. (B) Cells were treated with DMSO, 5 μ M SAHA or 200 nM LAQ824 for 24 and 48 hours and subjected to trypan blue exclusion assay.

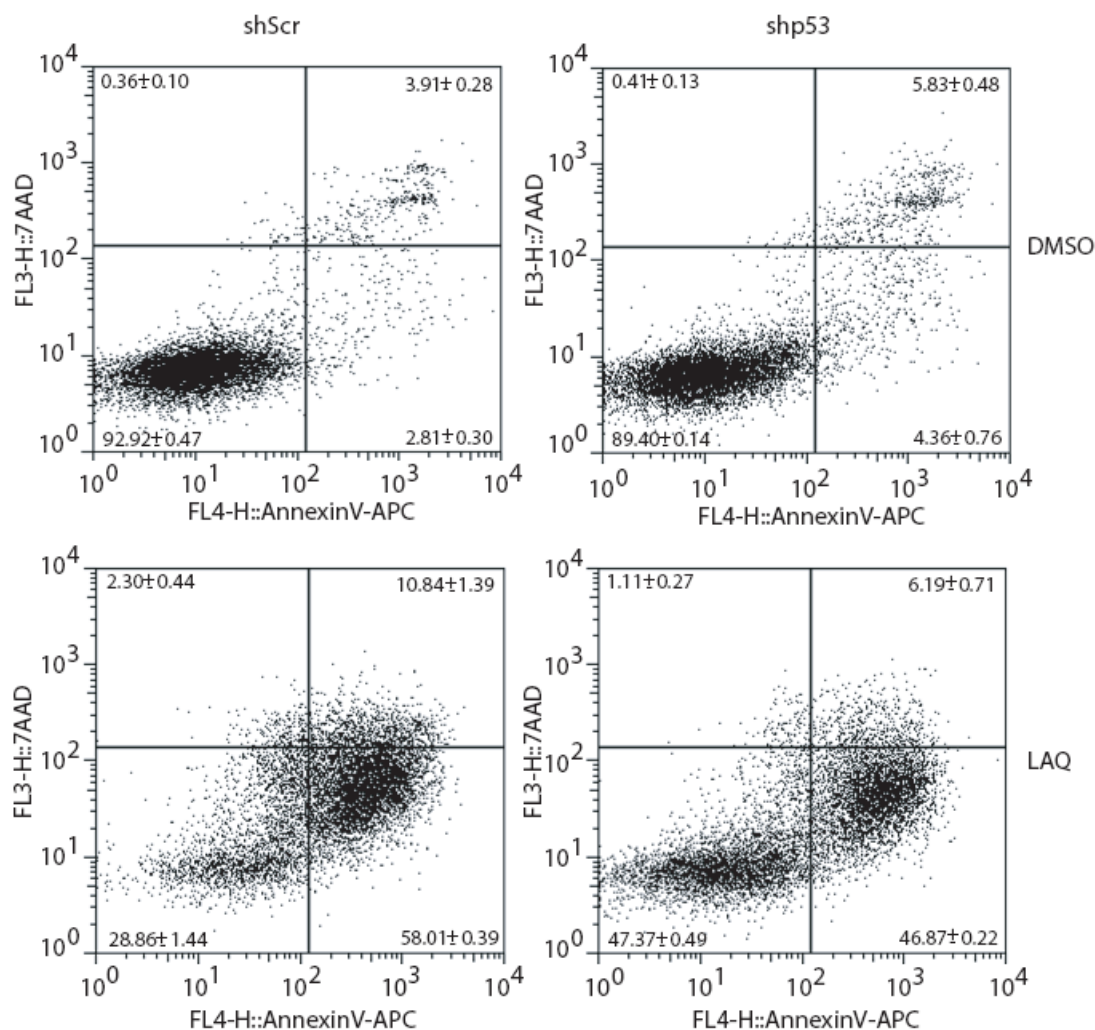


Figure 56. Knockdown of mutant p53 in SW480 cells prevents HDACi-induced apoptosis. SW480 cells were infected with either scrambled control (shScr) or p53 targeting (shp53) lentivirus as described in Figure 54. Cells were treated with DMSO or 200 nM LAQ824 for 24 hours and subjected to Annexin V-APC/7AAD staining and analysis by flow cytometry. Data are represented as mean \pm SD, n=3.

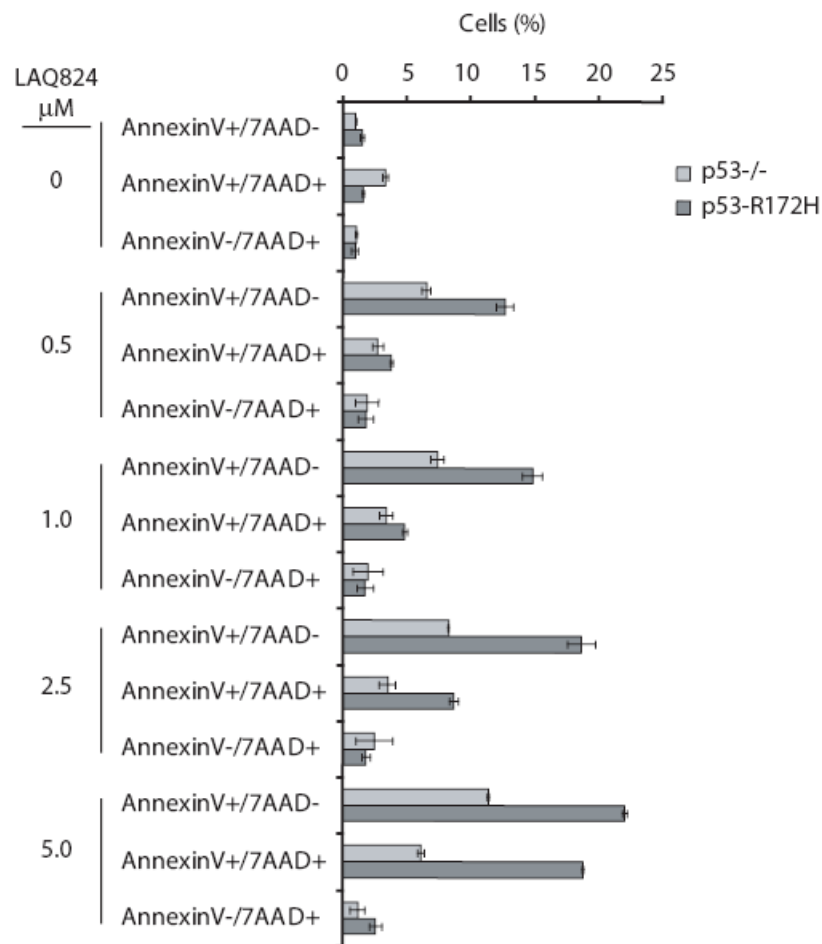


Figure 57. Knock-in of mutant p53 in MEFs enhances HDACi-induced apoptosis. MEFs derived from p53^{-/-} or knock-in p53^{R172H} were treated with the indicated concentration of LAQ824 for 24 hours and subjected to Annexin V-APC/7AAD staining and analysis by flow cytometry. Graphical representation of the different cell populations are presented as the mean \pm SD, n=3.

C-Terminal Acetylation Is Essential for Mutant p53-Mediated Apoptosis in Response to SAHA or LAQ824 Treatment

Inhibition of HDACs by SAHA or LAQ824 resulted in a significant increase in the levels of acetylated p53 (Figures 48, 50, and 52). To investigate the possible

involvement of p53 acetylation in its transcription-independent proapoptotic functions, three lysine residues (K320, K373, and K382), which are known to be acetylated by HDACi treatment (Terui et al., 2003), in human p53^{R175H} protein were mutated to arginine individually and in triplicate (K3R) to mimic the unacetylated state. These p53 constructs were cloned into retroviral vectors and infected into HCT116 p53^{-/-} cells to comparable levels (Figure 58A). The apoptotic index was then measured by caspase-3 activity after LAQ824 treatment (Figure 58B). Single mutations at the individual lysine residues yielded marginal decreases in apoptosis. However, mutation of all three lysines completely abolished the proapoptotic activity of p53^{R175H} in response to HDAC inhibition.

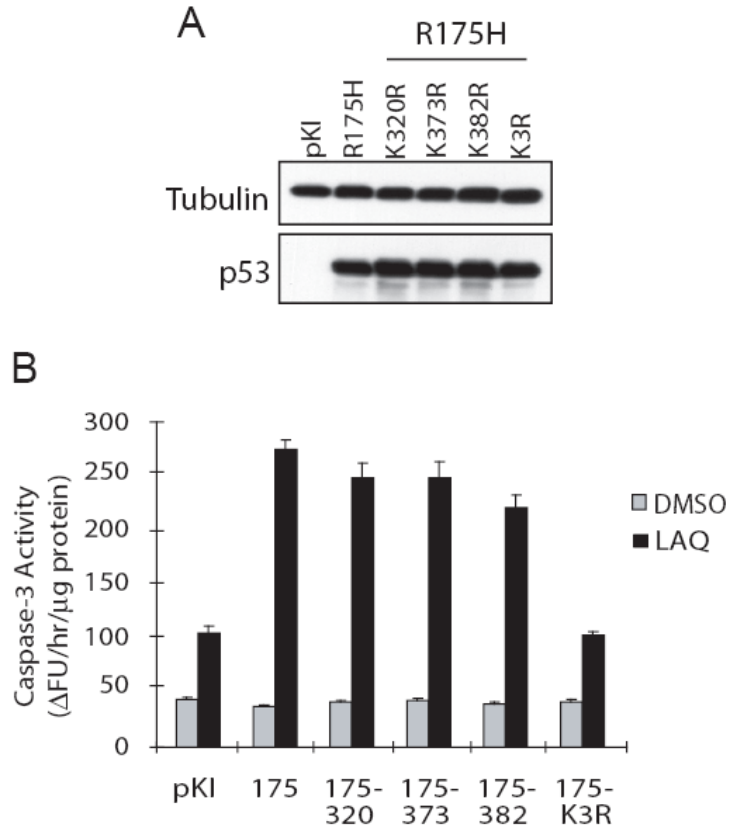


Figure 58. Substitution of K320, K373 and K382 to R attenuates the proapoptotic activity of mutant p53 in response to HDACi. HCT116 p53^{-/-} cells were infected with retroviral empty (pKI), p53^{R175H}, p53^{R175H/K320R}, p53^{R175H/K373R}, p53^{R175H/K382R} or p53^{R175H/K3R} constructs. (A) p53 expression was confirmed by western blot analysis. (B) The ability of p53 mutants to enhance HDACi-induced apoptosis was assessed by caspase-3 activation; mean \pm SD, n=3.

To further validate the importance of these residues in transactivational deficient p53, Myc-tagged p53^{QS} or p53^{QS/K3R} (all three lysines, K320/373/382, were mutated to arginine) was stably expressed in HCT116 p53^{-/-} cells (Figure 59A). Immunoblot analysis with anti-acetylated K382-p53 antibody confirmed the absence of HDACi-induced K382 acetylation in cells expressing the p53^{QS/K3R} mutant (Figure 59A). Consistently, the ability of p53^{QS} to induce caspase-3 activation and apoptotic cell death in response to SAHA or LAQ824 was drastically impaired by the K3R mutations (Figures 59B and 60).

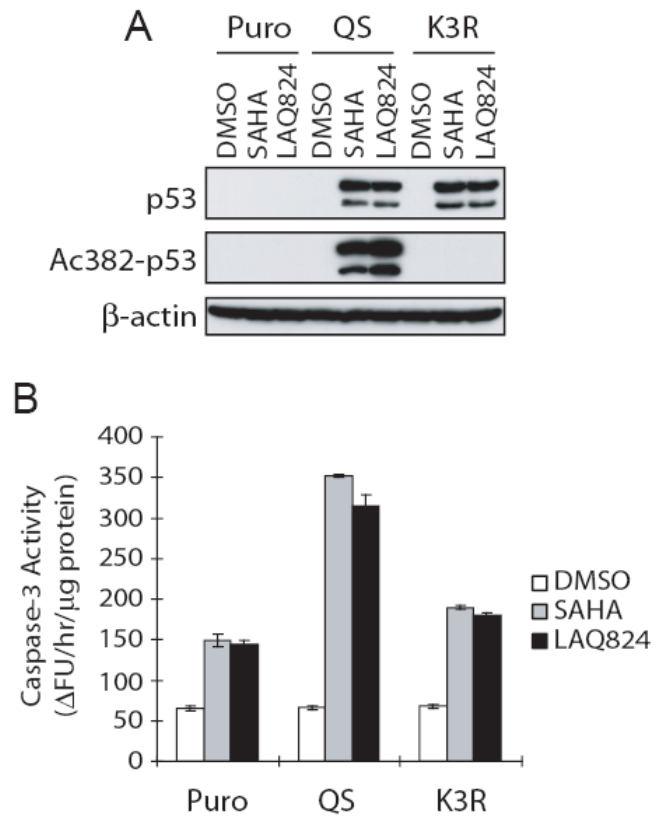


Figure 59. Mutation of K320, K373 and K382 to R attenuates the proapoptotic activity of p53^{QS} initiated by HDACi in HCT116 cells. HCT116 p53^{-/-} cells stably transfected with empty (Puro), Myc-p53^{QS} or Myc-p53^{QS/K3R} were treated with DMSO, 5 μ M SAHA or 200 nM LAQ824 for 18 hours and subjected to (A) immunoblot analysis and (B) caspase-3 assay; mean \pm SD, n=3.

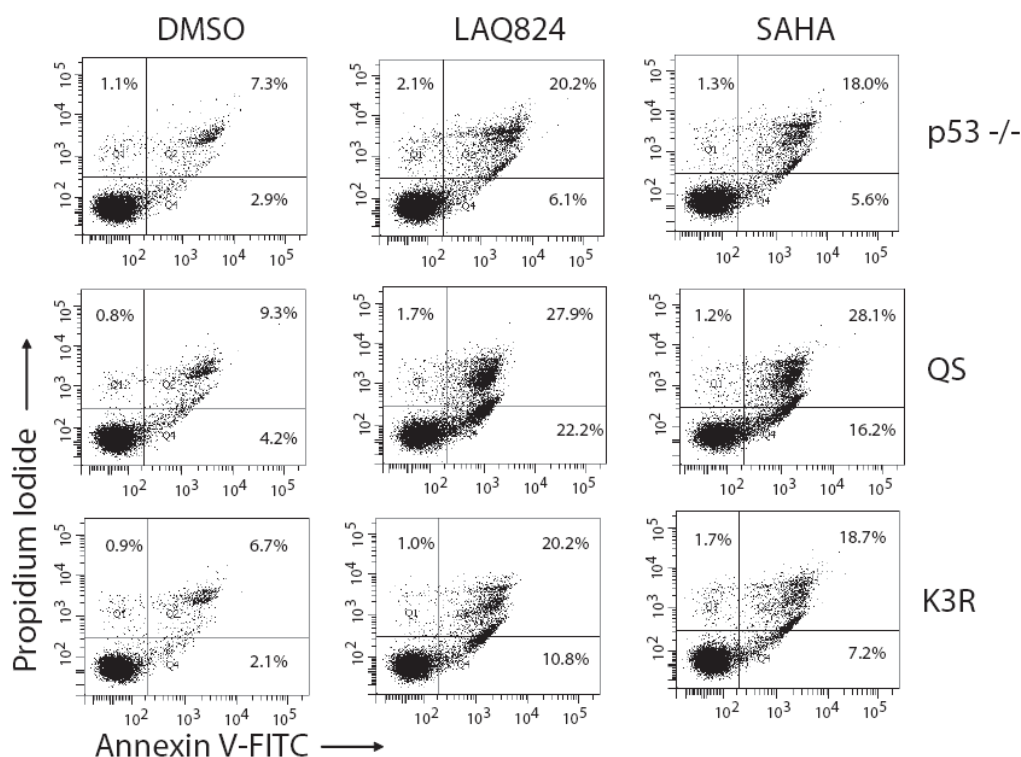


Figure 60. p53-dependent HDACi-induced cell death is repressed by mutation of three C-terminal lysine residues. HCT116 p53^{-/-} stably transfected with empty (Puro), Myc-p53^{QS} or Myc-p53^{QS/K3R} were treated with DMSO, 5 μ M SAHA or 200 nM LAQ824 for 18 hours and subjected to Annexin V-FITC/PI staining and flow cytometry analysis.

SirT1 is a class III HDAC whose activity also influences p53 acetylation status, but is not inhibited by LAQ824 or SAHA. To determine if SirT1 has a functional role in LAQ824 mediated apoptosis, H1299 p53^{-/-} and p53^{D281G} cells were transfected with empty control or SirT1 expressing plasmids. Overexpression of SirT1 resulted in a minimal reduction in acetylated K382-p53 (Figure 61A) and did not impact caspase-3 activation in either cell line treated with LAQ824 (Figure 61B).

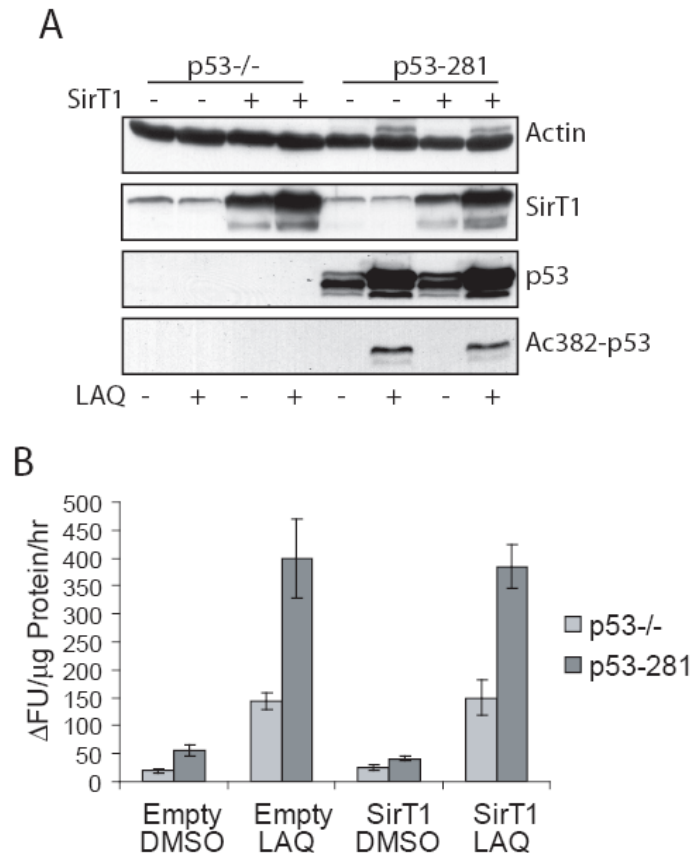


Figure 61. Overexpression of SirT1 does not impact p53 acetylation or p53-dependent HDACi-induced apoptosis. H1299 p53^{-/-} and p53^{D281G} cells were transfected with empty pCDNA3.1 or pCDNA3.1-SirT1 expression vectors and then treated with DMSO or 200 nM LAQ824 for 24 hours and subjected to (A) western blot and (B) caspase-3 assay.

Because SirT1 can be specifically inhibited by the compound EX527 (Napper et al., 2005), we treated p53^{-/-} and p53^{D281G} H1299 cells with EX527, LAQ824, or the combination of both compounds. Although EX527 alone had no effect on K382 acetylation, when combined with LAQ824 there was a significant increase in p53 acetylation (Figure 62). However, EX527 failed to enhance LAQ824-mediated apoptosis (Figure 63). This could possibly be due to a saturation of the acetylated p53 needed for

apoptosis induction after LAQ824 treatment. Overall, it appears that SirT1 does not play a significant role in LAQ824-mediated cytotoxicity.

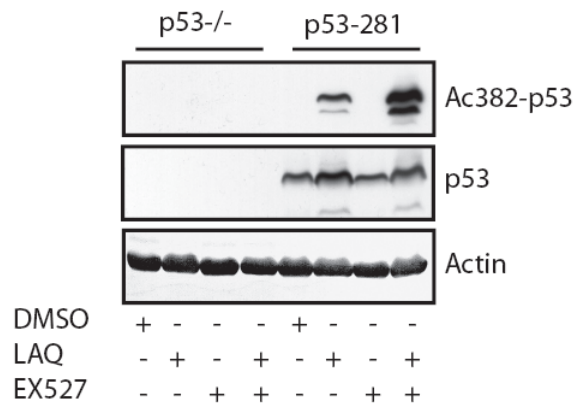


Figure 62. Pharmacological inhibition of SirT1 enhances p53 acetylation. H1299 p53^{-/-} and p53^{D281G} cells were treated with DMSO, 1 μ M EX527, 200 nM LAQ824 or the combination of 1 μ M EX527 and 200 nM LAQ824 for 24 hours and subjected to western blot analysis.

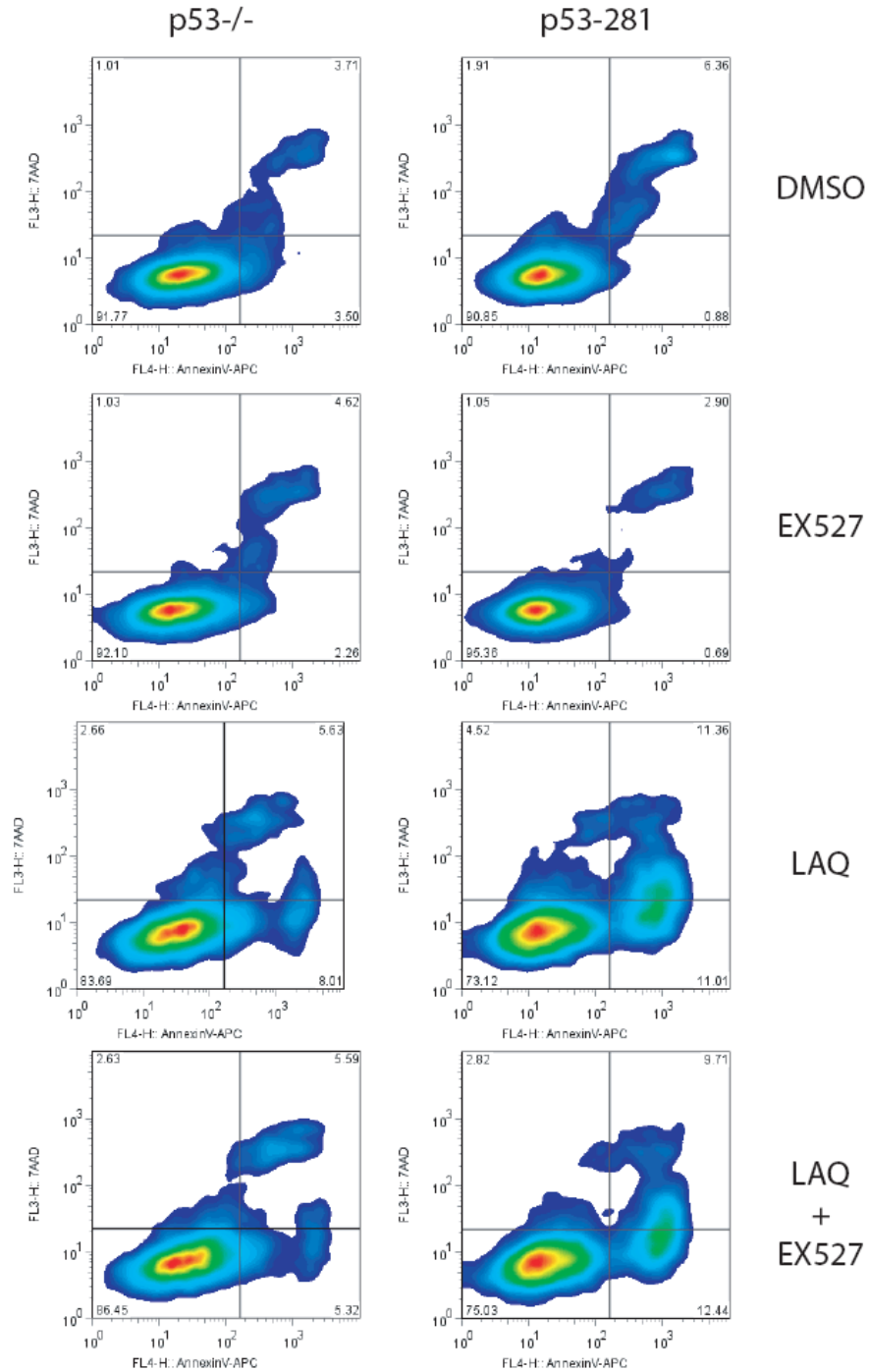


Figure 63. SirT1 inhibition does not affect p53-dependent HDACi-induced apoptosis. H1299 p53^{-/-} and p53^{D281G} cells were treated with DMSO, 1 μ M EX527, 200 nM LAQ824 or the combination of 1 μ M EX527 and 200 nM LAQ824 for 24 hours and subjected to Annexin V-APC/7AAD staining and flow cytometry analysis.

SAHA- or LAQ824-Mediated ROS Production Is Dependent on p53 Acetylation and Bax

ROS production plays a central role in HDACi-mediated cell death (Ruefli et al., 2001). In addition, ROS is a mediator of p53-induced apoptosis (Polyak et al., 1997). We therefore examined the correlation between ROS generation and p53 transactivation-independent proapoptotic function after HDACi treatment. To this end, HCT116 p53^{-/-} cells stably expressing p53^{QS} or empty control vector (Puro) were treated with SAHA or LAQ824 and the ROS levels were determined as previously described (Chandel et al., 2001). LAQ824 increased the ROS level in p53^{QS} but not control cells (Figure 64A). The antioxidant NAC reversed this ROS generation (Figure 64A) and decreased cell death (Figure 64B) induced by SAHA or LAQ824 in p53^{QS} cells.

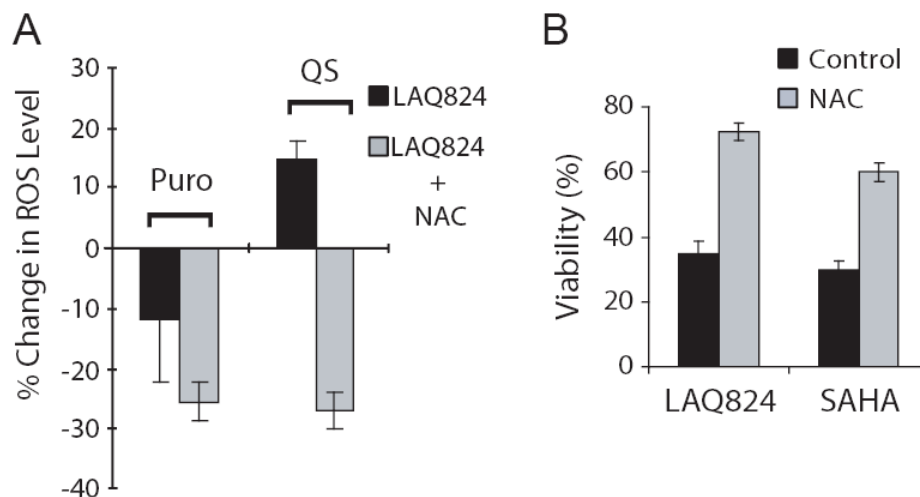


Figure 64. HDACi-induced p53-dependent ROS production promotes cell death. (A) HCT116 p53^{-/-} cells stably expressing control Puro or p53^{QS} were treated with 200 nM LAQ824 in the presence or absence of 10 mM NAC for 12 hours and the changes in intracellular ROS level were determined. (B) HCT116 p53^{-/-} cells stably expressing p53^{QS} were treated with 5 μ M SAHA or 200 nM LAQ824 in the presence or absence of 10 mM NAC for 48 hours. The percentage of cell viability was determined by trypan blue dye exclusion

assay; mean \pm SD, n=3.

Moreover, substitution of K320/373/382 to R (K3R) completely abolished the ability of p53^{QS} to induce ROS generation after LAQ824 treatment (Figures 65A & B). These results suggest that SAHA and LAQ824 require p53 acetylation but not its transcriptional activity to induce ROS production that is essential for HDACi-mediated cytotoxicity.

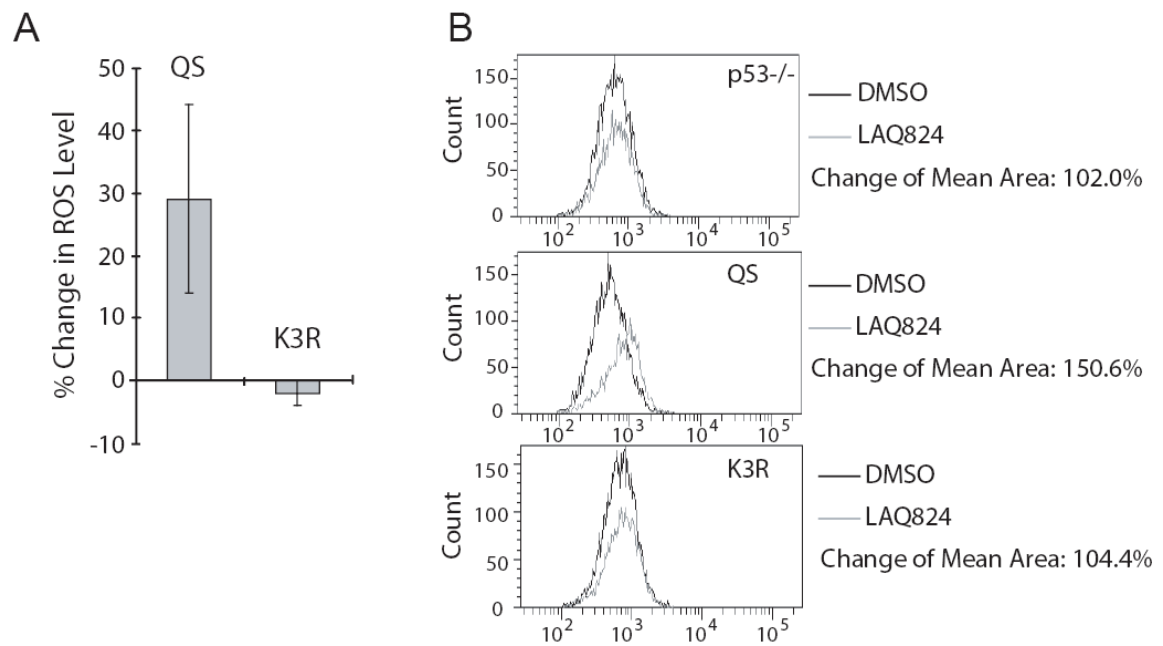


Figure 65. K3R mutation prevents HDACi-induced p53-dependent ROS production. HCT116 p53^{-/-} stably transfected with p53^{QS} or p53^{QS/K3R} were treated with 200 nM LAQ824 and intracellular ROS levels were determined by analysis of DCF staining using (A) cell-free analysis by spectrofluorometry or (B) live cell analysis by flow cytometry.

Mitochondria are the main source of cellular ROS and Bcl-2 family proteins are essential mediators of the amount of ROS production by HDACi (Ott et al., 2007a). To

determine the role of Bax in HDACi-induced ROS production and cell death, we took advantage of HCT116 Bax knockout cells (Zhang et al., 2000). Similarly to p53^{-/-} HCT116 cells, Bax^{-/-} HCT116 cells were also resistant to SAHA- or LAQ824-induced cell death regardless of normal p53 expression (Figure 66).

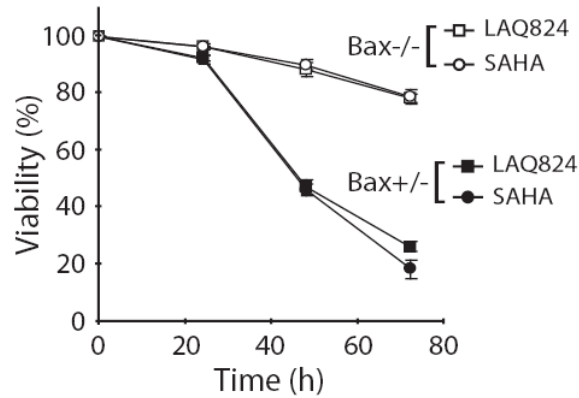


Figure 66. Bax expression promotes HDACi-induced cell death. Bax ^{+/-} and Bax ^{-/-} HCT116 cells were treated with 5 μ M SAHA or 200 nM LAQ824 for the indicated periods of time and subjected to trypan blue dye exclusion assay.

Furthermore, the upregulation of ROS levels induced by SAHA or LAQ824 was not observed in Bax-null HCT116 cells (Figures 67A & B). Taken together, these data suggest that p53 exerts its transcription-independent function upstream of Bax signaling to control ROS generation and apoptosis in response to HDACi treatment.

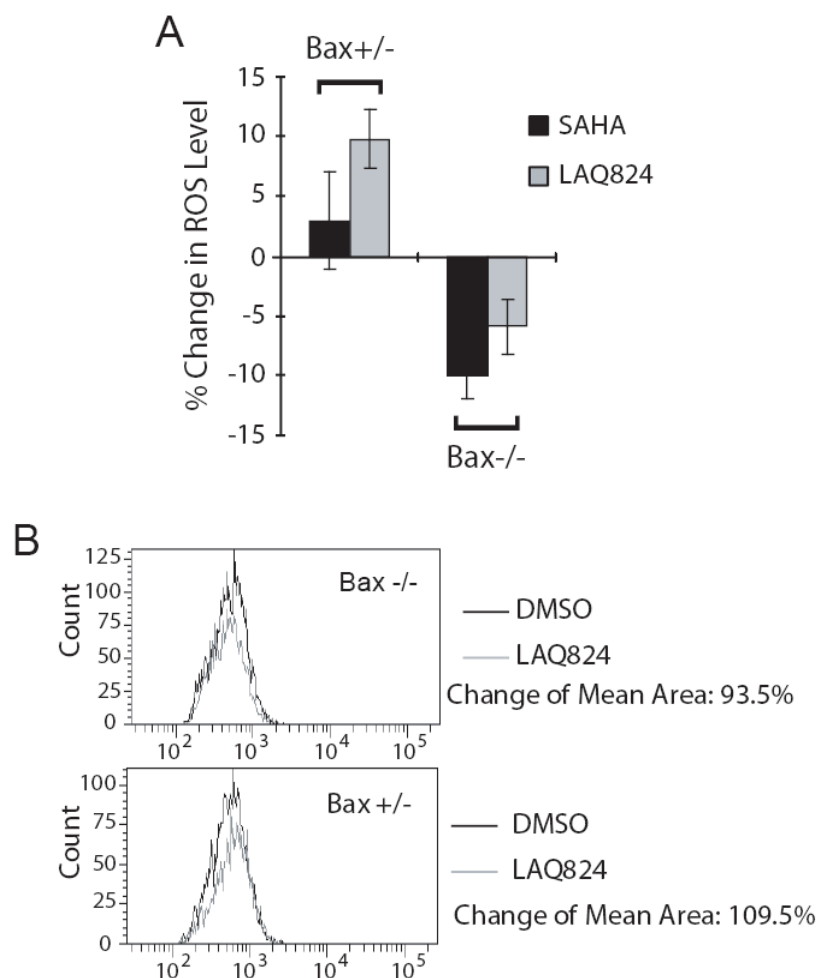


Figure 67. Bax expression promotes ROS generation in response to HDACi. Bax +/- and Bax -/- HCT116 cells were treated with 5 μ M SAHA or 200 nM LAQ824 for 12 hours and the changes in intracellular ROS level were determined by DCF using (A) cell-free analysis by spectrofluorometry or (B) live cell analysis by flow cytometry.

Acetylated p53 Binding to Ku70 Activates Bax by Disrupting the Bax/Ku70 Complex

To gain further insight into the transactivation-independent proapoptotic function of p53 in response to HDACi, we took a proteomics approach combined with the tandem affinity purification (TAP) (Wang et al., 2004) to identify p53^{QS} binding proteins. The N-terminal TAP-tagged p53^{QS} fusion protein was stably expressed in HCT116 p53^{-/-} cells

and its ability to enhance HDACi-mediated cytotoxicity was confirmed (Figure 68).

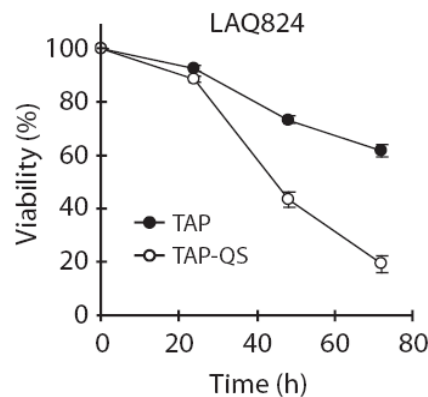


Figure 68. TAP-p53^{QS} mediated HDACi-induced cell death. HCT116 p53 ^{-/-} cells stably expressing control TAP or TAP-p53^{QS} were treated with 200 nM LAQ824 for indicated periods of time. The percentage of cell viability was determined by trypan blue dye exclusion assay.

These cells were then treated with LAQ824 and subjected to TAP purification and SDS-PAGE/mass spectrometry analysis. As a result, we identified Ku70 in the p53^{QS} complex (Figure 69). This association was not disrupted by DNase or ethidium bromide, indicating that the p53/Ku70 interaction is not mediated by DNA (data not shown). To determine if p53 acetylation is required for its binding to Ku70, we performed co-immunoprecipitation analysis in p53^{-/-} HCT116 cells stably transfected with empty control vector (Puro), Myc-p53^{QS}, or Myc-p53^{QS/K3R} constructs (Figure 70). A specific interaction between Myc-p53^{QS} and endogenous Ku70 was observed after LAQ824 treatment. However, substitution of K320/373/382 to R (K3R) in p53^{QS} did not affect this interaction, suggesting that acetylation at these lysines is dispensable for p53 binding to Ku70.

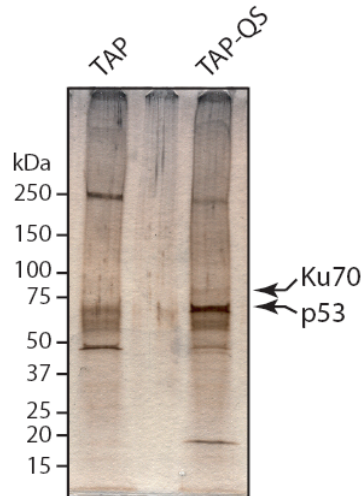


Figure 69. Identification of Ku70 in complex with TAP-p53^{QS}. Cell lysates prepared from HCT116 TAP and TAP-p53^{QS} cells treated with 200 nM LAQ824 for 20 hours were subjected to TAP purification followed by SDS-PAGE and mass spectrometry analysis.

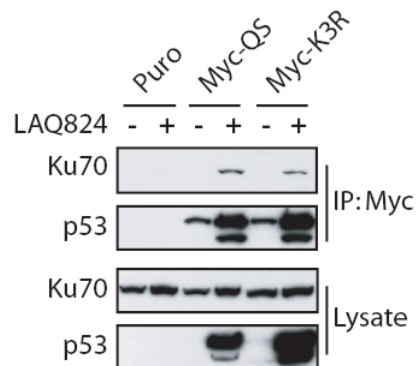


Figure 70. Ku70/p53 binding is independent of p53 acetylation at K320/373/382. HCT116 p53^{-/-} cells stably transfected with empty (Puro), Myc-p53^{QS} or Myc-p53^{QS/K3R} were treated with DMSO or 200 nM LAQ824 for 16 hours. Cell lysates were subjected to immunoprecipitation with anti-Myc antibody.

The observed increase in p53/Ku70 interaction after LAQ824 treatment is likely due to the increased protein levels of exogenous p53, because stabilization of p53 with MG132 also led to increased p53/Ku70 interaction (Figure 71). Moreover, *in vitro* GST

pull-down assays using GST-Ku70 and purified unacetylated or acetylated recombinant p53 revealed that the interaction between p53 and Ku70 is direct and acetylation-independent (Figure 72). Additionally, endogenous p53 could co-immunoprecipitated with endogenous Ku70 in both LAQ824 treated and untreated SW480 cells (Figure 73), providing further evidence that the p53/Ku70 interaction occurs at physiological protein levels in an acetylation-independent manner.

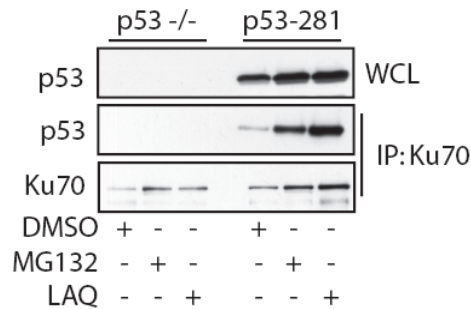


Figure 71. Increased p53 and Ku70 expression and complex formation is induced by proteasome inhibition or HDACi. H1299 p53^{-/-} and p53^{D281G} cells were treated with DMSO, 1 μ M MG132 or 200 nM LAQ824 for 24 hours and subjected to immunoprecipitation for Ku70.

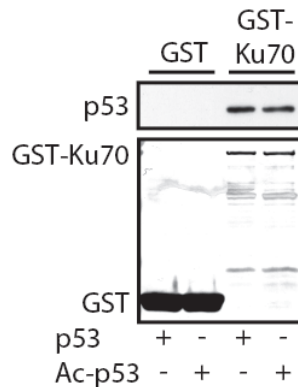


Figure 72. *In vitro* pull-down of GST-Ku70 and purified p53. Purified recombinant GST and GST-Ku70 was immobilized on Glutathione Sepharose 4B agarose

and incubated with purified non-acetylated or acetylated p53 for 2 hours, washed, and subjected to western analysis to determine p53 binding.

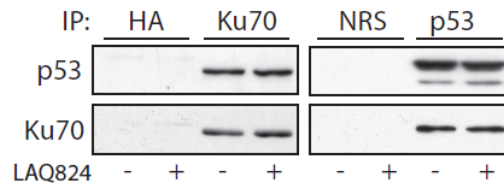


Figure 73. Ku70 associates with endogenous mutant p53 with and without HDACi treatment. SW480 cells were treated with DMSO or 200 nM LAQ824 for 24 hours and subjected to immunoprecipitation with HA or Ku70 monoclonal antibodies and normal rabbit serum (NRS) or p53 polyclonal antibodies.

The interaction between Ku70 and Bax prevents Bax conformational change and mitochondrial translocation (Cohen et al., 2004a; Li et al., 2007). Moreover, it has been shown that Ku70 acetylation reduces the Bax/Ku70 association and plays a role in HDACi-induced cell death (Subramanian et al., 2005). Thus, we speculated that the interaction between acetylated p53 and Ku70 may be involved in Bax activation induced by HDACi. After LAQ824 treatment, a drastic conformational activation of Bax measured by exposure of the 6A7 epitope was observed in p53^{QS} cells compared to p53^{QS/K3R} and Puro control cells that coincided with increased p53 acetylation (Figure 74). Analysis of Bcl-2 family proteins in these cell lines revealed that LAQ824 treatment resulted in an increase in BimEL, with a lesser extent to Bak and Mcl-1, and a slight decrease in Bcl-2 (Figure 74). However, there are no clear differences in these Bcl-2 family members between p53^{QS}, p53^{QS/K3R}, and control Puro expressing cells, suggesting that acetylated p53 has other modes of action besides regulating Bcl-2 family protein

expression for the induction of apoptosis in response to HDACi.

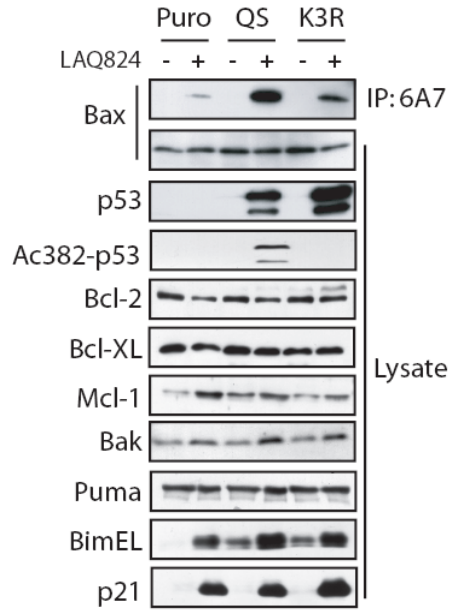


Figure 74. Acetylation of p53^{QS} is required for HDACi-induced Bax activation without impact on Bcl-2 family member expression. HCT116 p53^{-/-}, p53^{QS} and p53^{K3R} cells were treated with DMSO or 200 nM LAQ824 for 18 hours and subjected to Bax 6A7 IP and western blot analysis.

Interestingly, the Bax/Ku70 interaction was increased at 12 hours following LAQ824 treatment (Figures 75). However, while the enhanced Bax/Ku70 complex was maintained up to 36 hours in p53^{-/-} (Puro) cells, the Bax/Ku70 association was decreased at 24 hours in p53^{QS} cells. Similar results were obtained in p53^{-/-} and p53^{+/+} HCT116 cells (Figure 76).

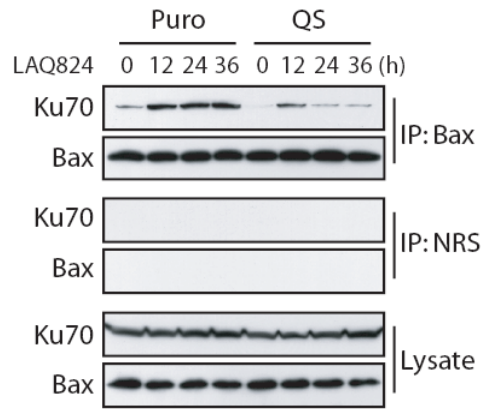


Figure 75. Ku70/Bax complex stability after HDACi treatment is regulated by p53. HCT116 p53^{-/-} cells stably expressing empty (Puro) or Myc-p53^{QS} were treated with 200 nM LAQ824 for the indicated times and subjected to immunoprecipitation with anti-Bax polyclonal antibody or control NRS.

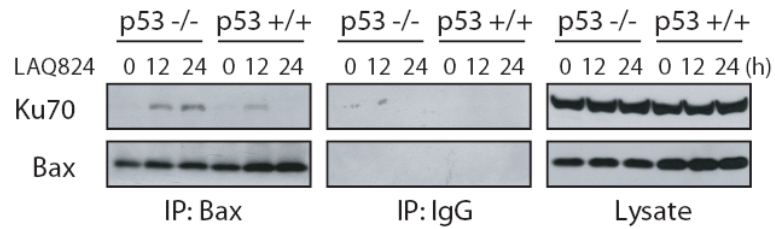


Figure 76. Endogenous wild type p53 retains the ability to disrupt the Bax/Ku70 complex. HCT116 p53^{-/-} and p53^{+/+} cells were treated with 200 nM of LAQ824 for the indicated times and subjected to immunoprecipitation with anti-Bax polyclonal antibody or control IgG.

Interestingly, the interaction between Ku70 and Bax was increased in p53^{QS/K3R} cells during LAQ824 treatment and the p53^{QS/K3R} protein was detected in the Bax/Ku70 complex after 24 hours treatment (Figure 77). These findings suggest that HDACi treatment initially promotes an increase in the interaction between Bax and Ku70, but prolonged treatment leads to the disruption of this complex probably through acetylated

p53.

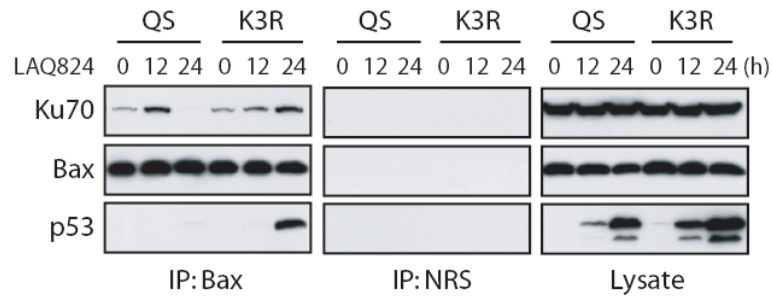


Figure 77. Acetylation deficient p53 expression prevents HDACi-induced disruption of Ku70/Bax complex. HCT116 p53^{-/-} cells stably expressing Myc-p53^{QS} or Myc-p53^{K3R} were treated with 200 nM of LAQ824 for the indicated times and subjected to immunoprecipitation with anti-Bax polyclonal antibody or control NRS.

Since p53 interacts with Ku70 independently of its acetylation status, the ability of acetylated p53 to disrupt the Bax/Ku70 interaction was assayed *in vitro*. The Bax/Ku70 immune complexes were isolated from HCT116 p53^{-/-} cell lysate and incubated with purified non-acetylated or acetylated recombinant p53 (Piluso et al., 2005) (Figure 78A). Only acetylated p53 was able to release Ku70 from the Bax/Ku70 complex when compared to control vehicle and unacetylated p53. Similar results were also obtained in a GST-Ku70 pull-down assay; acetylated p53 could prevent Bax binding to Ku70, whereas non-acetylated p53 had no effect (Figure 78B). These results clearly indicate that p53 acetylation is important for Bax release from Ku70.

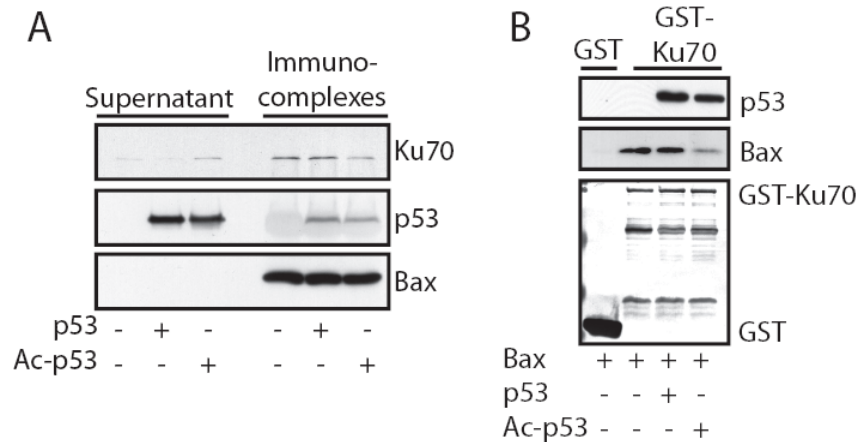


Figure 78. Purified acetylated p53 disrupts Bax/Ku70 complexes *in vitro*. (A) HCT116 p53^{-/-} cell lysate was immunoprecipitated with anti-Bax polyclonal antibody and the resulting isolated immunocomplexes were incubated with purified non-acetylated or acetylated p53 for 30 minutes. The supernatant was collected and the immunocomplexes were washed 3 times and subjected to western analysis. (B) Purified recombinant GST and GST-Ku70 were isolated by Glutathione Sepharose 4B agarose pull-down, after which purified Bax, non-acetylated or acetylated p53 were incubated together for 2 hours. Samples were washed and subjected to immunoblot analysis.

To elucidate the role of Ku70 in HDACi-induced apoptosis, H1299 p53^{-/-} and p53^{D281G} cells were infected with control shScr or shKu70 lentivirus. Knockdown of Ku70 was assayed by Western blot (Figure 79A). These cells were then treated with LAQ824 for 24 hours and subjected to apoptosis analysis with Annexin V-APC/7AAD staining and flow cytometry (Figure 79B). Knockdown of Ku70 in p53^{-/-} cells promotes an enhanced apoptotic response over shScr control cells. However, knockdown of Ku70 in p53^{D281G} cells did not significantly impact LAQ824 induced apoptosis. This result suggests that the Bax/Ku70 complex is essential to the prevention of HDACi-induced apoptosis in p53^{-/-} cells. However, when p53 is present and able to disrupt the Bax/Ku70 complex, reduction in Ku70 expression is redundant and unnecessary to promote

HDACi-induced apoptosis.

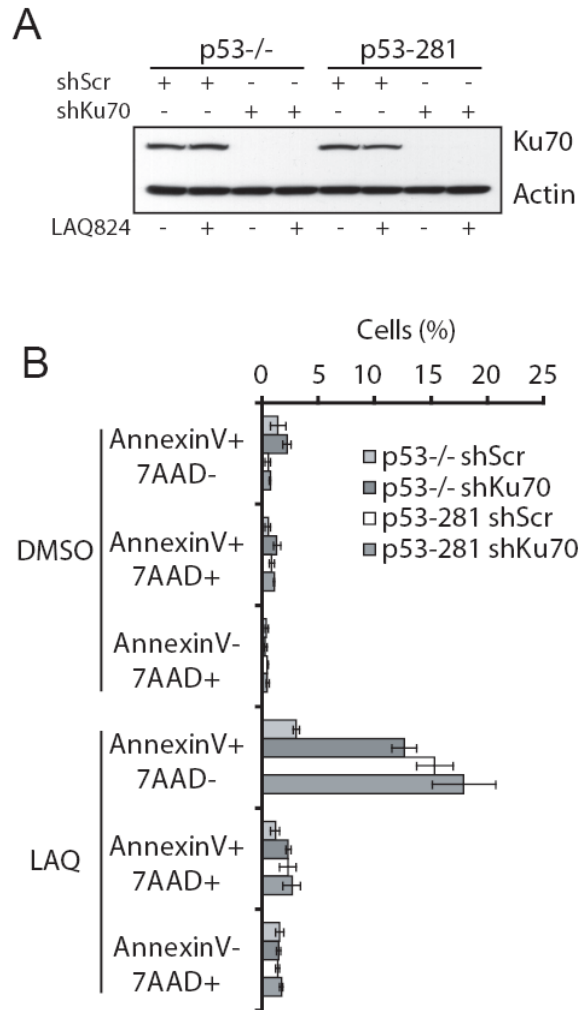


Figure 79. Ku70 knockdown preferentially enhances HDACi-induced apoptosis in p53^{-/-} cells. H1299 p53^{-/-} and p53^{D281G} cells were infected with shScr or shKu70 lentivirus. 24 hours after infection cells were treated with DMSO or 200 nM LAQ for an additional 24 hours and analyzed for (A) Ku70 knockdown by western blot and (B) apoptotic response measured by Annexin V-APC/7AAD staining and flow cytometry. Data represented as mean \pm SD, DMSO treated n=3, LAQ treated n=5.

To determine the cellular localization of the Bax/Ku70 complex, co-immunoprecipitation experiments were performed using heavy membrane, cytosolic, and

nuclear fractions isolated from p53^{-/-} HCT116 cells transfected with control empty vector (Puro), or Myc-tagged p53^{QS} or p53^{QS/K3R} (Figure 80). The Bax/Ku70 complex was increased in both cytosolic and nuclear fractions of Puro and p53^{QS/K3R} cells after LAQ824 treatment. Since the complex formation was only assayed at 24 hours after treatment, the accumulation of the Bax/Ku70 complex was not observed in p53^{QS} cells as it was at 12 hours seen in Figure 74. Furthermore, the Ku70/p53 complex was most readily detected in the nuclear fraction corresponding to the prominent localization of p53 and Ku70 to this cellular compartment. However, p53 could also be found localized to the cytosolic and heavy membrane fractions and in a complex with Ku70 at reduced levels compared to that in the nuclear fraction after LAQ824 treatment. The localization of p53^{QS}, Bax, and Ku70 was also confirmed by immunofluorescence (Figures 81A & B).

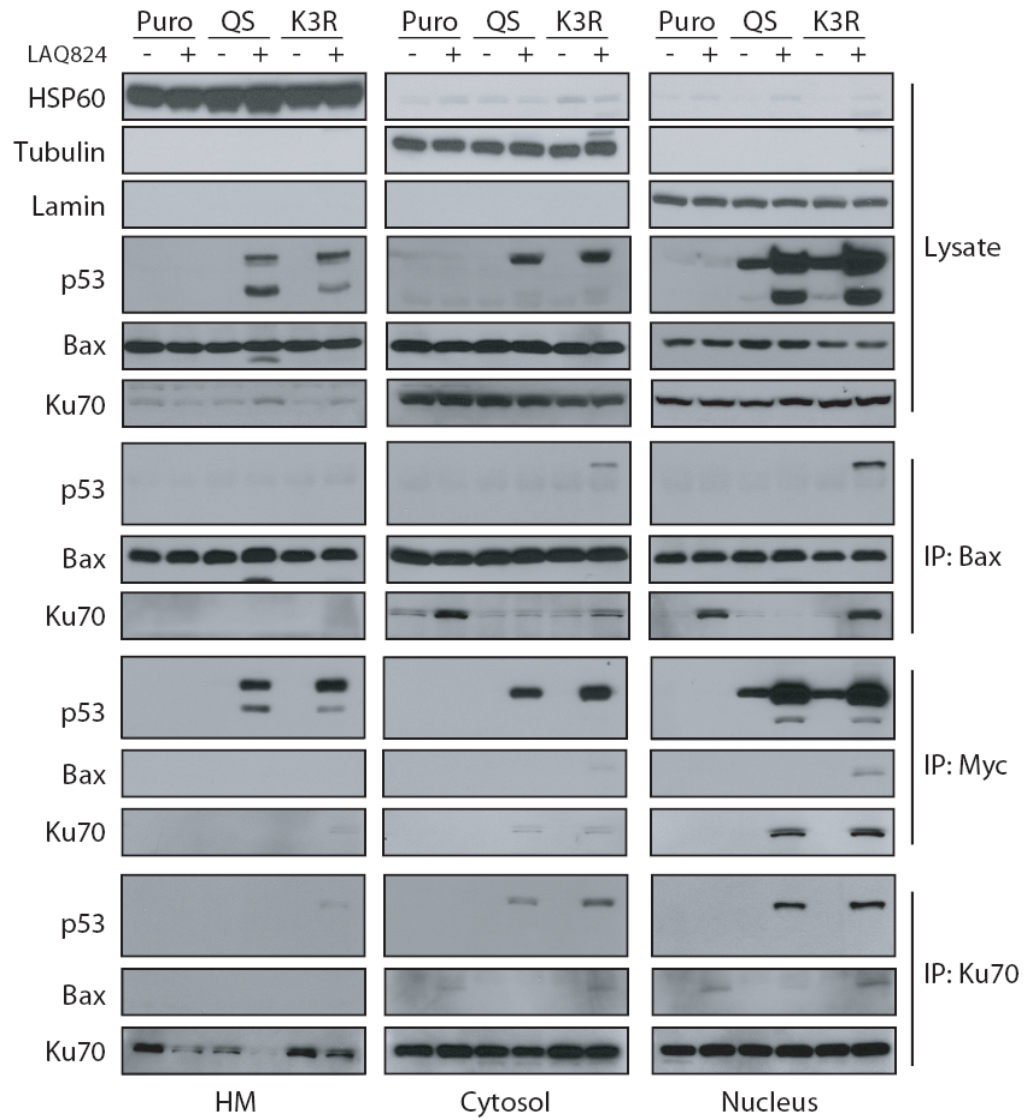


Figure 80. Cellular localization of p53/Ku70/Bax interactions. (A) HCT116 p53^{-/-} cells stably expressing Puro, Myc-p53^{QS} or Myc-p53^{QS/K3R} were treated with DMSO or 200 nM LAQ824 for 24 hours and subjected to cell fractionation and immunoprecipitation with the indicated antibodies.

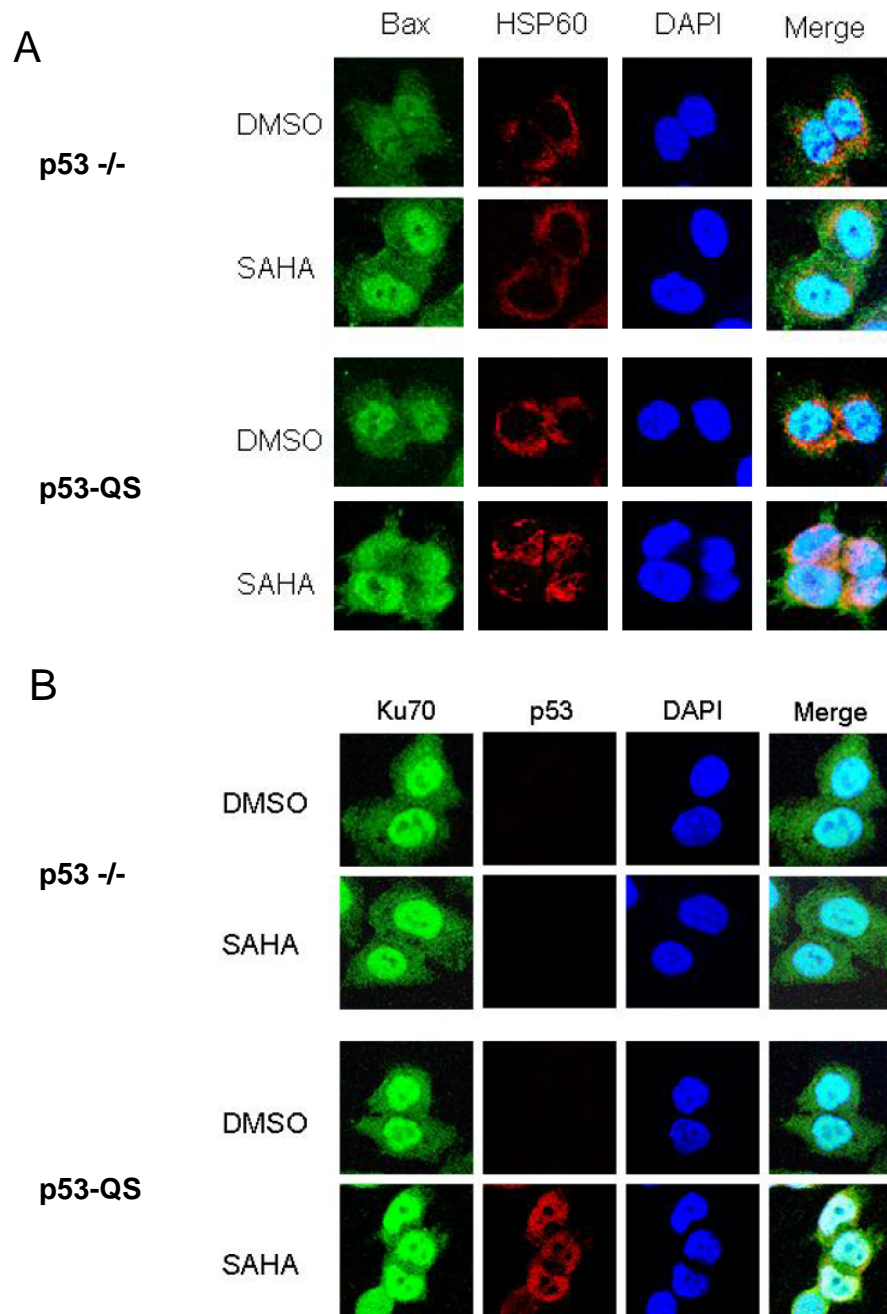


Figure 81. Cellular localization of p53, Ku70 and Bax in HCT116 cells. (A) HCT116 p53^{-/-} or p53^{QS} cells were treated with 5 μ M SAHA for 18 hours and then immunostained for Bax and HSP60 with DAPI counter staining. (B) HCT116 p53^{-/-} or p53^{QS} cells were treated with 5 μ M SAHA for 18 hours and then immunostained for Ku70 and p53 with DAPI counter staining.

To determine whether p53^{QS} affects the intracellular redistribution of Bax after HDACi treatment, we performed subcellular fractionation and immunoblot analysis. Ku70 localization was not affected by p53^{QS} after HDACi treatment, while the nuclear Bax was decreased in p53^{QS} cells but not in p53^{-/-} cells (Figure 82). Conversely, the cytosolic Bax was increased in p53^{QS} cells after HDACi treatment. These results support the hypothesis that Bax dissociation from Ku70 and subsequent translocation to the cytoplasm after HDACi treatment is mediated by acetylated p53. Consistent with the retained association of Ku70 with Bax, HDACi-induced Bax conformational activation at the mitochondria was attenuated in p53^{-/-} and p53^{QS/K3R} compared to p53^{QS} cells (Figure 83A), which correlated with an apparent increase in Bax translocation to the mitochondrial fraction specifically in HDACi treated p53^{QS} cells (Figure 83B).

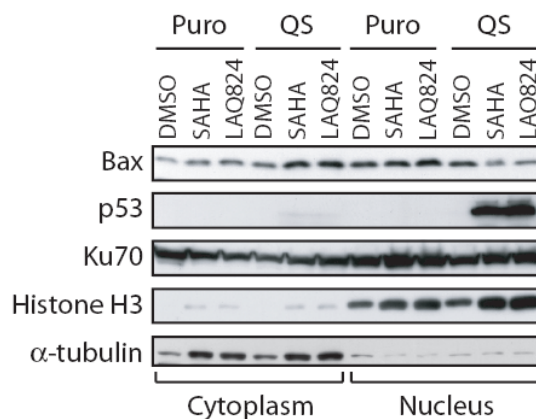


Figure 82. p53-dependent HDACi-induced Bax translocation from the nucleus to the cytoplasm. HCT116 p53^{-/-} cells stably expressing Puro or p53^{QS} were treated with DMSO, 5 μM SAHA or 200 nM LAQ824 for 18 hours and subjected to subcellular fractionation and immunoblot analysis.

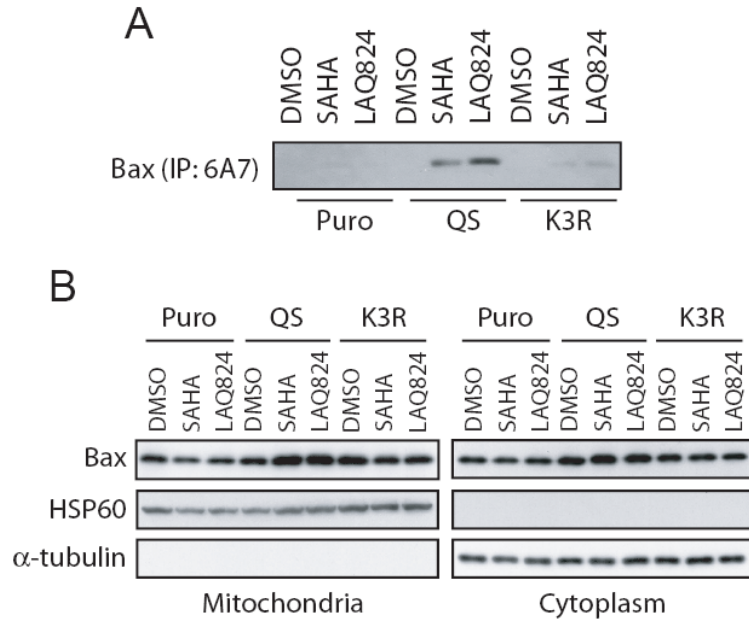


Figure 83. HDACi-induced acetylated p53-dependent Bax activation and mitochondrial accumulation. (A) Immunoprecipitation using Bax 6A7 antibody in mitochondrial fractions of HCT116 p53^{-/-}, p53^{QS} and p53^{QS/K3R} cells treated with DMSO, 5 μ M SAHA or 200 nM LAQ824 for 18 hours. (B) HCT116 p53^{-/-} cells stably transfected with Puro, Myc-p53^{QS} or Myc-p53^{QS/K3R} were treated with DMSO, 5 μ M SAHA or 200 nM LAQ824 for 16 hours and subjected to subcellular fractionation and immunoblot analysis.

Discussion

HDACi represent a new class of chemotherapeutic agents that have shown promise in pre-clinical and clinical trials and work by modifying the acetylation status of histone and non-histone proteins. Acetylation is accepted as an important posttranslational modification that impacts protein structure and/or function but relatively few non-histone proteins have been identified as regulated by acetylation. Of those identified are several important apoptotic molecules such as Ku70, p53, Rb, and p73 (Chan et al., 2001; Cohen et al., 2004a; Costanzo et al., 2002). The findings presented in

this report indicate that HDACi can induce apoptosis by a p53 transactivation-independent apoptotic mechanism. Expression of various mutants of p53 in p53^{-/-} backgrounds enhances HDACi-induced apoptosis, and knockdown of endogenous mutant p53 results in an abrogated apoptotic response. Both mutant and wild type p53 bind Ku70 and promote Bax dissociation from Ku70 in an acetylation dependent manner. Additionally, HDACi can induce substantial apoptosis in p53^{-/-} but not mutant p53 expressing cells when Ku70 is knocked down. Therefore, we propose a model where the interaction between Ku70 and p53 is acetylation independent; however, acetylation of p53 is absolutely required for Bax dissociation from Ku70, conformational activation, and enhanced apoptosis upon HDACi treatment (Figure 84). Given that p53 is frequently mutated in human cancers, our findings that mutant p53 sensitizes cancer cells to HDACi-induced apoptosis via Bax activation further explain why this class of inhibitors displays preferential anti-neoplastic potential.

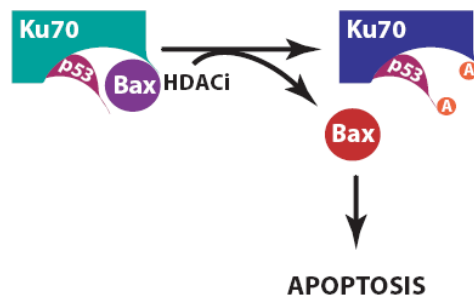


Figure 84. Proposed model for p53/Ku70/Bax interaction disrupted by HDACi-induced acetylation. In normal resting cells p53/Ku70/Bax can likely be found in complex. After HDACi, Bax is displaced from Ku70 by acetylated p53 and becomes activated to induce apoptosis.

Transcription-Independent p53 Mechanism of HDACi-induced Apoptosis

The transcriptional activity of p53 is able to efficiently enhance apoptosis in response to various cellular stresses. Genotoxic stresses result in activation of DNA damage response pathways that activate kinases such as ATM, ATR, CHK1 and CHK2 or methyltransferases such as Set7/9 that respectively phosphorylate or methylate p53 which in turn promotes the acetylation of p53 at C-terminal lysines resulting in enhanced protein stability and sequence specific DNA binding (Ivanov et al., 2007; Toledo and Wahl, 2006). However, through the course of our experiments into the nature of p53-dependent HDACi-induced apoptosis we found that the transcriptional activity of p53 was dispensable but acetylation of C-terminal lysines was not. There are conflicting reports as to what role p53 transcription has on HDACi-induced apoptosis. Much of this could be attributed to global changes in transcription due mainly because of histone acetylation and maybe less so through acetylation of other factors such as p53 or other transcription factors. Indeed, our experiments have found that expression of wild type p53 marginally enhanced HDACi-induced caspase-3 activity over the response from expression of mutant p53^{QS} (Figure 46), although this does not necessarily correlate exactly with the cell death response (Figure 45). This could indicate several possible scenarios. First, expression of wild type p53 could preferentially promote apoptosis through transcriptional regulation. However, our results have shown that wild type p53 still functions to disrupt the Bax/Ku70 complex when acetylated by HDACi treatment, suggesting that this pathway is active even in the presence of functional transactivation activity. This gives favor to the alternative scenario where wild type acetylated p53-dependent HDACi-induced apoptosis functions in a predominantly transcription-

independent manner to illicit such a response, likely through disruption of the Bax/Ku70 complex as shown above. This would explain why there was limited additive effect on HDACi-induced caspase-3 activation in wild type p53 over p53^{QS} expressing cells. However, this does not explain why there is enhanced viability in wild type p53 cells compared to p53^{QS} after HDACi treatment. This could possibly be caused by the increased stability of p53^{QS} over wild type p53 as the Mdm2 binding site is disrupted in this mutant. Nonetheless, more experiments will be needed to rectify the roles of acetylated wild type p53 in transcription-dependent and independent HDACi-induced apoptosis.

We sought to confirm that mutant p53^{QS} lacks HDACi-induced transcriptional activity by luciferase reporter gene assays (Figure 44). Expression of wild type p53 dramatically enhanced transcription from the p21 and MDM2 promoters, but p53^{QS} also exhibited low levels of target promoter activation compared to p53^{-/-} cells. However, this residual transactivation potential of p53^{QS} likely plays a minor role in HDACi-induced apoptosis for several reasons. First, microarray analysis revealed that there was no specific increase in p53 responsive target gene expression after treatment with HDACi (data not shown). The expression of p21 was also found to be similar between p53^{-/-}, p53^{QS}, and p53^{QS/K3R} stably expressing cell lines (Figure 74). Indeed, it has been shown that p21 expression is enhanced by HDACi in a p53-independent manner (Huang et al., 2000). Although p53^{QS} has been reported to retain the potential for transcriptional activation of Bax and repression of Bcl-2 (Johnson et al., 2005), we were unable to detect changes in apoptotic proteins controlled by p53^{QS} such as Bax, Bcl-2, and Puma after HDACi treatment in (Figure 74). Additionally, studies with transfection of prominent

naturally occurring p53 mutants into p53 deficient HCT116, K562, and H1299 cells, and knockdown of endogenous mutant p53 in SW480 and HT-29 cells further support our hypothesis that p53 retains a transcription-independent proapoptotic function mediated by acetylation.

p53 Localization and HDACi-Induced Apoptosis

Although it is widely accepted that p53 has a transcription-independent mechanism of apoptosis induction, several controversies remain. For instance, it has been shown that p53 translocates to mitochondria where it interacts with anti- or proapoptotic Bcl-2 family proteins including Bcl-XL, Bcl-2, and Bak to induce Bax and Bak activation, thereby leading to the release of cytochrome c from mitochondria to the cytoplasm (Chipuk et al., 2004; Deng et al., 2006; Leu et al., 2004; Mihara et al., 2003). However, other reports suggest that the level of mitochondrial p53 does not correlate with the level of apoptosis (Essmann et al., 2005; Mahyar-Roemer et al., 2004). Upon HDACi treatment, relatively low levels of p53^{QS} and p53^{QS/K3R} were detected in the mitochondrial fraction (Figure 80). Furthermore, there were no differences between wild type p53, p53^{QS}, and p53^{QS/K3R} for their ability to interact with Bcl-XL (Figure 85), even though p53^{QS/K3R} cannot significantly enhance HDACi-induced apoptosis, suggesting that the mitochondrial translocation of mutant p53 may not play a major role in HDACi-mediated apoptosis.

However, the small increase in HDACi-induced apoptosis in p53^{QS/K3R} over p53^{-/-} cells may rely on its ability to bind Bcl-XL at the mitochondria. Alternatively, this observation could possibly be due to another mechanism such as residual transcriptional

activity of p53^{QS/K3R}. Although our results suggest that p53^{QS} and p53^{QS/K3R} have no impact Bcl-2 family or p21 expression profiles in response to HDACi treatment compared to p53^{-/-} cells, other unexamined proteins may be affected which could theoretically also lead to the slight increase in apoptosis seen in the p53^{QS/K3R} cell line. The exact mechanism by which this occurs remains to be resolved.

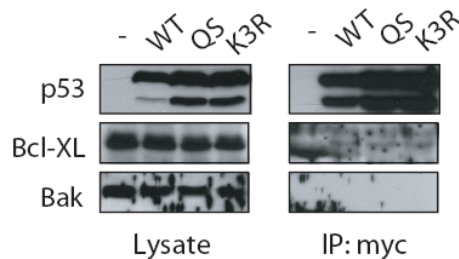


Figure 85. Wild type and mutant p53 interaction with Bcl-XL and Bak. p53^{-/-} HCT116 cells were transiently transfected with the indicated p53 expression plasmids. 36 hours after transfection, the cells were treated with LAQ for additional 12 hours. The cells were then lysed and subjected to IP with myc monoclonal antibody, followed by immunoblot analysis with anti-Bcl-XL or Bak polyclonal antibodies.

As a transcription factor, p53 principally localizes in the nucleus, but certain apoptotic stimuli can promote p53 accumulation in the cytosolic and mitochondrial fractions. Ku70 is an important mainly nuclear protein involved in non-homologous end joining DNA repair and V(D)J recombination (Gu et al., 1997). Therefore, it is not surprising that the nucleus is the compartment that contains the highest concentration of p53 associated with Ku70 (Figure 80). Alternatively, Bax has been classified as a predominantly cytosolic protein that loosely associates with the mitochondrial membrane in unstressed cells. Enigmatically, Bax has also been classified as a nuclear protein and

under certain stresses interacts with p53 (Nishita et al., 1998; Raffo et al., 2000). Similarly, our results indicate that a substantial amount of Bax can be found in the nucleus of HCT116 cells (Figures 80 and 81A). It seems that HDACi-induced p53 acetylation disrupts the Ku70/Bax complex, which results in Bax conformational change and translocation from the nucleus to the cytoplasm. However, cytosolic p53 is also equally capable of disrupting Bax from Ku70. The individual contributions of Ku70/Bax dissociation in the separate cellular compartments remain to be determined.

Bax has been traditionally characterized as a cytoplasmic and mitochondrial protein, however increasing evidence has suggested that Bax can also be found in the nucleus but its function there is almost completely unknown. Here we provide evidence that nuclear Bax activation can be initiated in response to HDACi treatment through displacement from Ku70 by acetylated p53 and translocation to the cytoplasm. Nuclear Bax localization may predispose cells to enhanced HDACi-induced apoptosis as Ku70/Bax interactions there have an increased probability of dissociation due to p53 enrichment. This localization of Bax may also have impacts on other proapoptotic signals originating in the nucleus such as genotoxic stresses. However, future research is still required to fully understand this seemingly misplaced population of Bax.

p53 Acetylation and Bax/Ku70 Disruption

LAQ824 treatment results in a transient increase in the association between Ku70 and Bax (Figures 75-78). This phenomenon could be due to several reasons. First, this apoptotic stimulus may encourage a cellular response designed to prevent Bax activation by sequestration through Ku70 binding. The increased association could be caused by a

variety of different mechanisms such as posttranslational modifications or intracellular distribution. Another intriguing possibility is that the increase in Ku70/Bax complex could serve a dual role as Ku70 promotes the deubiquitination of Bax in addition to its sequestration (Amsel et al., 2008). Thus, this interaction could prime cells for apoptosis by increasing the pool of unmodified Bax able to promote apoptosis after release from Ku70. In either case, prolonged HDACi treatment that results in the disruption of the Ku70/Bax complex can enhance the apoptotic response.

Expression of wild type and mutant p53 capable of being acetylated promotes the dissociation of Bax from Ku70 after a prolonged HDACi treatment (Figures 75-78). Moreover, acetylated but not unacetylated recombinant p53 is able to disrupt the Ku70/Bax complex in vitro (Figures 78A & B). However, the acetylation deficient p53^{QS/K3R} mutant retains the ability to interact with Ku70 and accumulates in the Ku70/Bax complex during HDACi treatment (Figures 77 and 80). Since acetylation of p53 alters its structure (Giordano and Avantaggiati, 1999), it is plausible that once p53 is acetylated changes in conformation lead to competition for the Bax binding site on Ku70. Acetylation and altered structure of p53 could also lead to conformational changes in Ku70, which may depend its own acetylation, and the release of Bax as previously proposed (Cohen et al., 2004a). Furthermore, past studies into the acetylation of Ku70 and the regulation of apoptosis were carried out primarily in 293T cells which would preclude p53 transactivational activity but not necessarily its transcriptional-independent role in HDACi-induced apoptosis (Cohen et al., 2004a; Li et al., 2007). Our results suggest that the cellular status of both p53 and Ku70 determines apoptotic responses (Figure 79B) and is warrant to be further evaluated as biomarkers for clinical therapeutic

response to HDACi such as SAHA and LAQ824. Additional assays designed to determine how acetylated p53 affects the Ku70/Bax complex at the structural level will give additional insights into this novel interplay between proteins with hopes of developing advanced therapeutic strategies to benefit cancer patients.

Materials and Methods

Cell Culture and Transfection

HCT116 cells were grown in McCoy's 5A medium, K562, SW480 and HT-29 cells were grown in RPMI, and H1299 and MEFs were grown in DMEM. Medium was supplemented with 10% FBS and 1X penicillin/streptomycin. MEFs p53^{-/-} and p53-R172H/R172H were a kind gift from Guillermina Lozano and were described previously (Lang et al., 2004). Cell transfection using Lipofectamine 2000 (Invitrogen) was described previously (Yamaguchi et al., 2004). The plasmids expressing p53 WT, QS, R175H, R273H, and D281G mutants were described previously (Hinds et al., 1990; Yamaguchi et al., 2004). The p53^{QS/K3R} mutant was prepared by two-step PCR and subcloned into pcDNA3-Myc vector. To establish stable transfectants, plasmids were co-transfected with pBabe-puro or pEF6-Myc-His (Invitrogen) empty vector and selection was performed with puromycin (puro) or blasticidin S HCl (Bsd). The p53^{R175H} lysine mutants were prepared by site directed mutagenesis (Stratagene) using pcDNA3-p53 as the template, cloned into the retroviral pKI vector between the BglII/XhoI restriction sites and transfected into 293 Amphi cells to produce recombinant retroviruses. The lentiviral pLK0.1 empty, scrambled, Ku70 shRNA (TRCN0000039611) and p53 shRNA (TRCN0000010814) constructs were purchased from Sigma. The shRNA lentivirus was

produced in 293FT cells using the ViraPower kit from Invitrogen as per the manufacture's recommendations.

Reagents

LAQ824 and SAHA were kindly provided by Novartis Pharmaceuticals Inc. (East Hanover, NJ) and Merck & Co., Inc. (Flemington, NJ), respectively. Anti-Ku70 monoclonal and polyclonal antibodies and anti-Myc polyclonal antibodies were purchased from Santa Cruz Biotechnology. Anti-p53 (CM1) polyclonal and monoclonal (DO-1) antibodies, anti-Bax polyclonal and monoclonal antibodies, anti-actin, tubulin and Myc monoclonal antibodies were described previously (Yamaguchi et al., 2004; Yamaguchi and Wang, 2006). Anti-Myc or Flag agarose beads were purchased from Sigma. Anti-histone H3 and anti-acetylated p53 (K382) antibodies were purchased from Cell Signaling. Anti-HSP60 and anti-Lamin B antibodies were purchased from BD Biosciences and Calbiochem, respectively.

Tandem Affinity Purification of p53^{QS} Binding Protein

The p53^{QS} cDNA was subcloned into the EcoRI and XhoI sites of pcDNA3-TAP vector (Wang et al., 2004). The N-terminal TAP-tagged p53^{QS} was stably expressed in p53^{-/-} HCT116 cells and treated with 200 nM LAQ824 for 20 hours. The cells were lysed in TAP lysis buffer (0.5% Triton X-100, 0.5 mM DTT, protease inhibitor cocktail, phosphatase inhibitor cocktail, 100 nM LAQ824) and sonicated. After centrifugation, the cell lysate was subjected to purification/mass spectrometry analysis as described previously (Wang et al., 2004).

GST-Ku70/Bax/p53 In Vitro Binding Assay

GST or GST-Ku70 conjugated Glutathione Sepharose 4B were resuspended in 1% Chaps buffer and 100 μ l were aliquoted into separate tubes. Approximately 0.5 μ g Bax was added with or without 200 ng purified non-acetylated or acetylated p53 and brought up to a total volume of 300 μ l with 1% Chaps buffer, then incubated with rocking at 4° C for 2 hours. Beads were spun down at 1,000g for 1 minute, supernatant was removed and beads were washed 3X with 1 mL ice-cold 1% Chaps buffer. Laemmli buffer was then added to the beads, boiled for 3 minutes, vortexed, and then run on an SDS-PAGE.

Immunoprecipitation

Immunoprecipitation of active Bax by anti-Bax 6A7 antibody and co-immunoprecipitation of Ku70 and Bax were carried out as described previously (Li et al., 2007; Yamaguchi et al., 2004). For co-immunoprecipitation of Ku70 and p53, cells were treated or untreated with HDACi for the indicated times and lysed in 1% Chaps lysis buffer. After sonication and centrifugation, the lysates were immunoprecipitated with Myc monoclonal, Ku70 monoclonal or p53 (CM1) polyclonal antibodies followed by immunoblot analysis with the indicated antibodies. For Figure 80, 500 μ g proteins were incubated overnight with 4 μ l of anti-Bax serum, 1 μ g of Myc (Sigma) or 1 μ g of Ku70 (Santa Cruz) polyclonal antibodies. Samples were further incubated with 15 μ l of Protein A agarose at 4°C for 2 hours, washed three times and analyzed by immunoblot.

Subcellular Fractionation

To isolate the mitochondria-enriched heavy membrane (HM) fraction, cells were homogenized in isotonic mitochondrial buffer (210 mM sucrose, 70 mM mannitol, 10 mM Hepes pH 7.4, 1 mM EDTA) containing protease inhibitor cocktail and centrifuged at $1,000 \times g$ for 10 min to discard nuclei and unbroken cells. The resulting supernatant was centrifuged at $10,000 \times g$ for 15 min to pellet the heavy membrane fraction and the supernatant was centrifuged further at $100,000 \times g$ for 30 min to obtain cytosolic fraction, which was transferred to new tubes and the NaCl and CHAPS concentrations were adjusted to 150 mM and 1%, respectively. To isolate the nuclear fraction, cells were resuspended in 5 volume of Buffer A (10 mM Tris-HCl pH8.0, 10 mM NaCl, 1 mM EDTA, 0.5 mM DTT, and protease inhibitor cocktail). After incubation on ice for 15 min, Triton X-100 was added to a final concentration of 0.2%. The lysates were then vortexed for 5 sec followed by centrifugation at $10,000 \times g$ for 10min. The crude nuclear pellets were washed with Buffer A twice, resuspended in Buffer B (10 mM Tris-HCl pH8.0, 150 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 0.5% Triton X-100, and protease inhibitor cocktail) and sonicated. The sonicated lysates were then centrifuged at $15,000 \times g$ for 10 min and the supernatant was used as nuclear fraction. For Ku70/Bax/p53 co-immunoprecipitation experiments, the nuclear and heavy membrane fractions were lysed in 1% Chaps and sonicated.

Caspase Assay, ROS Measurement and Annexin V staining

Caspase-3 activity was measured using the fluorometric caspase-3 activity assay kit according to the manufacturer's instructions (Sigma). ROS level was determined as

described previously (Chandel et al., 2001). Briefly, cells treated or untreated with HDACi for 12 to 16 hours were incubated in PBS containing 10 μ M dichlorodihydrofluorescein diacetate (H₂DCFDA, Molecular Probes) for 10 min at room temperature and lysed in ROS lysis buffer. H₂DCFDA oxidation into DCF was measured using a spectrofluorometer (excitation 485 nm, emission 535 nm). Data are normalized by protein concentration and expressed as relative change from untreated cells. For flow cytometry measurement of ROS, cells were treated or untreated with 200nM of LAQ for 14 hours then trypsinized, washed with PBS once and incubated in PBS containing 10 μ M H₂DCFDA for 20 minutes at room temperature. The cells were analyzed by flow cytometry and the results were analyzed by BD FACSDiva software (BD Bioscience). The results were shown as a change of mean area of fluorescence signals (measured by the software). Annexin V-FITC/PI or Annexin V-APC/7AAD staining was completed as per the manufacturer's recommendations (BD Biosciences) and analyzed by BD FACSCalibur and FlowJo software.

Luciferase Assays

Cells were seeded on 24-well plates and grown to 70% confluency. Cells were then transfected with 100 ng of reporter constructs and 10 ng internal control renilla luciferase internal control plasmid pRL-SV40 using Lipofectamine2000 transfection protocol. 24 hours after transfection cells were treated with 5 μ M SAHA or 200 nM LAQ for an additional 12 hours. Cells were then lysed and subjected to Dual-Luciferase assay as per the manufacturer's recommendations (Promega).

Protein Purification

Human Ku70 cDNA was subcloned into pGEX-4T-1 vector using BamHI and XhoI restriction enzyme cloning sites. pGEX-4T-1-Ku70 or empty vector was transformed into the BL21 strain of *E. coli* and grown overnight at 37° C in LB. One mL of this turbid culture was used to inoculate 200 mL of LB which was then grown to an OD₆₀₀ between 0.6-0.8. At this time, IPTG was added to a final concentration of 0.5 mM and then incubated for 6 hours at room temperature with shaking. *E. coli* was isolated by centrifugation at 25,000g for 10 minutes and resuspended in 2 mL PBS supplemented with protease inhibitors (Sigma). The resulting *E. coli* suspension was sonicated, and Triton X-100 was added to a final concentration of 1% and incubated for 30 minutes at room temperature with rocking. Cell debris was removed by centrifugation at 20,000g for 20 minutes at 4° C. The supernatant was collected and incubated with Glutathione Sepharose 4B (Amersham) for 30 minutes at room temperature with rocking. After wash 3X with 1 mL PBS and 1X with 1 mL 1% Chaps lysis buffer, the GST protein was eluted with 10 mM of reduced glutathione in 50 mM Tris-HCl (pH=8.0) and dialyzed against PBS (pH=7.4).

Recombinant full-length Bax protein was purified using the IMPACT system (New England Biolabs) as previously described (Suzuki et al., 2000). The pYTB1-Bax construct, which expresses a Bax and intein tag fusion protein, was transformed into *E. coli* BL21. Recombinant proteins were isolated by chitin affinity chromatography according to the manufacturer's protocol. The Bax protein was cleaved off from the intein tag by dithiothreitol and dialyzed in 10 mM HEPES (pH=7.4), 100 mM NaCl, 0.2 mM EDTA.

Purification of non-acetylated and acetylated p53 proteins was performed as previously described (Piluso et al., 2005). Briefly, *Sf21* armyworm cells were co-infected with HA-p53 and either empty or p300-6XHis baculoviruses. Forty-eight hours after infection, nuclear lysates were prepared, and total HA-p53 was purified with 12CA5 monoclonal antibody conjugated to protein A-Sepharose (Pierce) and washed three times in ten volumes of buffer D (20mM Hepes, pH 7.9, 20% glycerol, and 0.2mM EDTA) containing 0.5 M KCl. A final fourth wash used buffer D containing 0.1 M KCl. HA-p53 was then eluted with synthetic 12CA5 peptide. HA-p53 isolated from HA-p53/p300-6XHis co-infected lysate was subsequently depleted of non-acetylated p53 using Pab421-protein A beads that specifically recognizes non-acetylated p53.

In Vitro Disruption of Endogenous Bax/Ku70 Complex

Bax was immunoprecipitated from HCT116 p53^{-/-} cells using polyclonal Bax antibody immobilized on Protein A agarose beads. Immune complexes were washed 3X with 1mL ice-cold 1% Chaps lysis buffer and then resuspended in 20 µl 1% Chaps lysis buffer. Approximately 200 ng purified non-acetylated or acetylated p53 was then added and incubated with shaking at room temperature for 10 minutes. The immune complexes were then spun down and the supernatant was collected to detect unbound proteins. The beads were again washed 3X with 1 mL ice-cold 1% Chaps lysis buffer and then resuspended in Laemmli buffer. The supernatant and immune complexes were analyzed for released and bound proteins, respectively.

Immunostaining

Cells were cultured in chamber slides and treated with SAHA for 18 hours. The cells were then washed with PBS, fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.5% Triton X-100 for 15 min, and incubated with 3% BSA for 1 h. The cells were then incubated with the primary antibodies, anti-Myc monoclonal antibody (Sigma) plus anti-Ku70 polyclonal antibody (Santa Cruz), or anti-HSP60 monoclonal antibody (BD Bioscience) plus anti-Bax polyclonal antibody (Cell signaling), overnight at 4 °C, washed, and further incubated with anti-mouse IgG-FITC and anti-rabbit IgG-Texas Red secondary antibodies. Nuclei were stained with mount medium containing DAPI. Fluorescence images were captured with a fluorescence microscope.

Chapter Five: Scientific Significance & Future Directions

Interestingly, even with the vast amounts of data accumulated on the topic of Bax activation in the regulation of apoptosis, there persists a relative uncertainty as to the exact molecular mechanisms that govern the process. One of the most prominent questions is how can BH3-only molecules such as Bim directly promote Bax activation if the molecular interaction between the proteins is undetectable in most systems? This could be due to a transient ‘hit-and-run’ interaction where no stable complex is created. This may also be the byproduct of the assay system employed. However, other factors such as membrane lipid composition, structural forces and membrane curvature may also play a role. The one thing that is clear is that Bax is an exquisitely regulated protein. Cells seem to exist on the edge of apoptosis induction with antiapoptotic molecules preventing the aberrant activation of Bax. When an apoptotic insult does occur and the insult is strong enough, Bax can become activated by a number of different proteins and mechanisms to initiate cell death. Although most researchers have been hoping to discover an all-encompassing pathway of apoptosis to exploit, it has become clear that apoptosis depends on many different factors such as cell type, apoptotic stimulus and the microenvironment. Research into the multiple modes of Bax regulation will therefore likely be paired with individualized treatments in the future based on known pathways of Bax activation, the complement of Bax regulatory elements expressed in the target cell population, and the mechanism of action of the drug.

Src Regulation of Anoikis

Contextual clues from the extracellular matrix provide survival signals to adherent cells, and once the cell becomes dissociated from these signals it dies by anoikis. Oncogenic signaling can lead to the inhibition of anoikis, a necessary regulatory mechanism to overcome in order for cancers of attachment dependent origin to metastasize. Anoikis inhibition can promote cell survival after detachment from their normal ECM, while detached in the circulatory and lymphatic system and after attachment to the incorrect ECM. Indeed, the research presented above illustrates the robust ability of Src oncogenic kinase to prevent anoikis through several independent mechanisms that ultimately converge on regulation of Bax activation.

Src activation of classic Akt and Erk1/2 survival signaling pathways regulate the expression profiles of Bcl-2 family proteins Mcl-1 and Bim. Specifically, we have found that forced detachment of non-transformed NIH3T3 cells results in a dramatic decrease in Mcl-1 and increase in Bim expression. Src transformation represses these two events which promotes cell survival during detachment. Src-Akt signaling results in suppression of BimEL transcription, likely through phosphorylation of Foxo3a which retains this positive regulator of Bim transcription in the cytoplasm. Furthermore, Src-Erk1/2 signaling can also suppress Bim expression through phosphorylation which promotes an increase in its proteasomal degradation. Interestingly, our research has identified Mcl-1 as a critical regulator of anoikis. This protein is maintained at high expression levels by cellular attachment and by Src-Akt signaling which functionally suppresses the kinase activity of GSK-3 β which in turn prevents Mcl-1 phosphorylation at serine 159 and its targeting to the proteasome. Oncogene induced elongation of the half-life of Mcl-1

potently prevents anoikis. These findings are further confirmed in that cells are not dramatically sensitized to anoikis by combined inhibition of Bcl-XL and Bcl-2 through ABT-737 treatment, but repression of Mcl-1 expression alone potently enhances the anoikis response. Also, knockdown of Mcl-1 can dramatically reduce the propensity of cancer cells to metastasize *in vivo*.

As a novel compound designed to potentiate apoptotic responses ABT-737 has illustrated the need to target Mcl-1, if present at appreciable levels, in order to be effective. It is therefore necessary to continue the search for novel inhibitors of Mcl-1 to provide enhanced treatment strategies that can target individual or groups of Bcl-2 family proteins. One can envision the use of Mcl-1 small molecule inhibitors in the treatment of cancers that could prevent metastatic progression. Unfortunately, inhibition of Mcl-1 expression alone is unable to promote anoikis which also requires the increased expression of proapoptotic Bim to activate Bax. Development of Bax activating factors is also needed for therapeutic potentiation of apoptosis. These may include factors that promote the expression of BH3-only proteins, which could possibly be accomplished through inhibition of Foxo3a cytosolic retention. Also, small molecule compounds capable of displacing helix $\alpha 9$ and/or promoting Bax conformational activation could also prove beneficial. However, such compounds would likely require tumor specific targeting to avoid toxicity to normal cells. Another option, assuming the p53 pathway is intact, is to combine traditional chemotherapeutic agents that function through DNA damage and those that inhibit Bcl-2, Bcl-XL or Mcl-1. This would provide both the induction of proapoptotic molecules as well as the inhibition of their repression and may prove effective in some cancers. Ultimately, promotion of Bax activity specifically in tumor

cells remains highly promising but it is evident that enhanced therapeutic options still need to be developed.

Furthermore, our studies have also discovered that Src can prevent anoikis in a manner independent of Akt and Erk1/2 regulation of Mcl-1 and Bim expression. Our lab has previously discovered a novel Bax interacting factor, Bif-1, that has the potential to enhance Bax activation in response to cellular stresses. However, the molecular mechanisms regulating the interaction between Bax and Bif-1 are not clearly defined. We have discovered that Bif-1 is a direct phosphorylation target of Src kinase. Interestingly, the phosphorylation of Bif-1 mediated by Src was found to inhibit its interaction with Bax during cellular detachment and this was associated with lower anoikis potential. This finding illuminates another mechanism by which Src can prevent anoikis through its direct kinase activity.

Phosphorylation of Bif-1 by Src at tyrosine 80 occurs in the internal amphipathic helix domain which is essential for binding and tubulating membranes. Since phosphorylation imparts a negative charge to this region it may affect the helix organization and/or function of this critical domain. This may provide clues as to the causation of Bif-1 association with Bax. It should be determined what effects phosphorylation of Bif-1 by Src has on its membrane binding and tubulation potential and then determine if these properties influence the association between Bif-1 and Bax. It is possible that the association between these two proteins relies on the ability of Bif-1 to modify membrane structure which could in turn lead to Bax activation. If it is found that Bif-1 regulation of membrane dynamics is required for Bax binding and activation, the functional impacts should be delineated to determine at what step Bax activation is

affected. This could be found to be important for Bax conformational alteration, recruitment to the mitochondrial membrane or the insertional step. Furthermore, as Bif-1 is involved in autophagy the functional impacts on this pathway should also be evaluated. Bif-1 may be required for autophagosome maturation by regulation of membrane dynamics, a process that may potentially be impacted through Src, or Src family kinase, mediated phosphorylation at tyrosine 80.

Src kinase inhibitors are therefore promising therapeutic agents against certain cancers that rely on this signaling for metastatic progression. Inhibition of Src not only prevents survival signaling through downstream mediators such as Akt and Erk1/2 that regulate Mcl-1 and Bim, assuming other mutations that independently regulate these pathways are not activated, but also prevent the inhibition of other anti-apoptosis mechanisms such as phosphorylated Bif-1. Elucidation of other direct targets of Src that can prevent apoptosis need to be determined to fully understand its role in apoptosis prevention. Furthermore, it should be noted that many cancers have multiple oncogenic alterations that may bypass or enhance Src signaling. Src expression systems are useful at delineating the pathways that are controlled, but it should be recognized that independent activation of other signaling pathways in cancer may prevent significant therapeutic responses in a clinical setting.

Ku70 and p53 Interaction

Interestingly, the expression of transactivation deficient p53 can enhance HDACi-induced apoptosis. Whereas activation of p53 and its stabilization through hyperacetylation by HDACi treatment can conceivably impact transcription of

proapoptotic genes, the discovery of transactivation incompetent p53 mediated apoptosis in response to HDACi is somewhat less intuitive. Exploring the regulation of this event we have discovered the novel interaction between p53 and Ku70. This interaction results in the release of Bax from its inhibitory complex with Ku70 upon acetylation of p53 at three critical C-terminus lysine residues. Indeed, knockdown of Ku70 in p53-null but not p53^{R175H} H1299 cells robustly enhanced apoptosis, which indicates that Bax retention by Ku70 inhibits HDACi-induced apoptosis in the absence of acetylated p53. Although acetylation of Ku70 has been shown to be important for the release of Bax and the induction of HDACi-induced apoptosis, it remains to be determined what role acetylation of Ku70 plays in p53-mediated disruption of the Ku70/Bax complex.

Increasing evidence implicates the Ku70/Bax complex as a critical cellular determinant of apoptosis that can be disrupted by HDACi treatment as well as proteins such as NBS1 and p18-cyclin E fragment caused by genotoxic stress (Iijima et al., 2008; Mazumder et al., 2007; Subramanian et al., 2007). Therefore, it is likely that many proteins that bind Ku70 as well as direct Ku70 modification can disrupt Bax and promote apoptosis. However, our findings are important to the understanding of the role p53 plays in apoptosis independent of its transcriptional activity. It remains to be determined if other cellular stresses that promote p53 acetylation such as DNA damage can also promote the dissociation of Bax from Ku70 to enhance the apoptotic response. This newly identified apoptotic function of acetylated p53 may work in concert or independently of its previously described role at the mitochondria in the inhibition of Bcl-XL and Bcl-2 to activate Bax.

Cell lines that exhibit higher levels of Bax in the nucleus may be more susceptible

to acetylated p53 disruption from the Ku70 complex and may have a larger role in the initiation of apoptosis. A lower prevalence of Bax in the nuclear compartment may exhibit delayed apoptosis due to the time lapse required for the translocation of acetylated p53 to the cytoplasm. Also, since p53 was previously shown to activate Bax directly (Chipuk et al., 2004), the prevalence of nuclear Bax could result in its activation and recruitment to the mitochondria independently of its dissociation with Ku70 as described here. Indeed, p53 association with nuclear Bax has already been found to correlate with cisplatin induced apoptosis in cells expressing wild type p53 (Raffo et al., 2000). These results should also be confirmed by additional experiments including those that use mutant p53 expressing cell lines. It also remains to be determined whether nuclear Bax specifically associates with Ku70 in the nucleus. Immunodepletion of Ku70 could be performed to determine the amount of Bax associated with Ku70 in the nucleus. Furthermore, Bax localization should be assessed in Ku70 knockdown and knock-out cell lines because Ku70 may be required for nuclear Bax.

Another issue is the role of Ku80 in this complex as it is commonly associated with Ku70 in the nuclear compartment. It should be determined what effects nuclear Ku70/Ku80 complex formation has on the inhibition of nuclear Bax activation in both acetylated p53-dependent and independent models. Reciprocally, does nuclear Bax modify the function of Ku70 in NHEJ or V(D)J recombination? Bax bound to Ku70 could act as a direct surveillance mechanism of DNA damage and repair processes. If genotoxic stresses are found to activate Bax in the nucleus, then nuclear Bax could theoretically act more efficiently in the apoptotic cascade as signals would not have to amplify through transcription and translation relaying signals from the nucleus to the

cytoplasm to activate Bax. Furthermore, Ku-mediated NHEJ DNA repair has been found to be inhibited by Bcl-2 (Wang et al., 2008). It is possible that Bax somehow plays a role in this process, and release of Bax from Ku70 might block the inhibitory effect of Bcl-2 on Ku thereby allowing Ku to act in DNA repair. Similarly, the binding potential between Ku70, Bax, and Bcl-2 should be evaluated to determine the functional effects of their presence and/or absence in Ku70-mediated NHEJ and V(D)J recombination.

There also remains the issue of how this novel interaction between p53 and Ku70 impacts their respective functions. There is the possibility that binding to Ku70 can change p53 DNA binding affinity, transactivation or transrepression capabilities or other protein-protein interactions. It is tempting to speculate that Ku70 localization to sites of DNA damage may recruit p53 to signaling complexes that enhance its activation. This interaction may tether the independent functions of these two proteins as a mechanism of DNA integrity surveillance. Further research is required to answer these and many other questions that arise out of the association between these two important proteins.

HDAC Inhibitor Regulation of Apoptosis

Histone acetyltransferases and histone deacetylases regulate cellular transcriptional machinery by controlling the levels of acetylation of the core histones as well as non-histone proteins including transcription factors such as p53 (Glozak et al., 2005; Minucci and Pelicci, 2006). HDAC inhibitors (HDACi) SAHA and LAQ824 are potent inhibitors of class I and II HDACs and are currently under development as chemotherapeutic agents. The FDA has even approved SAHA for the treatment of cutaneous T cell lymphoma, a form of non-Hodgkin's lymphoma (Martinez-Iglesias et

al., 2008). HDAC inhibitors are considered to be promising chemotherapeutic agents due to their ability to induce differentiation, cell cycle arrest and apoptosis in a variety of cancer cells through altered expression of various cell cycle and apoptosis proteins (Bhalla, 2005; Minucci and Pelicci, 2006). HDACi mediated hyperacetylation of core histones causes transcriptional activation of genes which aid in their proapoptotic and cell cycle arrest capabilities (Johnstone, 2002). Reactive oxygen species (ROS) generation also contributes to the apoptotic response incurred by HDACi. The genome wide effect of HDACi and their varied non-histone targets has made the elucidation of their mechanism of action challenging. As our understanding increases of how this class of anti-neoplastic drugs works so does their potential for beneficial use in the clinic.

Our experiments have helped shed light onto a novel mechanism of HDACi-induced apoptosis that relies on acetylated p53-dependent disruption of Bax/Ku70 complexes. We have also found that this effect of p53 is independent of its transcriptional activity. This has potential therapeutic implications for the treatment of neoplastic malignancies using HDAC inhibitors as a platform. Mutations of p53 are one of the most common occurrences found in cancer and cause these cancers to be refractory to traditional chemotherapeutic agents that induce genotoxic stress. It could therefore be envisioned that HDAC inhibitors might be used in personalized therapies to enhance their effectiveness. Analysis of a patient's p53 status in cancer could identify those patients that would respond favorably to HDACi therapy based on expression of p53, which will likely be independent of its mutational status. However, those patients that lack p53 expression would be theorized to respond much less favorably to this treatment. To confirm that this hypothesis is correct, additional analysis of p53 expression and

mutational status in patient samples should be correlated to clinical responses to HDACi treatments. Similarly, *in vivo* experiments using p53^{-/-} and mutant p53 knock-in mice could also be used to compare cancer progression and survival rates to determine if p53 status imparts a survival advantage after challenge with HDACi treatment. Positive results in these experiments would then be justification for moving experiments into the clinic to determine if this personalized care can increase the efficacy of HDAC inhibitors.

References Cited

(1966). Nobel Prize. *Br Med J* 2, 964.

Akiyama, T., Bouillet, P., Miyazaki, T., Kadono, Y., Chikuda, H., Chung, U. I., Fukuda, A., Hikita, A., Seto, H., Okada, T., Inaba, T., Sanjay, A., Baron, R., Kawaguchi, H., Oda, H., Nakamura, K., Strasser, A., and Tanaka, S. (2003). Regulation of osteoclast apoptosis by ubiquitylation of proapoptotic BH3-only Bcl-2 family member Bim. *Embo J* 22, 6653-6664.

Alt, F. W., Kellems, R. E., Bertino, J. R., and Schimke, R. T. (1978). Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. *J Biol Chem* 253, 1357-1370.

Amsel, A. D., Rathaus, M., Kronman, N., and Cohen, H. Y. (2008). Regulation of the proapoptotic factor Bax by Ku70-dependent deubiquitylation. *Proc Natl Acad Sci U S A* 105, 5117-5122.

Annis, M. G., Soucie, E. L., Dlugosz, P. J., Cruz-Aguado, J. A., Penn, L. Z., Leber, B., and Andrews, D. W. (2005). Bax forms multispinning monomers that oligomerize to permeabilize membranes during apoptosis. *Embo J* 24, 2096-2103.

Ashkenazi, A. (2002). Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nat Rev Cancer* 2, 420-430.

Bennett, M., Macdonald, K., Chan, S. W., Luzio, J. P., Simari, R., and Weissberg, P. (1998). Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis. *Science* 282, 290-293.

- Bhalla, K. N. (2005). Epigenetic and chromatin modifiers as targeted therapy of hematologic malignancies. *J Clin Oncol* 23, 3971-3993.
- Bissonnette, N., Wasylyk, B., and Hunting, D. J. (1997). The apoptotic and transcriptional transactivation activities of p53 can be dissociated. *Biochem Cell Biol* 75, 351-358.
- Blake, R. A., Broome, M. A., Liu, X., Wu, J., Gishizky, M., Sun, L., and Courtneidge, S. A. (2000). SU6656, a selective src family kinase inhibitor, used to probe growth factor signaling. *Mol Cell Biol* 20, 9018-9027.
- Bowman, T., Broome, M. A., Sinibaldi, D., Wharton, W., Pledger, W. J., Sedivy, J. M., Irby, R., Yeatman, T., Courtneidge, S. A., and Jove, R. (2001). Stat3-mediated Myc expression is required for Src transformation and PDGF-induced mitogenesis. *Proc Natl Acad Sci U S A* 98, 7319-7324.
- Brooks, C. L., and Gu, W. (2003). Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation. *Curr Opin Cell Biol* 15, 164-171.
- Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* 96, 857-868.
- Brunton, V. G., Avizienyte, E., Fincham, V. J., Serrels, B., Metcalf, C. A., 3rd, Sawyer, T. K., and Frame, M. C. (2005). Identification of Src-specific phosphorylation site on focal adhesion kinase: dissection of the role of Src SH2 and catalytic functions and their consequences for tumor cell behavior. *Cancer Res* 65, 1335-1342.
- Caelles, C., Heimberg, A., and Karin, M. (1994). p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes. *Nature* 370, 220-224.

- Calalb, M. B., Zhang, X., Polte, T. R., and Hanks, S. K. (1996). Focal adhesion kinase tyrosine-861 is a major site of phosphorylation by Src. *Biochem Biophys Res Commun* 228, 662-668.
- Campbell, S. L., Khosravi-Far, R., Rossman, K. L., Clark, G. J., and Der, C. J. (1998). Increasing complexity of Ras signaling. *Oncogene* 17, 1395-1413.
- Cartron, P. F., Priault, M., Oliver, L., Meflah, K., Manon, S., and Vallette, F. M. (2003). The N-terminal end of Bax contains a mitochondrial-targeting signal. *J Biol Chem* 278, 11633-11641.
- Certo, M., Del Gaizo Moore, V., Nishino, M., Wei, G., Korsmeyer, S., Armstrong, S. A., and Letai, A. (2006). Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell* 9, 351-365.
- Chan, H. M., Krstic-Demonacos, M., Smith, L., Demonacos, C., and La Thangue, N. B. (2001). Acetylation control of the retinoblastoma tumour-suppressor protein. *Nat Cell Biol* 3, 667-674.
- Chandel, N. S., Schumacker, P. T., and Arch, R. H. (2001). Reactive oxygen species are downstream products of TRAF-mediated signal transduction. *J Biol Chem* 276, 42728-42736.
- Chen, D., Kon, N., Li, M., Zhang, W., Qin, J., and Gu, W. (2005). ARF-BP1/Mule is a critical mediator of the ARF tumor suppressor. *Cell* 121, 1071-1083.
- Chen, S., Dai, Y., Harada, H., Dent, P., and Grant, S. (2007). Mcl-1 down-regulation potentiates ABT-737 lethality by cooperatively inducing Bak activation and Bax translocation. *Cancer Res* 67, 782-791.

- Chipuk, J. E., Kuwana, T., Bouchier-Hayes, L., Droin, N. M., Newmeyer, D. D., Schuler, M., and Green, D. R. (2004). Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science* *303*, 1010-1014.
- Chipuk, J. E., Maurer, U., Green, D. R., and Schuler, M. (2003). Pharmacologic activation of p53 elicits Bax-dependent apoptosis in the absence of transcription. *Cancer Cell* *4*, 371-381.
- Chung, K. C., Sung, J. Y., Ahn, W., Rhim, H., Oh, T. H., Lee, M. G., and Ahn, Y. S. (2001). Intracellular calcium mobilization induces immediate early gene pip92 via Src and mitogen-activated protein kinase in immortalized hippocampal cells. *J Biol Chem* *276*, 2132-2138.
- Clohessy, J. G., Zhuang, J., and Brady, H. J. (2004). Characterisation of Mcl-1 cleavage during apoptosis of haematopoietic cells. *Br J Haematol* *125*, 655-665.
- Clohessy, J. G., Zhuang, J., de Boer, J., Gil-Gomez, G., and Brady, H. J. (2006). Mcl-1 interacts with truncated Bid and inhibits its induction of cytochrome c release and its role in receptor-mediated apoptosis. *J Biol Chem* *281*, 5750-5759.
- Cobb, B. S., Schaller, M. D., Leu, T. H., and Parsons, J. T. (1994). Stable association of pp60src and pp59fyn with the focal adhesion-associated protein tyrosine kinase, pp125FAK. *Mol Cell Biol* *14*, 147-155.
- Cohen, H. Y., Lavu, S., Bitterman, K. J., Hekking, B., Imahiyerobo, T. A., Miller, C., Frye, R., Ploegh, H., Kessler, B. M., and Sinclair, D. A. (2004a). Acetylation of the C terminus of Ku70 by CBP and PCAF controls Bax-mediated apoptosis. *Mol Cell* *13*, 627-638.

- Cohen, H. Y., Miller, C., Bitterman, K. J., Wall, N. R., Hekking, B., Kessler, B., Howitz, K. T., Gorospe, M., de Cabo, R., and Sinclair, D. A. (2004b). Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. *Science* 305, 390-392.
- Coll, M. L., Rosen, K., Ladeda, V., and Filmus, J. (2002). Increased Bcl-xL expression mediates v-Src-induced resistance to anoikis in intestinal epithelial cells. *Oncogene* 21, 2908-2913.
- Collett, M. S., Erikson, E., Purchio, A. F., Brugge, J. S., and Erikson, R. L. (1979). A normal cell protein similar in structure and function to the avian sarcoma virus transforming gene product. *Proc Natl Acad Sci U S A* 76, 3159-3163.
- Costanzo, A., Merlo, P., Pediconi, N., Fulco, M., Sartorelli, V., Cole, P. A., Fontemaggi, G., Fanciulli, M., Schiltz, L., Blandino, G., Balsano, C., and Levrero, M. (2002). DNA damage-dependent acetylation of p73 dictates the selective activation of apoptotic target genes. *Mol Cell* 9, 175-186.
- Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378, 785-789.
- Cuddeback, S. M., Yamaguchi, H., Komatsu, K., Miyashita, T., Yamada, M., Wu, C., Singh, S., and Wang, H. G. (2001). Molecular cloning and characterization of Bif-1: A novel SH3 domain- containing protein that associates with Bax. *J Biol Chem* 276, 20559-20565.
- Cully, M., You, H., Levine, A. J., and Mak, T. W. (2006). Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. *Nat Rev Cancer* 6, 184-192.

- Daugas, E., Susin, S. A., Zamzami, N., Ferri, K. F., Irinopoulou, T., Larochette, N., Prevost, M. C., Leber, B., Andrews, D., Penninger, J., and Kroemer, G. (2000). Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. *Faseb J* *14*, 729-739.
- Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M. J., Bottomley, W., Davis, N., Dicks, E., Ewing, R., Floyd, Y., Gray, K., Hall, S., Hawes, R., Hughes, J., Kosmidou, V., Menzies, A., Mould, C., Parker, A., Stevens, C., Watt, S., Hooper, S., Wilson, R., Jayatilake, H., Gusterson, B. A., Cooper, C., Shipley, J., Hargrave, D., Pritchard-Jones, K., Maitland, N., Chenevix-Trench, G., Riggins, G. J., Bigner, D. D., Palmieri, G., Cossu, A., Flanagan, A., Nicholson, A., Ho, J. W., Leung, S. Y., Yuen, S. T., Weber, B. L., Seigler, H. F., Darrow, T. L., Paterson, H., Marais, R., Marshall, C. J., Wooster, R., Stratton, M. R., and Futreal, P. A. (2002). Mutations of the BRAF gene in human cancer. *Nature* *417*, 949-954.
- Deng, X., Gao, F., Flagg, T., Anderson, J., and May, W. S. (2006). Bcl2's flexible loop domain regulates p53 binding and survival. *Mol Cell Biol* *26*, 4421-4434.
- Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B., and Martinou, J. C. (1999). Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. *J Cell Biol* *144*, 891-901.
- Dijkers, P. F., Medema, R. H., Lammers, J. W., Koenderman, L., and Coffey, P. J. (2000). Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. *Curr Biol* *10*, 1201-1204.
- Ding, H. F., Lin, Y. L., McGill, G., Juo, P., Zhu, H., Blenis, J., Yuan, J., and Fisher, D. E. (2000). Essential role for caspase-8 in transcription-independent apoptosis triggered by p53. *J Biol Chem* *275*, 38905-38911.
- Dornan, D., Wertz, I., Shimizu, H., Arnott, D., Frantz, G. D., Dowd, P., O'Rourke, K., Koeppen, H., and Dixit, V. M. (2004). The ubiquitin ligase COP1 is a critical negative regulator of p53. *Nature* *429*, 86-92.

- Douma, S., Van Laar, T., Zevenhoven, J., Meuwissen, R., Van Garderen, E., and Peeper, D. S. (2004). Suppression of anoikis and induction of metastasis by the neurotrophic receptor TrkB. *Nature* 430, 1034-1039.
- Dumont, P., Leu, J. I., Della Pietra, A. C., 3rd, George, D. L., and Murphy, M. (2003). The codon 72 polymorphic variants of p53 have markedly different apoptotic potential. *Nat Genet* 33, 357-365.
- el-Deiry, W. S. (1998). Regulation of p53 downstream genes. *Semin Cancer Biol* 8, 345-357.
- Erikson, R. L., Purchio, A. F., Erikson, E., Collett, M. S., and Brugge, J. S. (1980). Molecular events in cells transformed by Rous Sarcoma virus. *J Cell Biol* 87, 319-325.
- Erster, S., Mihara, M., Kim, R. H., Petrenko, O., and Moll, U. M. (2004). In vivo mitochondrial p53 translocation triggers a rapid first wave of cell death in response to DNA damage that can precede p53 target gene activation. *Mol Cell Biol* 24, 6728-6741.
- Essmann, F., Pohlmann, S., Gillissen, B., Daniel, P. T., Schulze-Osthoff, K., and Janicke, R. U. (2005). Irradiation-induced translocation of p53 to mitochondria in the absence of apoptosis. *J Biol Chem* 280, 37169-37177.
- Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L., and Henson, P. M. (1992). Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol* 148, 2207-2216.

- Farsad, K., Ringstad, N., Takei, K., Floyd, S. R., Rose, K., and De Camilli, P. (2001). Generation of high curvature membranes mediated by direct endophilin bilayer interactions. *J Cell Biol* 155, 193-200.
- Fincham, V. J., Brunton, V. G., and Frame, M. C. (2000). The SH3 domain directs actomyosin-dependent targeting of v-Src to focal adhesions via phosphatidylinositol 3-kinase. *Mol Cell Biol* 20, 6518-6536.
- Frame, M. C., Fincham, V. J., Carragher, N. O., and Wyke, J. A. (2002). v-Src's hold over actin and cell adhesions. *Nat Rev Mol Cell Biol* 3, 233-245.
- Fridman, J. S., and Lowe, S. W. (2003). Control of apoptosis by p53. *Oncogene* 22, 9030-9040.
- Frisch, S. M. (1999). Evidence for a function of death-receptor-related, death-domain-containing proteins in anoikis. *Curr Biol* 9, 1047-1049.
- Frisch, S. M., and Francis, H. (1994). Disruption of epithelial cell-matrix interactions induces apoptosis. *JCell Biol* 124, 619-626.
- Fukazawa, H., Noguchi, K., Masumi, A., Murakami, Y., and Uehara, Y. (2004). BimEL is an important determinant for induction of anoikis sensitivity by mitogen-activated protein/extracellular signal-regulated kinase kinase inhibitors. *Mol Cancer Ther* 3, 1281-1288.
- Gallop, J. L., Jao, C. C., Kent, H. M., Butler, P. J., Evans, P. R., Langen, R., and McMahon, H. T. (2006). Mechanism of endophilin N-BAR domain-mediated membrane curvature. *Embo J* 25, 2898-2910.
- Garcia-Saez, A. J., Coraiola, M., Serra, M. D., Mingarro, I., Muller, P., and Salgado, J. (2006). Peptides corresponding to helices 5 and 6 of Bax can independently form large lipid pores. *Febs J* 273, 971-981.

- Gardai, S. J., Hildeman, D. A., Frankel, S. K., Whitlock, B. B., Frasch, S. C., Borregaard, N., Marrack, P., Bratton, D. L., and Henson, P. M. (2004). Phosphorylation of Bax Ser184 by Akt regulates its activity and apoptosis in neutrophils. *J Biol Chem* 279, 21085-21095.
- Gilley, J., Coffey, P. J., and Ham, J. (2003). FOXO transcription factors directly activate bim gene expression and promote apoptosis in sympathetic neurons. *J Cell Biol* 162, 613-622.
- Gilmore, A. P. (2005). Anoikis. *Cell Death Differ* 12 Suppl 2, 1473-1477.
- Gilmore, A. P., Metcalfe, A. D., Romer, L. H., and Streuli, C. H. (2000). Integrin-mediated survival signals regulate the apoptotic function of Bax through its conformation and subcellular localization. *J Cell Biol* 149, 431-446.
- Giordano, A., and Avantaggiati, M. L. (1999). p300 and CBP: partners for life and death. *J Cell Physiol* 181, 218-230.
- Glozak, M. A., Sengupta, N., Zhang, X., and Seto, E. (2005). Acetylation and deacetylation of non-histone proteins. *Gene* 363, 15-23.
- Gomez-Bougie, P., Bataille, R., and Amiot, M. (2005). Endogenous association of Bim BH3-only protein with Mcl-1, Bcl-xL and Bcl-2 on mitochondria in human B cells. *Eur J Immunol* 35, 971-976.
- Green, D. R. (2005). Apoptotic pathways: ten minutes to dead. *Cell* 121, 671-674.
- Green, D. R., and Evan, G. I. (2002). A matter of life and death. *Cancer Cell* 1, 19-30.

- Gu, W., and Roeder, R. G. (1997). Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell* *90*, 595-606.
- Gu, Y., Jin, S., Gao, Y., Weaver, D. T., and Alt, F. W. (1997). Ku70-deficient embryonic stem cells have increased ionizing radiosensitivity, defective DNA end-binding activity, and inability to support V(D)J recombination. *Proc Natl Acad Sci U S A* *94*, 8076-8081.
- Gu, Y., Sekiguchi, J., Gao, Y., Dikkes, P., Frank, K., Ferguson, D., Hasty, P., Chun, J., and Alt, F. W. (2000). Defective embryonic neurogenesis in Ku-deficient but not DNA-dependent protein kinase catalytic subunit-deficient mice. *Proc Natl Acad Sci U S A* *97*, 2668-2673.
- Guo, B., Zhai, D., Cabezas, E., Welsh, K., Nouraini, S., Satterthwait, A. C., and Reed, J. C. (2003). Humanin peptide suppresses apoptosis by interfering with Bax activation. *Nature* *423*, 456-461.
- Guo, W., and Giancotti, F. G. (2004). Integrin signalling during tumour progression. *Nat Rev Mol Cell Biol* *5*, 816-826.
- Hall, C. L., Lange, L. A., Prober, D. A., Zhang, S., and Turley, E. A. (1996). pp60(c-src) is required for cell locomotion regulated by the hyaluronanreceptor RHAMM. *Oncogene* *13*, 2213-2224.
- Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. *Cell* *100*, 57-70.
- Harada, H., Quearry, B., Ruiz-Vela, A., and Korsmeyer, S. J. (2004). Survival factor-induced extracellular signal-regulated kinase phosphorylates BIM, inhibiting its association with BAX and proapoptotic activity. *Proc Natl Acad Sci U S A* *101*, 15313-15317.

- Haupt, Y., Rowan, S., Shaulian, E., Vousden, K. H., and Oren, M. (1995). Induction of apoptosis in HeLa cells by trans-activation-deficient p53. *Genes Dev* 9, 2170-2183.
- Henderson, C., Mizzau, M., Paroni, G., Maestro, R., Schneider, C., and Brancolini, C. (2003). Role of caspases, Bid, and p53 in the apoptotic response triggered by histone deacetylase inhibitors trichostatin-A (TSA) and suberoylanilide hydroxamic acid (SAHA). *J Biol Chem* 278, 12579-12589.
- Hinds, P. W., Finlay, C. A., Quartin, R. S., Baker, S. J., Fearon, E. R., Vogelstein, B., and Levine, A. J. (1990). Mutant p53 DNA clones from human colon carcinomas cooperate with ras in transforming primary rat cells: a comparison of the "hot spot" mutant phenotypes. *Cell Growth Differ* 1, 571-580.
- Hoeflich, K. P., Luo, J., Rubie, E. A., Tsao, M. S., Jin, O., and Woodgett, J. R. (2000). Requirement for glycogen synthase kinase-3 β in cell survival and NF-kappaB activation. *Nature* 406, 86-90.
- Hollestelle, A., Elstrodt, F., Nagel, J. H., Kallemeijn, W. W., and Schutte, M. (2007). Phosphatidylinositol-3-OH kinase or RAS pathway mutations in human breast cancer cell lines. *Mol Cancer Res* 5, 195-201.
- Honda, R., and Yasuda, H. (1999). Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. *Embo J* 18, 22-27.
- Hsu, Y. T., and Youle, R. J. (1997). Nonionic detergents induce dimerization among members of the Bcl-2 family. *J Biol Chem* 272, 13829-13834.
- Huang, L., Sowa, Y., Sakai, T., and Pardee, A. B. (2000). Activation of the p21WAF1/CIP1 promoter independent of p53 by the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) through the Sp1 sites. *Oncogene* 19, 5712-5719.

- Hubner, A., Barrett, T., Flavell, R. A., and Davis, R. J. (2008). Multisite phosphorylation regulates Bim stability and apoptotic activity. *Mol Cell* 30, 415-425.
- Hynes, R. O. (1999). Cell adhesion: old and new questions. *Trends Cell Biol* 9, M33-37.
- Hynes, R. O. (2002). Integrins: bidirectional, allosteric signaling machines. *Cell* 110, 673-687.
- Iijima, K., Muranaka, C., Kobayashi, J., Sakamoto, S., Komatsu, K., Matsuura, S., Kubota, N., and Tauchi, H. (2008). NBS1 regulates a novel apoptotic pathway through Bax activation. *DNA Repair (Amst)*.
- Irby, R. B., Mao, W., Coppola, D., Kang, J., Loubeau, J. M., Trudeau, W., Karl, R., Fujita, D. J., Jove, R., and Yeatman, T. J. (1999). Activating SRC mutation in a subset of advanced human colon cancers. *Nat Genet* 21, 187-190.
- Ivanov, G. S., Ivanova, T., Kurash, J., Ivanov, A., Chuikov, S., Gizatullin, F., Herrera-Medina, E. M., Rauscher, F., 3rd, Reinberg, D., and Barlev, N. A. (2007). Methylation-acetylation interplay activates p53 in response to DNA damage. *Mol Cell Biol* 27, 6756-6769.
- Jacobs, C., and Rubsamen, H. (1983). Expression of pp60c-src protein kinase in adult and fetal human tissue: high activities in some sarcomas and mammary carcinomas. *Cancer Res* 43, 1696-1702.
- Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J., and Thun, M. J. (2007). Cancer statistics, 2007. *CA Cancer J Clin* 57, 43-66.
- Jia, S. F., Worth, L. L., and Kleinerman, E. S. (1999). A nude mouse model of human osteosarcoma lung metastases for evaluating new therapeutic strategies. *Clin Exp Metastasis* 17, 501-506.

- Jockusch, B. M., Bubeck, P., Giehl, K., Kroemker, M., Moschner, J., Rothkegel, M., Rudiger, M., Schluter, K., Stanke, G., and Winkler, J. (1995). The molecular architecture of focal adhesions. *Annu Rev Cell Dev Biol* 11, 379-416.
- Johnson, T. M., Hammond, E. M., Giaccia, A., and Attardi, L. D. (2005). The p53^{QS} transactivation-deficient mutant shows stress-specific apoptotic activity and induces embryonic lethality. *Nat Genet* 37, 145-152.
- Johnstone, R. W. (2002). Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. *Nat Rev Drug Discov* 1, 287-299.
- Kaplan, K. B., Swedlow, J. R., Morgan, D. O., and Varmus, H. E. (1995). c-Src enhances the spreading of src^{-/-} fibroblasts on fibronectin by a kinase-independent mechanism. *Genes Dev* 9, 1505-1517.
- Karbowski, M., Jeong, S. Y., and Youle, R. J. (2004). Endophilin B1 is required for the maintenance of mitochondrial morphology. *J Cell Biol* 166, 1027-1039.
- Karbowski, M., Lee, Y. J., Gaume, B., Jeong, S. Y., Frank, S., Nechushtan, A., Santel, A., Fuller, M., Smith, C. L., and Youle, R. J. (2002). Spatial and temporal association of Bax with mitochondrial fission sites, Drp1, and Mfn2 during apoptosis. *J Cell Biol* 159, 931-938.
- Kim, H., Rafiuddin-Shah, M., Tu, H. C., Jeffers, J. R., Zambetti, G. P., Hsieh, J. J., and Cheng, E. H. (2006). Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. *Nat Cell Biol* 8, 1348-1358.
- Kokontis, J. M., Wagner, A. J., O'Leary, M., Liao, S., and Hay, N. (2001). A transcriptional activation function of p53 is dispensable for and inhibitory of its apoptotic function. *Oncogene* 20, 659-668.
- Koshikawa, N., Maejima, C., Miyazaki, K., Nakagawara, A., and Takenaga, K. (2006).

Hypoxia selects for high-metastatic Lewis lung carcinoma cells overexpressing Mcl-1 and exhibiting reduced apoptotic potential in solid tumors. *Oncogene* *25*, 917-928.

Krajewski, S., Krajewska, M., Shabaik, A., Miyashita, T., Wang, H.-G., and Reed, J. C. (1994). Immunohistochemical determination of in vivo distribution of bax, a dominant inhibitor of bcl-2. *AmJPathol* *145*, 1323-1333.

Kurzrock, R., Kloetzer, W. S., Talpaz, M., Blick, M., Walters, R., Arlinghaus, R. B., and Gutterman, J. U. (1987). Identification of molecular variants of p210bcr-abl in chronic myelogenous leukemia. *Blood* *70*, 233-236.

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

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

Lang, G. A., Iwakuma, T., Suh, Y. A., Liu, G., Rao, V. A., Parant, J. M., Valentin-Vega, Y. A., Terzian, T., Caldwell, L. C., Strong, L. C., El-Naggar, A. K., and Lozano, G. (2004). Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome. *Cell* *119*, 861-872.

Lei, K., and Davis, R. J. (2003). JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis. *Proc Natl Acad Sci U S A* *100*, 2432-2437.

Leng, R. P., Lin, Y., Ma, W., Wu, H., Lemmers, B., Chung, S., Parant, J. M., Lozano, G., Hakem, R., and Benchimol, S. (2003). Pirh2, a p53-induced ubiquitin-protein

ligase, promotes p53 degradation. *Cell* 112, 779-791.

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

Leu, J. I., Dumont, P., Hafey, M., Murphy, M. E., and George, D. L. (2004). Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. *Nat Cell Biol* 6, 443-450.

Levinson, A. D., Oppermann, H., Levintow, L., Varmus, H. E., and Bishop, J. M. (1978). Evidence that the transforming gene of avian sarcoma virus encodes a protein kinase associated with a phosphoprotein. *Cell* 15, 561-572.

Li, M., Luo, J., Brooks, C. L., and Gu, W. (2002). Acetylation of p53 inhibits its ubiquitination by Mdm2. *J Biol Chem* 277, 50607-50611.

Li, Y., Yokota, T., Gama, V., Yoshida, T., Gomez, J. A., Ishikawa, K., Sasaguri, H., Cohen, H. Y., Sinclair, D. A., Mizusawa, H., and Matsuyama, S. (2007). Bax-inhibiting peptide protects cells from polyglutamine toxicity caused by Ku70 acetylation. *Cell Death Differ*.

Lieber, M. R., Ma, Y., Pannicke, U., and Schwarz, K. (2003). Mechanism and regulation of human non-homologous DNA end-joining. *Nat Rev Mol Cell Biol* 4, 712-720.

Lindsten, T., Ross, A. J., King, A., Zong, W. X., Rathmell, J. C., Shiels, H. A., Ulrich, E., Waymire, K. G., Mahar, P., Frauwirth, K., Chen, Y., Wei, M., Eng, V. M., Adelman, D. M., Simon, M. C., Ma, A., Golden, J. A., Evan, G., Korsmeyer, S. J., MacGregor, G. R., and Thompson, C. B. (2000). The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. *Mol Cell* 6, 1389-1399.

- Loza-Coll, M. A., Perera, S., Shi, W., and Filmus, J. (2005). A transient increase in the activity of Src-family kinases induced by cell detachment delays anoikis of intestinal epithelial cells. *Oncogene* 24, 1727-1737.
- Luciano, F., Jacquel, A., Colosetti, P., Herrant, M., Cagnol, S., Pages, G., and Auberger, P. (2003). Phosphorylation of Bim-EL by Erk1/2 on serine 69 promotes its degradation via the proteasome pathway and regulates its proapoptotic function. *Oncogene* 22, 6785-6793.
- Luo, J., Li, M., Tang, Y., Laszkowska, M., Roeder, R. G., and Gu, W. (2004). Acetylation of p53 augments its site-specific DNA binding both in vitro and in vivo. *Proc Natl Acad Sci U S A* 101, 2259-2264.
- Luo, J., Su, F., Chen, D., Shiloh, A., and Gu, W. (2000). Deacetylation of p53 modulates its effect on cell growth and apoptosis. *Nature* 408, 377-381.
- Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. (1998). Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* 94, 481-490.
- Lutter, M., Fang, M., Luo, X., Nishijima, M., Xie, X., and Wang, X. (2000). Cardiolipin provides specificity for targeting of tBid to mitochondria. *Nat Cell Biol* 2, 754-761.
- Mahyar-Roemer, M., Fritzsche, C., Wagner, S., Laue, M., and Roemer, K. (2004). Mitochondrial p53 levels parallel total p53 levels independent of stress response in human colorectal carcinoma and glioblastoma cells. *Oncogene* 23, 6226-6236.
- Manis, J. P., Gu, Y., Lansford, R., Sonoda, E., Ferrini, R., Davidson, L., Rajewsky, K., and Alt, F. W. (1998). Ku70 is required for late B cell development and immunoglobulin heavy chain class switching. *J Exp Med* 187, 2081-2089.

- Marani, M., Tenev, T., Hancock, D., Downward, J., and Lemoine, N. R. (2002). Identification of novel isoforms of the BH3 domain protein Bim which directly activate Bax to trigger apoptosis. *Mol Cell Biol* 22, 3577-3589.
- Martin, M. J., Melnyk, N., Pollard, M., Bowden, M., Leong, H., Podor, T. J., Gleave, M., and Sorensen, P. H. (2006). The insulin-like growth factor I receptor is required for Akt activation and suppression of anoikis in cells transformed by the ETV6-NTRK3 chimeric tyrosine kinase. *Mol Cell Biol* 26, 1754-1769.
- Martinez-Iglesias, O., Ruiz-Llorente, L., Sanchez-Martinez, R., Garcia, L., Zambrano, A., and Aranda, A. (2008). Histone deacetylase inhibitors: mechanism of action and therapeutic use in cancer. *Clin Transl Oncol* 10, 395-398.
- Masuda, M., Takeda, S., Sone, M., Ohki, T., Mori, H., Kamioka, Y., and Mochizuki, N. (2006). Endophilin BAR domain drives membrane curvature by two newly identified structure-based mechanisms. *Embo J* 25, 2889-2897.
- Maurer, U., Charvet, C., Wagman, A. S., Dejardin, E., and Green, D. R. (2006). Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1. *Mol Cell* 21, 749-760.
- Mazumder, S., Plesca, D., Kinter, M., and Almasan, A. (2007). Interaction of a cyclin E fragment with Ku70 regulates Bax-mediated apoptosis. *Mol Cell Biol* 27, 3511-3520.
- Miao, J., Chen, G. G., Yun, J. P., Chun, S. Y., Zheng, Z. Z., Ho, R. L., Chak, E. C., Xia, N. S., and Lai, P. B. (2007). Identification and characterization of BH3 domain protein Bim and its isoforms in human hepatocellular carcinomas. *Apoptosis* 12, 1691-1701.

- Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P., and Moll, U. M. (2003). p53 has a direct apoptogenic role at the mitochondria. *Mol Cell* 11, 577-590.
- Minucci, S., and Pelicci, P. G. (2006). Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. *Nat Rev Cancer* 6, 38-51.
- Miyashita, T., Harigai, M., Hanada, M., and Reed, J. C. (1994). Identification of a p53-dependent negative response element in the bcl-2 gene. *Cancer Res* 54, 3131-3135.
- Moarefi, I., LaFevre-Bernt, M., Sicheri, F., Huse, M., Lee, C. H., Kuriyan, J., and Miller, W. T. (1997). Activation of the Src-family tyrosine kinase Hck by SH3 domain displacement. *Nature* 385, 650-653.
- Momand, J., Jung, D., Wilczynski, S., and Niland, J. (1998). The MDM2 gene amplification database. *Nucleic Acids Res* 26, 3453-3459.
- Montgomery, A. M., Reisfeld, R. A., and Cheresch, D. A. (1994). Integrin alpha v beta 3 rescues melanoma cells from apoptosis in three-dimensional dermal collagen. *Proc Natl Acad Sci U S A* 91, 8856-8860.
- Muller, C., Paupert, J., Monferran, S., and Salles, B. (2005). The double life of the Ku protein: facing the DNA breaks and the extracellular environment. *Cell Cycle* 4, 438-441.
- Naismith, J. H., and Sprang, S. R. (1998). Modularity in the TNF-receptor family. *Trends Biochem Sci* 23, 74-79.

- Napper, A. D., Hixon, J., McDonagh, T., Keavey, K., Pons, J. F., Barker, J., Yau, W. T., Amouzegh, P., Flegg, A., Hamelin, E., Thomas, R. J., Kates, M., Jones, S., Navia, M. A., Saunders, J. O., DiStefano, P. S., and Curtis, R. (2005). Discovery of indoles as potent and selective inhibitors of the deacetylase SIRT1. *J Med Chem* 48, 8045-8054.
- Nechushtan, A., Smith, C. L., Hsu, Y. T., and Youle, R. J. (1999). Conformation of the Bax C-terminus regulates subcellular location and cell death. *Embo J* 18, 2330-2341.
- Nishita, M., Inoue, S., Tsuda, M., Tateda, C., and Miyashita, T. (1998). Nuclear translocation and increased expression of Bax and disturbance in cell cycle progression without prominent apoptosis induced by hyperthermia. *Exp Cell Res* 244, 357-366.
- Niu, G., Bowman, T., Huang, M., Shivers, S., Reintgen, D., Daud, A., Chang, A., Kraker, A., Jove, R., and Yu, H. (2002). Roles of activated Src and Stat3 signaling in melanoma tumor cell growth. *Oncogene* 21, 7001-7010.
- Nomura, M., Shimizu, S., Sugiyama, T., Narita, M., Ito, T., Matsuda, H., and Tsujimoto, Y. (2003). 14-3-3 Interacts directly with and negatively regulates pro-apoptotic Bax. *J Biol Chem* 278, 2058-2065.
- Ohkubo, S., Tanaka, T., Taya, Y., Kitazato, K., and Prives, C. (2006). Excess HDM2 impacts cell cycle and apoptosis and has a selective effect on p53-dependent transcription. *J Biol Chem* 281, 16943-16950.
- Ohtsuka, T., Ryu, H., Minamishima, Y. A., Macip, S., Sagara, J., Nakayama, K. I., Aaronson, S. A., and Lee, S. W. (2004). ASC is a Bax adaptor and regulates the p53-Bax mitochondrial apoptosis pathway. *Nat Cell Biol* 6, 121-128.

- Olive, K. P., Tuveson, D. A., Ruhe, Z. C., Yin, B., Willis, N. A., Bronson, R. T., Crowley, D., and Jacks, T. (2004). Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. *Cell* 119, 847-860.
- Oltersdorf, T., Elmore, S. W., Shoemaker, A. R., Armstrong, R. C., Augeri, D. J., Belli, B. A., Bruncko, M., Deckwerth, T. L., Dinges, J., Hajduk, P. J., Joseph, M. K., Kitada, S., Korsmeyer, S. J., Kunzer, A. R., Letai, A., Li, C., Mitten, M. J., Nettesheim, D. G., Ng, S., Nimmer, P. M., O'Connor, J. M., Oleksijew, A., Petros, A. M., Reed, J. C., Shen, W., Tahir, S. K., Thompson, C. B., Tomaselli, K. J., Wang, B., Wendt, M. D., Zhang, H., Fesik, S. W., and Rosenberg, S. H. (2005). An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* 435, 677-681.
- Opferman, J. T., Letai, A., Beard, C., Sorcinelli, M. D., Ong, C. C., and Korsmeyer, S. J. (2003). Development and maintenance of B and T lymphocytes requires antiapoptotic MCL-1. *Nature* 426, 671-676.
- Ott, M., Gogvadze, V., Orrenius, S., and Zhivotovsky, B. (2007a). Mitochondria, oxidative stress and cell death. *Apoptosis* 12, 913-922.
- Ott, M., Norberg, E., Walter, K. M., Schreiner, P., Kemper, C., Rapaport, D., Zhivotovsky, B., and Orrenius, S. (2007b). The mitochondrial TOM complex is required for tBid/Bax-induced cytochrome c release. *J Biol Chem* 282, 27633-27639.
- Ouyang, H., Nussenzweig, A., Kurimasa, A., Soares, V. C., Li, X., Cordon-Cardo, C., Li, W., Cheong, N., Nussenzweig, M., Iliakis, G., Chen, D. J., and Li, G. C. (1997). Ku70 is required for DNA repair but not for T cell antigen receptor gene recombination In vivo. *J Exp Med* 186, 921-929.
- Park, R. K., Erdreich-Epstein, A., Liu, M., Izadi, K. D., and Durden, D. L. (1999). High affinity IgG receptor activation of Src family kinases is required for modulation of the Shc-Grb2-Sos complex and the downstream activation of the nicotinamide adenine dinucleotide phosphate (reduced) oxidase. *J Immunol* 163, 6023-6034.

- Peter, B. J., Kent, H. M., Mills, I. G., Vallis, Y., Butler, P. J., Evans, P. R., and McMahon, H. T. (2004). BAR domains as sensors of membrane curvature: the amphiphysin BAR structure. *Science* 303, 495-499.
- Petros, A. M., Olejniczak, E. T., and Fesik, S. W. (2004). Structural biology of the Bcl-2 family of proteins. *Biochim Biophys Acta* 1644, 83-94.
- Pierrat, B., Simonen, M., Cueto, M., Mestan, J., Ferrigno, P., and Heim, J. (2001). SH3GLB, a new endophilin-related protein family featuring an SH3 domain. *Genomics* 71, 222-234.
- Pietrzak, M., and Puzianowska-Kuznicka, M. (2008). p53-dependent repression of the human MCL-1 gene encoding an anti-apoptotic member of the BCL-2 family: the role of Sp1 and of basic transcription factor binding sites in the MCL-1 promoter. *Biol Chem* 389, 383-393.
- Pietsch, E. C., Leu, J. I., Frank, A., Dumont, P., George, D. L., and Murphy, M. E. (2007). The tetramerization domain of p53 is required for efficient BAK oligomerization. *Cancer Biol Ther* 6, 1576-1583.
- Piluso, L. G., Wei, G., Li, A. G., and Liu, X. (2005). Purification of acetyl-p53 using p300 co-infection and the baculovirus expression system. *Protein Expr Purif* 40, 370-378.
- Polyak, K., Zweler, J. L., Kinler, K. W., and Vogelstein, B. (1997). A model for p53-induced apoptosis. *Nature* 389, 300-305.
- Pullan, S., Wilson, J., Metcalfe, A., Edwards, G. M., Goberdhan, N., Tilly, J., Hickman, J. A., Dive, C., and Streuli, C. H. (1996). Requirement of basement membrane for the suppression of programmed cell death in mammary epithelium. *JCell Science* 109, 631-642.

- Puthalakath, H., Huang, D. C., O'Reilly, L. A., King, S. M., and Strasser, A. (1999). The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol Cell* 3, 287-296.
- Qi, X. J., Wildey, G. M., and Howe, P. H. (2006). Evidence that Ser87 of BimEL is phosphorylated by Akt and regulates BimEL apoptotic function. *J Biol Chem* 281, 813-823.
- Raffo, A. J., Kim, A. L., and Fine, R. L. (2000). Formation of nuclear Bax/p53 complexes is associated with chemotherapy induced apoptosis. *Oncogene* 19, 6216-6228.
- Rahimi, N., Hung, W., Tremblay, E., Saulnier, R., and Elliott, B. (1998). c-Src kinase activity is required for hepatocyte growth factor-induced motility and anchorage-independent growth of mammary carcinoma cells. *J Biol Chem* 273, 33714-33721.
- Ravichandran, K. S. (2001). Signaling via Shc family adapter proteins. *Oncogene* 20, 6322-6330.
- Reed, J. C. (1995). Regulation of apoptosis by bcl-2 family proteins and its role in cancer and chemoresistance. *Curr Opin Oncol* 7, 541-546.
- Reginato, M. J., Mills, K. R., Paulus, J. K., Lynch, D. K., Sgroi, D. C., Debnath, J., Muthuswamy, S. K., and Brugge, J. S. (2003). Integrins and EGFR coordinately regulate the pro-apoptotic protein Bim to prevent anoikis. *Nat Cell Biol* 5, 733-740.
- Ren, Y., Meng, S., Mei, L., Zhao, Z. J., Jove, R., and Wu, J. (2004). Roles of Gab1 and SHP2 in paxillin tyrosine dephosphorylation and Src activation in response to epidermal growth factor. *J Biol Chem* 279, 8497-8505.

- Rosen, K., Coll, M. L., Li, A., and Filmus, J. (2001). Transforming growth factor-alpha prevents detachment-induced inhibition of c-Src kinase activity, Bcl-XL down-regulation, and apoptosis of intestinal epithelial cells. *J Biol Chem* 276, 37273-37279.
- Rosen, K., Rak, J., Leung, T., Dean, N. M., Kerbel, R. S., and Filmus, J. (2000). Activated Ras prevents downregulation of Bcl-X(L) triggered by detachment from the extracellular matrix. A mechanism of Ras-induced resistance to anoikis in intestinal epithelial cells. *J Cell Biol* 149, 447-456.
- Rosen, N., Bolen, J. B., Schwartz, A. M., Cohen, P., DeSeau, V., and Israel, M. A. (1986). Analysis of pp60c-src protein kinase activity in human tumor cell lines and tissues. *J Biol Chem* 261, 13754-13759.
- Roucou, X., Montessuit, S., Antonsson, B., and Martinou, J. C. (2002). Bax oligomerization in mitochondrial membranes requires tBid (caspase-8-cleaved Bid) and a mitochondrial protein. *Biochem J* 368, 915-921.
- Roy, S., Packman, K., Jeffrey, R., and Tenniswood, M. (2005). Histone deacetylase inhibitors differentially stabilize acetylated p53 and induce cell cycle arrest or apoptosis in prostate cancer cells. *Cell Death Differ* 12, 482-491.
- Ruas, M., and Peters, G. (1998). The p16INK4a/CDKN2A tumor suppressor and its relatives. *Biochim Biophys Acta* 1378, F115-177.
- Ruefli, A. A., Ausserlechner, M. J., Bernhard, D., Sutton, V. R., Tainton, K. M., Kofler, R., Smyth, M. J., and Johnstone, R. W. (2001). The histone deacetylase inhibitor and chemotherapeutic agent suberoylanilide hydroxamic acid (SAHA) induces a cell-death pathway characterized by cleavage of Bid and production of reactive oxygen species. *Proc Natl Acad Sci U S A* 98, 10833-10838.

- Sakaguchi, K., Herrera, J. E., Saito, S., Miki, T., Bustin, M., Vassilev, A., Anderson, C. W., and Appella, E. (1998). DNA damage activates p53 through a phosphorylation-acetylation cascade. *Genes Dev* 12, 2831-2841.
- Salcini, A. E., McGlade, J., Pelicci, G., Nicoletti, I., Pawson, T., and Pelicci, P. G. (1994). Formation of Shc-Grb2 complexes is necessary to induce neoplastic transformation by overexpression of Shc proteins. *Oncogene* 9, 2827-2836.
- Schaller, M. D., Hildebrand, J. D., Shannon, J. D., Fox, J. W., Vines, R. R., and Parsons, J. T. (1994). Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. *Mol Cell Biol* 14, 1680-1688.
- Schlaepfer, D. D., Broome, M. A., and Hunter, T. (1997). Fibronectin-stimulated signaling from a focal adhesion kinase-c-Src complex: involvement of the Grb2, p130cas, and Nck adaptor proteins. *Mol Cell Biol* 17, 1702-1713.
- Schlaepfer, D. D., and Hunter, T. (1996). Evidence for in vivo phosphorylation of the Grb2 SH2-domain binding site on focal adhesion kinase by Src-family protein-tyrosine kinases. *Mol Cell Biol* 16, 5623-5633.
- Sellers, T. A., Potter, J. D., Bailey-Wilson, J. E., Rich, S. S., Rothschild, H., and Elston, R. C. (1992). Lung cancer detection and prevention: evidence for an interaction between smoking and genetic predisposition. *Cancer Res* 52, 2694s-2697s.
- Shanmugathan, M., and Jothy, S. (2000). Apoptosis, anoikis and their relevance to the pathobiology of colon cancer. *Pathol Int* 50, 273-279.
- Sjolander, A., Yamamoto, K., Huber, B. E., and Lapetina, E. G. (1991). Association of p21ras with phosphatidylinositol 3-kinase. *Proc Natl Acad Sci U S A* 88, 7908-7912.

- Stehelin, D., Varmus, H. E., Bishop, J. M., and Vogt, P. K. (1976). DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. *Nature* 260, 170-173.
- Subramanian, C., Jarzembowski, J. A., Opipari, A. W., Jr., Castle, V. P., and Kwok, R. P. (2007). CREB-binding protein is a mediator of neuroblastoma cell death induced by the histone deacetylase inhibitor trichostatin A. *Neoplasia* 9, 495-503.
- Subramanian, C., Opipari, A. W., Jr., Bian, X., Castle, V. P., and Kwok, R. P. (2005). Ku70 acetylation mediates neuroblastoma cell death induced by histone deacetylase inhibitors. *Proc Natl Acad Sci U S A* 102, 4842-4847.
- Sugimura, M., Kobayashi, K., Sagae, S., Nishioka, Y., Ishioka, S., Terasawa, K., Tokino, T., and Kudo, R. (2000). Mutation of the SRC gene in endometrial carcinoma. *Jpn J Cancer Res* 91, 395-398.
- Suzuki, M., Youle, R. J., and Tjandra, N. (2000). Structure of bax. Coregulation of dimer formation and intracellular localization. *Cell* 103, 645-654.
- Takahashi, Y., Coppola, D., Matsushita, N., Cualing, H. D., Sun, M., Sato, Y., Liang, C., Jung, J. U., Cheng, J. Q., Mul, J. J., Pledger, W. J., and Wang, H. G. (2007). Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. *Nat Cell Biol* 9, 1142-1151.
- Takahashi, Y., Karbowski, M., Yamaguchi, H., Kazi, A., Wu, J., Sebti, S. M., Youle, R. J., and Wang, H. G. (2005). Loss of Bif-1 suppresses Bax/Bak conformational change and mitochondrial apoptosis. *Mol Cell Biol* 25, 9369-9382.
- Tang, Y., Zhao, W., Chen, Y., Zhao, Y., and Gu, W. (2008). Acetylation is indispensable for p53 activation. *Cell* 133, 612-626.

- Terrones, O., Antonsson, B., Yamaguchi, H., Wang, H. G., Liu, J., Lee, R. M., Herrmann, A., and Basanez, G. (2004). Lipidic pore formation by the concerted action of proapoptotic BAX and tBID. *J Biol Chem* 279, 30081-30091.
- Terui, T., Murakami, K., Takimoto, R., Takahashi, M., Takada, K., Murakami, T., Minami, S., Matsunaga, T., Takayama, T., Kato, J., and Niitsu, Y. (2003). Induction of PIG3 and NOXA through acetylation of p53 at 320 and 373 lysine residues as a mechanism for apoptotic cell death by histone deacetylase inhibitors. *Cancer Res* 63, 8948-8954.
- Thomas, S. M., and Brugge, J. S. (1997). Cellular functions regulated by Src family kinases. *Annu Rev Cell Dev Biol* 13, 513-609.
- Thompson, C. B. (1995). Apoptosis in the pathogenesis and treatment of disease. *Science* 267, 1456-1462.
- Toledo, F., and Wahl, G. M. (2006). Regulating the p53 pathway: in vitro hypotheses, in vivo veritas. *Nat Rev Cancer* 6, 909-923.
- Tomita, Y., Marchenko, N., Erster, S., Nemajerova, A., Dehner, A., Klein, C., Pan, H., Kessler, H., Pancoska, P., and Moll, U. M. (2006). WT p53, but not tumor-derived mutants, bind to Bcl2 via the DNA binding domain and induce mitochondrial permeabilization. *J Biol Chem* 281, 8600-8606.
- Tsujimoto, Y., Gorham, J., Cossman, J., Jaffe, E., and Croce, C. M. (1985). The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. *Science* 229, 1390-1393.
- Tuteja, R., and Tuteja, N. (2000). Ku autoantigen: a multifunctional DNA-binding protein. *Crit Rev Biochem Mol Biol* 35, 1-33.

- Valentijn, A. J., Metcalfe, A. D., Kott, J., Streuli, C. H., and Gilmore, A. P. (2003). Spatial and temporal changes in Bax subcellular localization during anoikis. *J Cell Biol* 162, 599-612.
- van Loo, G., Schotte, P., van Gurp, M., Demol, H., Hoorelbeke, B., Gevaert, K., Rodriguez, I., Ruiz-Carrillo, A., Vandekerckhove, J., Declercq, W., Beyaert, R., and Vandenabeele, P. (2001). Endonuclease G: a mitochondrial protein released in apoptosis and involved in caspase-independent DNA degradation. *Cell Death Differ* 8, 1136-1142.
- Vassilev, L. T. (2007). MDM2 inhibitors for cancer therapy. *Trends Mol Med* 13, 23-31.
- Vaziri, H., Dessain, S. K., Ng Eaton, E., Imai, S. I., Frye, R. A., Pandita, T. K., Guarente, L., and Weinberg, R. A. (2001). hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell* 107, 149-159.
- von Willebrand, M., Williams, S., Saxena, M., Gilman, J., Tailor, P., Jascur, T., Amarante-Mendes, G. P., Green, D. R., and Mustelin, T. (1998). Modification of phosphatidylinositol 3-kinase SH2 domain binding properties by Abl- or Lck-mediated tyrosine phosphorylation at Tyr-688. *J Biol Chem* 273, 3994-4000.
- Walker, E. H., Perisic, O., Ried, C., Stephens, L., and Williams, R. L. (1999). Structural insights into phosphoinositide 3-kinase catalysis and signalling. *Nature* 402, 313-320.
- Wang, G., Barrett, J. W., Nazarian, S. H., Everett, H., Gao, X., Bleackley, C., Colwill, K., Moran, M. F., and McFadden, G. (2004). Myxoma virus M11L prevents apoptosis through constitutive interaction with Bak. *J Virol* 78, 7097-7111.
- Wang, Q., Gao, F., May, W. S., Zhang, Y., Flagg, T., and Deng, X. (2008). Bcl2 negatively regulates DNA double-strand-break repair through a nonhomologous end-joining pathway. *Mol Cell* 29, 488-498.

- Weber, A., Paschen, S. A., Heger, K., Wilfling, F., Frankenberg, T., Bauerschmitt, H., Seiffert, B. M., Kirschnek, S., Wagner, H., and Hacker, G. (2007). BimS-induced apoptosis requires mitochondrial localization but not interaction with anti-apoptotic Bcl-2 proteins. *J Cell Biol* *177*, 625-636.
- Wei, L., Yang, Y., and Yu, Q. (2001). Tyrosine kinase-dependent, phosphatidylinositol 3'-kinase, and mitogen-activated protein kinase-independent signaling pathways prevent lung adenocarcinoma cells from anoikis. *Cancer Res* *61*, 2439-2444.
- Wei, L., Yang, Y., Zhang, X., and Yu, Q. (2004). Altered regulation of Src upon cell detachment protects human lung adenocarcinoma cells from anoikis. *Oncogene* *23*, 9052-9061.
- Wei, M. C., Lindsten, T., Mootha, V. K., Weiler, S., Gross, A., Ashiya, M., Thompson, C. B., and Korsmeyer, S. J. (2000). tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. *Genes Dev* *14*, 2060-2071.
- Weigelt, B., Peterse, J. L., and van 't Veer, L. J. (2005). Breast cancer metastasis: markers and models. *Nat Rev Cancer* *5*, 591-602.
- Weng, C., Li, Y., Xu, D., Shi, Y., and Tang, H. (2005). Specific cleavage of Mcl-1 by caspase-3 in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in Jurkat leukemia T cells. *J Biol Chem* *280*, 10491-10500.
- Whitfield, J., Neame, S. J., Paquet, L., Bernard, O., and Ham, J. (2001). Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release. *Neuron* *29*, 629-643.
- Willis, S. N., Fletcher, J. I., Kaufmann, T., van Delft, M. F., Chen, L., Czabotar, P. E., Ierino, H., Lee, E. F., Fairlie, W. D., Bouillet, P., Strasser, A., Kluck, R. M., Adams, J. M., and Huang, D. C. (2007). Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. *Science* *315*, 856-859.

- Windham, T. C., Parikh, N. U., Siwak, D. R., Summy, J. M., McConkey, D. J., Kraker, A. J., and Gallick, G. E. (2002). Src activation regulates anoikis in human colon tumor cell lines. *Oncogene* 21, 7797-7807.
- Wolf, B. B., and Green, D. R. (1999). Suicidal tendencies: apoptotic cell death by caspase family proteinases. *J Biol Chem* 274, 20049-20052.
- Woods, N. T., Yamaguchi, H., Lee, F. Y., Bhalla, K. N., and Wang, H. G. (2007). Anoikis, initiated by Mcl-1 degradation and Bim induction, is deregulated during oncogenesis. *Cancer Res* 67, 10744-10752.
- Yamaguchi, H., Chen, J., Bhalla, K., and Wang, H. G. (2004). Regulation of Bax activation and apoptotic response to microtubule-damaging agents by p53 transcription-dependent and -independent pathways. *J Biol Chem* 279, 39431-39437.
- Yamaguchi, H., and Hendrickson, W. A. (1996). Structural basis for activation of human lymphocyte kinase Lck upon tyrosine phosphorylation. *Nature* 384, 484-489.
- Yamaguchi, H., Paranawithana, S. R., Lee, M. W., Huang, Z., Bhalla, K. N., and Wang, H.-G. (2002). Epothilone B analogue (BMS-247550)-mediated cytotoxicity through induction of Bax conformational change in human breast cancer cells. *Cancer Res* 62, 466-471.
- Yamaguchi, H., and Wang, H. G. (2002). Bcl-XL protects BimEL-induced Bax conformational change and cytochrome C release independent of interacting with Bax or BimEL. *J Biol Chem* 277, 41604-41612.
- Yamaguchi, H., and Wang, H. G. (2006). Tissue transglutaminase serves as an inhibitor of apoptosis by cross-linking caspase 3 in thapsigargin-treated cells. *Mol Cell Biol* 26, 569-579.

- Yamaguchi, H., Woods, N. T., Dorsey, J. F., Takahashi, Y., Gjertsen, N. R., Yeatman, T., Wu, J., and Wang, H. G. (2008). SRC directly phosphorylates bif-1 and prevents its interaction with bax and the initiation of anoikis. *J Biol Chem* 283, 19112-19118.
- Yang, J. S., Zhang, L., Lee, S. Y., Gad, H., Luini, A., and Hsu, V. W. (2006). Key components of the fission machinery are interchangeable. *Nat Cell Biol* 8, 1376-1382.
- Yawata, A., Adachi, M., Okuda, H., Naishiro, Y., Takamura, T., Hareyama, M., Takayama, S., Reed, J. C., and Imai, K. (1998). Prolonged cell survival enhances peritoneal dissemination of gastric cancer cells. *Oncogene* 16, 2681-2686.
- Yeatman, T. J. (2004). A renaissance for SRC. *Nat Rev Cancer* 4, 470-480.
- Yu, C. L., Meyer, D. J., Campbell, G. S., Lerner, A. C., Carter-Su, C., Schwartz, J., and Jove, R. (1995). Enhanced DNA-binding activity of a Stat3-related protein in cells transformed by the Src oncoprotein. *Science* 269, 81-83.
- Yu, G. W., Rudiger, S., Veprintsev, D., Freund, S., Fernandez-Fernandez, M. R., and Fersht, A. R. (2006). The central region of HDM2 provides a second binding site for p53. *Proc Natl Acad Sci U S A* 103, 1227-1232.
- Yu, J., Zhang, L., Hwang, P. M., Kinzler, K. W., and Vogelstein, B. (2001). PUMA induces the rapid apoptosis of colorectal cancer cells. *Mol Cell* 7, 673-682.
- Zhang, L., Yu, J., Park, B. H., Kinzler, K. W., and Vogelstein, B. (2000). Role of BAX in the apoptotic response to anticancer agents. *Science* 290, 989-992.

Zhang, Q., Thomas, S. M., Xi, S., Smithgall, T. E., Siegfried, J. M., Kamens, J., Gooding, W. E., and Grandis, J. R. (2004). SRC family kinases mediate epidermal growth factor receptor ligand cleavage, proliferation, and invasion of head and neck cancer cells. *Cancer Res* *64*, 6166-6173.

Zhong, Q., Gao, W., Du, F., and Wang, X. (2005). Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis. *Cell* *121*, 1085-1095.

About the Author

Nicholas Taylor Woods attended the University of Nebraska at Lincoln from 2000-2004 where he earned his Bachelor of Science degree in Biochemistry. During this time he gained valuable laboratory experience in the labs of Dr. Donald Lee, Dr. Han Asard, and Dr. Mohammad Koohmaraie researching plant genetics, redox biochemistry, and microbiology, respectively. After graduation, he joined the Cancer Biology Ph.D. program at the H. Lee Moffitt Cancer Center and Research Institute. There he joined the lab of Dr. Hong-Gang Wang and completed his dissertation work in the field of apoptosis. The focus of his research projects was the regulation of Bax activation in the initiation of apoptosis.