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Regulation of Cancer Cell Survival Mediated by Endogenous Tumor Suppression: A Dissertation

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Regulation of Cancer Cell Survival Mediated by Endogenous Tumor Suppression

A Dissertation Presented by

Minakshi Guha

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

July 10, 2009

Department of Cancer Biology

Regulation of Cancer Cell Survival Mediated by Endogenous Tumor Suppression

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July 10, 2009

Acknowledgements

I'd like to thank my thesis advisor, Dr. Dario Altieri for being an exceptional mentor throughout my graduate career. He has provided his unconditional support and guidance for the completion of my doctoral thesis. I strongly believe my achievement as a graduate student would not have been possible without his excellent mentorship. His ability to dedicate his time and energy to managing each and every project in our lab has always amazed me. His breadth of knowledge and attention to detail has been exceptional, but at the same time he always encouraged me to work independently and pursue my own ideas. My wonderful lab members – Takehiko Dohi, Jagadish Ghosh, Byoung Heon Kang, Christopher Raskett, Fang Xia, Janet Plescia, Pedro Canovas, Boglarka Gyurkocza, and Iren Kurtser were all very supportive and helpful during my training as a graduate student. I will never forget how each one of them used to go out of their way to help me solve my project challenges or discuss about potential experimental ideas. I would like to acknowledge Connie Lee and Swarna Mehrotra, the two other students in my lab who have never made me feel alone in my journey through graduate school and who were able to uplift me with their kind words and gestures. I'd also like to thank my thesis advisory committee members for helpful discussions and supporting me throughout my graduate career.

I would like to acknowledge my family for their moral support and guidance that has allowed me to focus on my goal to complete this degree. My mom, dad, and my sister have always been there for me whenever I needed any advice and taught me to never give up and to keep persevering through the challenges of life. I would like to thank Kamal Datta for always being there for me in times when it was tough and always giving me that extra boost of confidence before any major presentation. I would also like to thank my friends through graduate school, Katerina Mardilovich, Ermelinda Porpiglia, and Dimitra Pirperis who have made this journey so sweet and memorable. Thank you girls for sharing your life and lunches with me! Thank you all for believing in me and helping me every step of the way…I could have not done it without each and every one of you.

Abstract

Cancer is the second leading cause of death among men and women after heart disease. Though our knowledge associated with the complexities of the cancer network has significantly improved over the past several decades, we have only recently started to get a more complete molecular understanding of the disease. To better comprehend signaling pathways that prevent disease development, we focused our efforts on investigating endogenous tumor suppression networks in controlling effectors of cancer cell survival and proliferation. Survivin is one such effector molecule that controls both cell proliferation and survival. In order to identify how this protein is overexpressed in cancer cells as opposed to normal cells, we looked at signaling molecules that negatively regulate this inhibitor of apoptosis protein. PTEN and caspase 2 are two of the identified proteins that utilize their enzymatic activity to suppress tumor growth by inhibiting downstream cell survival effectors, namely survivin. PTEN uses its phosphatase activity to suppress the PI3K/AKT pathway and maintain cellular homeostasis. In the absence of AKT activity, FOXO transcription factors are able to target downstream gene expression and regulate cell proliferation and survival. Here we have identified survivin as a novel gene target of FOXO, which binds to a specific promoter region of survivin and suppresses its transcription. Alternatively, caspase 2 uses its catalytic activity to suppress survivin gene expression by targeting the NFκB pathway. Caspase 2 acts by cleaving a novel substrate known as RIP1 that prevents NFκB from entering the nucleus, thus inhibiting target gene transcription. Interestingly, survivin is known to be a direct gene target of NFκB that controls cancer cell survival. In our investigation, we found that survivin is downregulated upon caspase 2 activation via the NFκB pathway, resulting in

decreased cell cycle kinetics, increased apoptotic threshold and suppressed tumor growth in mice. These studies conclude that survivin is a common effector molecule that is regulated by tumor suppressors to maintain cellular homeostasis. However, upon deactivation of the tumor suppressor pathway, survivin is deregulated and contributes significantly to disease progression. These observations may lead to potential therapeutic implications and novel targeting strategies that will help eradicate harmful cancer cells and spare surrounding healthy cells; often the most persistent problem of most conventional chemotherapy.

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Chapter 1. Introduction

One of the hallmarks of cancer involves deregulated cell death pathways, making it difficult to kill tumor cells. A common problem encountered during treatment by chemotherapy or radiation therapy is resistance of transformed cells to programmed cell death, or apoptosis, which results in poor prognosis and increased tumor recurrence (Viktorsson et al., 2005). Apoptosis can be initiated by the mitochondria pathway (intrinsic) or by the death receptor pathway (extrinsic). Upon receiving an apoptotic stimulus, cells undergo a series of changes associated with cell death signaling that ultimately results in caspase activation, substrate cleavage, and finally phagocytosis of the apoptotic cell. Treatment options that implement radiation therapy utilize high energy ionizing radiation to cause DNA double strand breaks that eventually result in cell death via the intrinsic apoptotic pathway. However, many cancer cells are able to evade treatment by exploiting alternate pathways for cell survival and therefore are able to override cell death signals. The two main gene families that regulate apoptosis include the Bcl-2 and the inhibitors of apoptosis (IAPs) family of proteins (Altieri, 2003). The Bcl-2 family can be subdivided into pro- and anti-apoptotic proteins that are characterized by 1 to 4 copies of Bcl2 homology (BH) domain and a C-terminal hydrophobic region. The pro-apoptotic molecules include Bax, Bad, Bak and Bid that increase mitochondria permeability upon apoptotic stimulation. Alternatively, anti-apoptotic molecules that resist mitochondria permeability include Bcl-2 and Bcl-x_L. The central feature of Bcl-2 proteins is to homodimerize or heterodimerize at the mitochondrial membrane leading to the transition from cell survival to cell death depending on the differential recruitment of these proteins (Altieri, 2003). Among the

Bcl-2 family members that contain BH domains 1-3 are Bax and Bak that seem to initiate most of the mitochondria mediated apoptosis. In contrast, the IAP family members inhibit apoptosis by targeting caspases and downstream Bcl-2 proteins (Altieri, 2003). The IAPs are characterized by containing 1-3 copies of a 70 amino acid zinc finger fold known as the baculovirus IAP repeat (BIR). Some of them also contain a caspase recruitment domain (CARD) that helps in caspase binding and inhibition. These IAPs include, XIAP, c-IAP1, c-IAP2 and survivin. With the exception of XIAP, all the other IAPs inhibit apoptosis independent of direct caspase inhibition (Altieri, 2008). Survivin in particular, associates with XIAP increasing its stability against degradation and synergistically inhibits caspase-dependent apoptosis (Dohi et al., 2004b). Moreover, proteins released by the mitochondria during permeability transition include pro-apoptotic Smac/DIABLO that can relieve the inhibition effect of IAPs on caspases, leading to apoptosis (Altieri, 2003). Other mechanisms of IAP regulation of apoptosis also involve activation of TGFβ or c-Jun kinase signaling pathway (Altieri, 2003). The role of IAPs in mediating the balance between cell survival and cell death is one of the essential features of these proteins making them important molecules in the study of tumor progression.

Survivin in Cancer

The ability of transformed cells to evade apoptosis and favor cell survival is a hallmark of tumor progression and resistance to therapy (Luo et al., 2009). IAPs are highly expressed in tumor cells and can block apoptosis by inhibiting effector caspases (Dohi and Altieri, 2005). Survivin, in particular, is overexpressed in many different

cancers, but remains undetectable in most normal tissues (Altieri, 2008). The differential expression of survivin can be attributed to deregulated gene expression in cancer cells. Survivin is an evolutionarily conserved protein that plays an essential role in both mitosis and inhibition of cell death. Moreover, survivin is indispensable during embryonic and fetal development since survivin knockout mice are embryonic lethal at day 3.5 with clear defects in mitosis (Uren et al., 2000). Understanding the multiple functional roles of survivin in cancer, including resistance to cancer therapy, promoting angiogenesis and preventing cells from apoptosis makes this a relevant target protein for cancer biologists to comprehend (Altieri, 2008; Tran et al., 2002).

Survivin plays a critical role in regulating cell division and proliferation by interacting with members of the chromosomal passenger complex (Altieri, 2008). Survivin facilitates chromosomal alignment during mitosis by targeting molecules of the chromosomal passenger complex such as Aurora kinase B (AURKB), inner centromere protein (INCENP), and borealin to kinetochores (Jeyaprakash et al., 2007). Proper microtubule attachment to the aligned chromosomes is essential for mitotic checkpoint, however, mislocalization of the chromosome passenger complex causes mitotic failure resulting in aneuploidy and missegregation (Altieri, 2008). Thus, survivin plays a role in spindle checkpoint as it is indicated as a sensor for kinetochore to microtubule attachement and tension that is prerequisite for chromosomal segregation. Moreover, some survivin localizes on the microtubules during spindle formation and plays a role in stabilizing microtubule dynamics (Rosa et al., 2006). Survivin depletion by siRNA results in increased incidence of microtubule nucleation and catastrophe, whereas overexpression of survivin results in suppressed microtubule dynamics (Rosa et al.,

2006). The regulation of microtubule dynamics by survivin also overlaps with the role of survivin in cell death. Survivin is phosphorylated on Thr34 by CDK1, or checkpoint dependent kinase 1 (O'Connor et al., 2002) that allows survivin to be stabilized and counteract apoptosis during cell division. Loss of phosphorylation on Thr34 by CDK1 results in dissociation of survivin from a caspase 9 complex resulting in mitotic failure and subsequent apoptosis (O'Connor et al., 2000). Thus, upon DNA damage or exposure to certain mitotic inhibitors, like nocodazole, survivin is able to prevent apoptosis when phosphorylated by CDK1 (O'Connor et al., 2000).

Survivin's role as an inhibitor of apoptosis in tumor cells comes from the observation that acute lowering of its expression by small interfering RNA (siRNA), ribozyme or dominant negative treatment results in apoptosis (Altieri, 2008). Therefore, upstream factors that regulate survivin gene expression and protein stability are crucial in understanding its cytoprotective function. Several tumor suppressor genes that regulate survivin expression at the transcriptional level include p53 (Hoffman et al., 2002; Mirza et al., 2002), adenomatous polyposis coli APC (Zhang et al., 2001), histone deacetylase SIRT1 (Wang et al., 2008b), and fragile histidine triad gene (FHIT) (Semba et al., 2006). On the other hand, oncogenic factors that control survivin gene expression include TCF4/β catenin (Kim et al., 2003), signal transduction and activator of transcription (STAT3) (Gritsko et al., 2006), and E2F transcription factors (Jiang et al., 2004). Survivin is also regulated by post-transcriptional mechanisms by stabilizing survivin mRNA or protein levels. Post-transcriptional regulators include mammalian target of rapamycin (mTOR) (Vaira et al., 2007), phosphatidylinositol-3-kinase-AKT pathway (PI3K/AKT) (Asanuma et al., 2005), CDK1 (O'Connor et al., 2000), and heat shock

protein 90 (HSP90) (Kang and Altieri, 2006) just to name a few. Thus, survivin acts as a nodal protein in cancer progression as many of these proteins or pathways are modified or mutated resulting in deregulated survivin expression and increased cell survival threshold.

The mechanism behind survivin induced cytoprotection relies both on intermolecular interactions as well as mitochondrial dynamics. One of the key features of survivin as an inhibitor of apoptosis protein is its ability to bind to XIAP, another member of the IAP family. Dohi et al. have shown that a mitochondria specific survivin pool is released from the intermembrane space of the mitochondria to the cytosol in response to DNA damage (Dohi et al., 2004a). As a consequence, survivin is able to prevent apoptosis by forming a complex with XIAP that enhances protein stability and synergistically inhibits caspase 9 activation (Dohi et al., 2004b). Survivin binding to XIAP can be regulated by phosphorylation of survivin on Ser20 by protein kinase A (PKA), that prevents complex formation and caspase inhibition (Dohi et al., 2007). In addition, survivin is regulated by chaperone molecules such as heat shock protein 90 (HSP90) which is involved in survivin import to the mitochondria (Kang et al., 2007). Inside the mitochondria, survivin is able to bind SMAC, a pro-apoptotic molecule that is known to inhibit XIAP's effect on caspase inhibition. Thus, by sequestering SMAC away from XIAP, survivin promotes an anti-apoptotic environment (Altieri, 2008).

Numerous studies have correlated increased survivin levels with poor prognosis and cancer progression. Survivin expression has been associated with increased cancer recurrence and poor survival rate in a variety of cancer types including neuroblastoma, lung and breast cancer (Ito et al., 2005; Shinohara et al., 2005; Yamashita et al., 2007).

More specifically, nuclear staining of survivin in tissue samples correlated with increased risk for recurrence and death among nonsmall cell lung carcinoma patients (Shinohara et al., 2005). Thus, molecular targeting of the survivin network in these patients may benefit disease outcome, by limiting cell proliferation and promoting cell death. In fact, several antagonists have already emerged over the past several years as potential therapeutic strategy to target survivin as a nodal protein that links cell death and cell division mediators (refer to Fig. 1-1). These include an antisense molecule, LY2181308, that specifically targets survivin, and a small molecule inhibitor of survivin transcription, YM155 that have both completed phase *I* clinical trials and are currently undergoing phase *II* clinical trials (Altieri, 2008). Our lab has also synthesized a peptidomimetic, also known as Shepherdin that serves as a HSP90 and survivin antagonist by interfering with the ATPase pocket of HSP90 and destabilizing its client proteins (Plescia et al., 2005). Preliminary studies with shepherdin showed promising anticancer activity and low toxicity in mice with human PC3 xenografts (Plescia et al., 2005). Although, many of these anticancer treatments are still in the preliminary stage of testing, there is great promise for future drug discovery efforts that target survivin and its client proteins. Further elucidating how survivin is regulated in cancer cells and identifying additional functions that it may have as a nodal protein, may point to better drug discovery efforts and understanding of survivin dynamics.

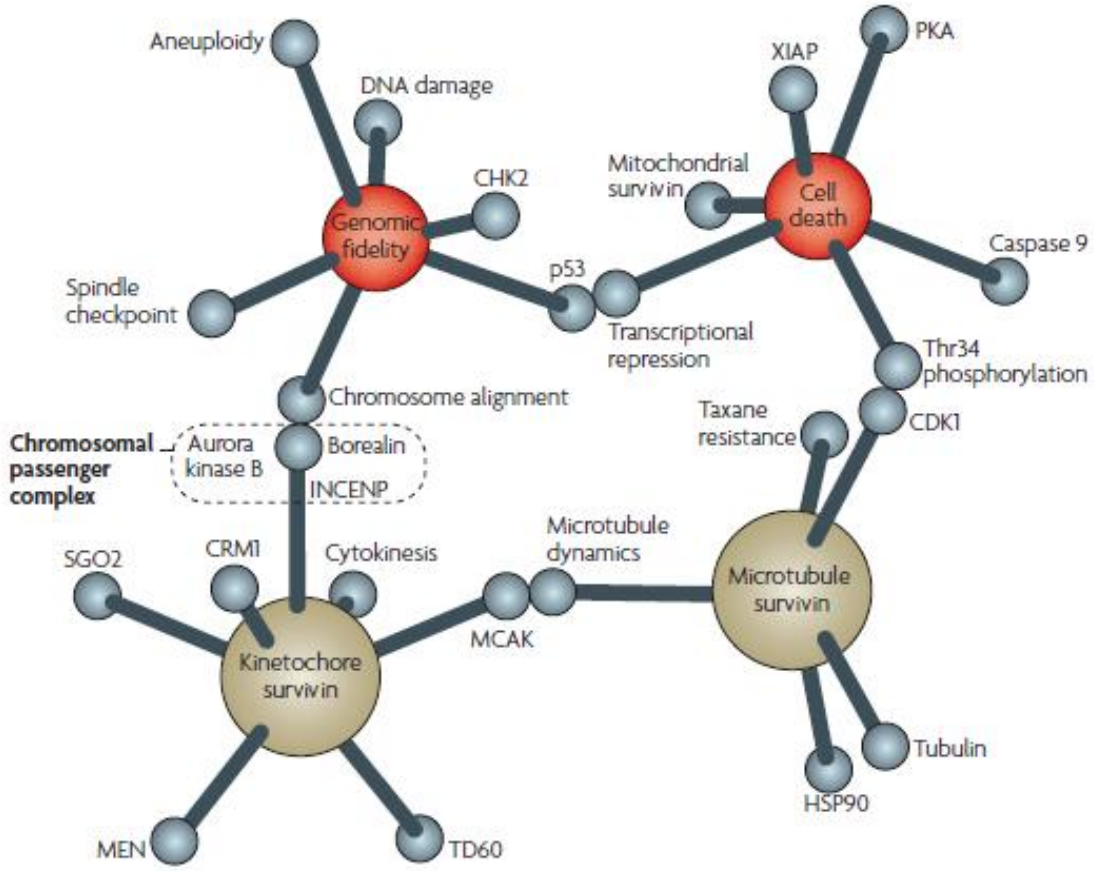


Figure 1-1 Survivin network

Survivin network linking cell death and cell division functions for overall tumor maintenance. (Altieri, 2008)

Tumor Suppression Network

Characteristics of cell transformation often involve the ability of cells to evade apoptosis, escape checkpoint controls and proliferate without restraints. Fortunately, evolution has installed innate tumor suppression mechanisms that keep cells in check despite being constantly exposed to DNA damaging factors from environmental stresses (Lowe et al., 2004). These tumor suppression mechanisms oppose cell proliferation and transformation by inducing either apoptosis or senescence. Senescence occurs when cells go into a state of permanent and irreversible cell-cycle arrest (Campisi, 2001) . This happens as a result of activation of certain oncogenes, such as Ras, that may trigger untransformed cells to undergo senescence. Therefore, endogenous tumor suppressors and cell cycle checkpoints are at play to prevent cells from being susceptible to oncogene-mediated transformation. These tumor suppressors include the classic Rb and p53 known regulators of senescence (Lowe et al., 2004). Escape from oncogene-induced senescence is one of the prerequisites of tumor progression when cells undergo transformation and bypass checkpoint controls that allow them to survive and proliferate, also known as immortalization (Lowe et al., 2004). Similarly, p53 is also known to induce apoptosis in response to a variety of cell stresses including DNA damage, hypoxia and nutrient deprivation. Apoptosis can be induced by oncogenic lesions both by the intrinsic and extrinsic pathways that ultimately lead to cell suicide. The intrinsic form of cell death involves permeabilization of the mitochondria that results in the release of several pro-apoptotic factors, such as cytochrome C, that forms a complex with Apaf-1 to activate caspase 9 (Li and Yuan, 2008). This leads to the caspase cascade involving downstream activation of effector caspases, such as caspase 3 and caspase 7. On the other

hand, the extrinsic apoptotic pathway involves the activation of cell-surface death receptors that rely on death ligands, such as TNFα, FasL and TRAIL. Upon interaction of the ligands to their respective receptors, a 'death-inducing signaling complex' (DISC) is formed that activates caspase 8. Recruitment of caspase 8 to the DISC complex then initiates autocatalysis of the enzyme leading to the caspase cascade and subsequent apoptosis (Li and Yuan, 2008). The focus of this dissertation involves elucidating apoptosis control that is regulated by the tumor suppression network. We have identified two important tumor suppressors that target survivin as one of the main effectors of this pathway to regulate cancer cell survival. Caspase 2 and PTEN are two such upstream regulators that drive tumor suppression in two completely independent pathways that ultimately converge on survivin as their main effector molecule.

Caspase-dependent Apoptosis

Caspase 2 is an evolutionarily conserved caspase, or cysteine protease that is predominantly known to mediate apoptosis (Zhivotovsky and Orrenius, 2005). The enzyme is associated with a p53-induced death domain containing protein, or PIDDosome complex, but its exact form of activation and downstream target proteins remain poorly understood (Tinel and Tschopp, 2004). Caspase 2 belongs to the Ich 1 subfamily and shares homology with initiator caspases involved in stress-induced apoptosis (Zhivotovsky and Orrenius, 2005). The enzyme contains a CARD domain or caspase recruitment domain that is closely related to that of caspase 9. One of the unique features of caspase 2 is that it resembles both upstream and downstream caspases. There are two mRNA species of caspase 2 – a long caspase-2L and a short caspase-2S. Overexpression of caspase-2L induces cell death while overexpression of caspase-2S suppresses cell death (Wang et al., 1994; Zhivotovsky and Orrenius, 2005). Both mRNA variants are differentially expressed in various tissue types and selectively expressed during different stages of embryonic development (Zhivotovsky and Orrenius, 2005). This indicates that caspase 2 has essential roles in both survival and programmed cell death and can vary from one cell type to another.

Localization of caspase 2 within the cell has also been found to be important in regulating its apoptotic function. Caspase 2 is found in the nucleus, Golgi complex, and soluble cytoplasm. There are two nuclear localization signals (NLS) in the prodomain of caspase 2 that regulates its nuclear import. Activation of the nuclear pool results in mitochondrial dysfunction, indicating a potential existence of a nuclear-mitochondria apoptotic pathway (Paroni et al., 2002; Robertson et al., 2002). However, overexpression

of a cytosolic form of caspase 2 with mutated NLS was also able to induce apoptosis. Therefore the role of caspase 2 in the nucleus has still been unclear. Caspase 2 localization in the Golgi complex has been attributed to cleavage of golgin-160 that disassembles this organelle during apoptosis (Mancini et al., 2000). Lastly, caspase 2 abundance in the cytosol is attributed to its ability to interact with several death adaptor molecules such as RAIDD, RIP1 and TRADD, thus suggesting a potential role in death receptor-mediated apoptosis (Lamkanfi et al., 2005; Zhivotovsky and Orrenius, 2005).

Caspase 2 is the most apical caspase involved in the intrinsic apoptotic pathway. Upon DNA damage, the enzyme is activated by autocatalysis and recruited to the PIDDosome complex (Gao et al., 2005; Tinel and Tschopp, 2004). Activated caspase 2 acts upstream of mitochondrial events and induces the release of cytochrome c and other proapoptotic proteins resulting in cell death (Guo et al., 2002; Lassus et al., 2002; Robertson et al., 2002). Other key players in this pathway include BH3 motif-only proapoptotic Bcl-2 family members that have been shown to be cleaved by caspase 2 and mediate the mitochondrial apoptotic pathway (Gao et al., 2005). A recent study revealed that Bid cleavage by caspase 2 is essential for cytochrome c release and that other Bcl-2 family members are important in regulating caspase 2 induced apoptosis (Gao et al., 2005). Finally, cyclin D3, a cell cycle regulator and the E2F1 transcription factor have also been implicated in mediating caspase 2- induced cell death during cell cycle progression (Mendelsohn et al., 2002; Nahle et al., 2002). Overexpression of cyclin D3 showed increased caspase 2 cleavage that contributes to cell death during S-phase entry (Mendelsohn et al., 2002). Similarly, E2F1 expression results in accumulated caspase 2 by a direct transcriptional mechanism that leads to cell death upon cell cycle re-entry

(Nahle et al., 2002). Although many proteins have been implicated in activation of caspase 2, it is now clear that caspase 2 is activated by dimerization and not processing of the zymogen (Krumschnabel et al., 2009). Thus a close proximity model for caspase 2 activation seems to be sufficient for its catalytic activity and subsequent processing and removal of the prodomain. In fact, the catalytic site of caspase 2, cysteine 320, was found to be essential for autoprocessing of the protein, consistent with the 'initiator' status of caspase 2 (Krumschnabel et al., 2009).

Caspase 2 activation in response to DNA damage, including UV radiation, cytokine deprivation and administration of TNF or TRAIL seems to be a prerequisite for apoptosis onset. One study showed that TNFα sensitizes cancer cells to chemotherapeutic agents by a mitochondrial apoptotic pathway that requires caspase 2 activation (Schmelz et al., 2004). It appears that caspase 2 activation is necessary for translocation of BAX to the mitochondria and release of pro-apoptotic proteins such as Smac/DIABLO and cytochrome C. Moreover, studies using recombinant caspase 2 showed that the processed enzyme was capable of outer mitochondrial membrane permeabilization and subsequent release of pro-apoptotic factors like cytochrome C (Robertson et al., 2004). Therefore, loss of caspase 2 may account for deregulated apoptosis in cancer and resistance to chemotherapy. Surprisingly, caspase 2-/- mice are viable and show limited phenotypic changes. Defects include excessive germ cells in the female ovaries and resistance to apoptosis following chemotherapeutic treatment of oocytes and lymphocytes (Morita et al., 2001). This indicates the existence of compensatory mechanisms that render the caspase 2 knockout mouse viable with very little defects. Recently however, caspase 2 has been indicated to play a role in tumor suppression as caspase 2 knockout mouse

embryonic fibroblasts (MEFs) induced tumor growth *in vivo* (Ho et al., 2009). These caspase 2-null MEFs showed increased resistance to apoptotic stimulus and cell cycle checkpoint defects upon gamma-irradiation (Ho et al., 2009). Despite activation of caspase 2 upon DNA damage, there still remains the question whether caspase 2 plays a role in cell survival mediated through NFκB or whether it contributes to cell death. Secondly, the mechanism of caspase 2 activation in vivo is still not well characterized. Thirdly, the physiological function of caspase 2 still remains to be clarified as mice deficient in caspase 2 are viable. And lastly, very few studies have addressed the participation of caspase 2 in human disease and the mechanism by which it regulates cancer cell survival. The objective of this study will be to elucidate some of the unknown pathophysiological functions of caspase 2 in vivo and to determine how this protein contributes to disease progression.

RIP1 regulation of NFκB

A protein that is known to interact with caspase 2, also known as receptor interacting protein, or RIP1 kinase, is a crucial regulator of cell death and cell survival via the NFκB signaling axis. RIP kinases are classified as serine/threonine kinases based on sequence similarities and substrate specificities (Festjens et al., 2007). RIP1 in particular contains a C-terminal domain that resembles the death domain (DD) superfamily and a caspase recruitment domain (CARD) allowing its recruitment to protein complexes following stimulation by a variety of stress signals (Festjens et al., 2007). RIP1 acts as an adaptor protein by binding to death receptors such as tumor necrosis factor receptor, TNF-R1, and TNF-related apoptosis-inducing ligand, TRAIL-

R1, and TRAIL-R2 (Hsu et al., 1996). The function of RIP1 in cellular homeostasis was confirmed by the fact that $RIP1^{-/-}$ mice appear normal at birth, but die at the age of 1-3 days (Kelliher et al., 1998). These mice display extensive apoptosis in both lymphoid and adipose tissue, thus making RIP1 indispensable in maintaining cellular integrity. Moreover, several studies have shown a role of RIP1 in activating a survival signaling pathway mediated by the nuclear transcription factor, NFκB (Festjens et al., 2007).

NFκB signaling pathway can be initiated by binding of TNFα to its receptor, TNF-R1, which induces the formation of two signaling complexes, one of which involves RIP1 and TNF receptor associated factor 2, or TRAF2. Recruitment of this complex (complex I) to the receptor results in the activation of NFκB survival signaling that leads to the subsequent induction of several anti-apoptotic genes, including c-FLIP, cIAP1 and cIAP2 (Festjens et al., 2007). Moreover, Kelliher and group showed that RIP1 deficient cells were unable to induce NFκB activation following stimulation by TNFα (Kelliher et al., 1998). RIP1 also links TNFR1 to the caspase cascade, forming a second complex that is involved in pro-apoptotic signaling. In this parallel signaling pathway, both caspase-8 and Fas associated death domain, or FADD, are recruited as part of complex II, leading to the consequent initiation of apoptosis (Festjens et al., 2007). In this caspase cascade, NFκB activation is blocked following TNFα mediated signaling by direct caspase 8 cleavage of RIP1 (Lin et al., 1999). Therefore complex II results in apoptosis when complex I-mediated activation of NFκB is weak. Thus, the cleavage of RIP1 is a major regulator of TNFα-induced apoptosis by determining a cell's fate to live or die.

The mechanism by which RIP1 induces NFκB activation is dependent on RIP1 ubiquitination and activation of an intermediate IκB signaling complex. Upon TNFα

stimulation, RIP1 becomes autophosphorylated and polyubiquitinated on both Lys48 and Lys63 (Festjens et al., 2007). Lys48 polyubiquitination leads to degradation of the protein by proteasomes, while Lys63 linkages result in the activation of the IκB kinase. In fact, Lys 63 polyubiquitination of RIP1 is required for the TNFα-induced activation of NFκB survival signaling (Ea et al., 2006). Moreover, a point mutation of RIP1 on Lys377 (K377R) blocks polyubiquitination by K63-linked polyubiquitin chains and prevents the recruitment of IκB kinase, IKK and TAK1 complex to the TNF receptor, thereby inhibiting NFκB activation (Ea et al., 2006). Therefore, polyubiquitination of RIP1 is essential for the activation of IKK that phophorylates IκB, an inhibitor of NF-κB, and targets it for degradation by the proteasomal pathway. NFκB is then liberated from the inhibitory complex and is translocated to the nucleus where it initiates target gene transcription involved in immunity, inflammation and survival (Chen, 2005).

PTEN/PI3K signaling in cancer

The second part of this thesis focuses on an alternate tumor suppressor pathway that helps regulate effector molecules of tumor growth. Over the past decade, the PTEN/phosphatidylinositol 3-kinase (PI3K) signaling pathway proved to be one of the most deregulated pathways in human cancers (Engelman et al., 2006). Constitutive activation of this pathway leads to genomic instability, increased cell proliferation and cell survival. PI3Ks consist of heterodimers composed of a regulatory subunit, known as p85, and a catalytic subunit known as p110 (Engelman et al., 2006). Activation of PI3K is initiated by binding to growth factor receptor tyrosine kinases (RTKs) or G-protein coupled receptors (GPCRs). Once activated, PI3K converts its substrate, also known as

$PI(4,5)P_2$ to $PI(3,4,5)P_3$, by adding a phosphate group to the '3' position of the inositol ring (Osaki et al., 2004). PTEN, a tumor suppressor protein, reverses this process by acting as a lipid phosphatase and thereby negatively regulating the PI3K pathway (refer to Fig. 1-2). When $PI(3,4,5)P_3$ (PIP_3) is in abundance, proteins with plekstrin homology (PH) domain are able to bind PIP_3 and initiate downstream signaling. Such proteins include the serine/threonine kinase, AKT, and the 3'-phosphoinositide-dependent kinase, PDK1 (Osaki et al., 2004). AKT translocates to the membrane and interacts with PIP_3 via its PH domain, enabling it to be phosphorylated by PDKs at two critical residues, Thr308 and Ser473 (refer to Fig. 1-2). There are three AKT isoforms also known as protein kinase B (PKB), AKT1 (PKBα), AKT2(PKBβ), and AKT3(PKBγ) that are differentially expressed in various tissues with AKT1 being most abundant in brain, heart and lung (Osaki et al., 2004). Phosphorylation of AKT by PDK leads to its active conformation and stabilization. AKT is then translocated to the cytoplasm where it can initiate cell survival and cell proliferation by targeting numerous downstream substrates of AKT.

The phosphatase and tensin homologue deleted on chromosome 10, or PTEN, is perhaps the most important negative regulator of the PI3K pathway. In fact, PTEN is the second most mutated tumor suppressor gene in human cancers just after p53 (Yin and Shen, 2008). In many human cancers, PTEN is deleted or mutated leading to activation of the PI3K/AKT pathway and subsequent increase in cell proliferation and resistance to apoptosis. In addition, germline mutations of PTEN were found to occur in 80% of patients with Cowden Syndrome, a disease that is associated with cancer predisposition (Eng, 2003). More importantly, somatic mutation or allelic deletion of PTEN is a

common event in glioblastomas, melanomas, and cancers of the prostate and endometrium (refer to Table 1-1) (Engelman et al., 2006). While mice homozygous for PTEN are embryonic lethal, mice heterozygous for PTEN develop neoplasia of the endometrium, which correlates well with 50% of human endometrial cancers that also harbor PTEN mutations (Stambolic et al., 2000).

PTEN acts as a powerful tumor suppressor having important roles in chromosomal stability, cell cycle progression, cell survival and motility (Yin and Shen, 2008). One of the first studies linking PTEN to genomic instability was done by Parson's group showing PTEN-null embryonic stem cells exhibiting checkpoint defects in response to ionizing radiation (Puc et al., 2005). PTEN silencing results in AKT activation that triggers phosphorylation of the checkpoint protein, CHK1, and subsequent sequesteration and degradation of CHK1 in the cytoplasm (Puc et al., 2005). This impairs the ability of cells to undergo DNA repair and results in double-strand chromosomal breaks. On the other hand, overexpression of nuclear PTEN has been associated with cell cycle arrest at the G0/G1 phase. Among AKT substrates that play a role in cell cycle are Forkhead transcription factors (FOXOs) and glycogen synthase kinase3 (GSK3) (Sansal and Sellers, 2004). Activation of AKT, or loss of PTEN, also results in cell survival since FOXO transcription factors target pro-apoptotic genes such as FAS and Bim (Brunet et al., 1999). Lastly, PTEN also effects cell migration and invasion by utilizing its protein phosphatase activity. Focal adhesion kinase (FAK) has been identified as a direct substrate of PTEN and known to be a mediator of cell surface interactions (Tamura et al., 1998). By reducing FAK tyrosine phosphorylation, PTEN regulates cell motility by

inhibiting cell migration and invasion, thus promoting tumor suppression (Tamura et al., 1998).

Crosstalk between p53 and PTEN occurs at multiple nodes and loss of both of these tumor suppressor functions results in malignancy in a synergistic manner. The tumor suppressor p53 binds to the promoter region of PTEN and activates its gene transcription, thereby functioning as a negative regulator of PI3K signaling (Stambolic et al., 2001). Moreover, upon DNA damage induction of p53 in tumor cells with wild type p53 resulted in increased PTEN mRNA levels. Increased PTEN level participates in a positive feedback loop by protecting p53 from mdm2-mediated degradation (Li and Ross, 2007). PTEN does so by binding to p53 and preventing mdm2 from ubiquitination and degradation of p53 (Mayo et al., 2002). PTEN also regulates p53 stability by negatively regulating PI3K and AKT activity leading to decreased phosphorylation of mdm2 (Mayo and Donner, 2002). Dephosphorylation of mdm2 sequesters the protein in the cytoplasm and prevents ubiquitination of nuclear p53. An increased level of p53 in the nucleus interacts and increases PTEN levels, thus contributing to the positive feedback loop. Another model that looks at p53 and PTEN interactions involves studies that were done in mice prostate tumors. Conditional disruption of p53 alone in the mouse prostate did not result in tumorigenesis, whereas PTEN inactivation resulted in progressive prostate cancer after about a period of 10 months (Chen et al., 2005). Interestingly, loss of both p53 and PTEN resulted in synergistic tumorigenesis that led to lethality as early as 7 months. Chen and group concluded that complete loss of PTEN resulted in senescence induced by p53. On the occasion that p53 is also inactivated together with PTEN, mice

no longer displayed senescence, but instead developed invasive prostate carcinoma in situ (Chen et al., 2005).

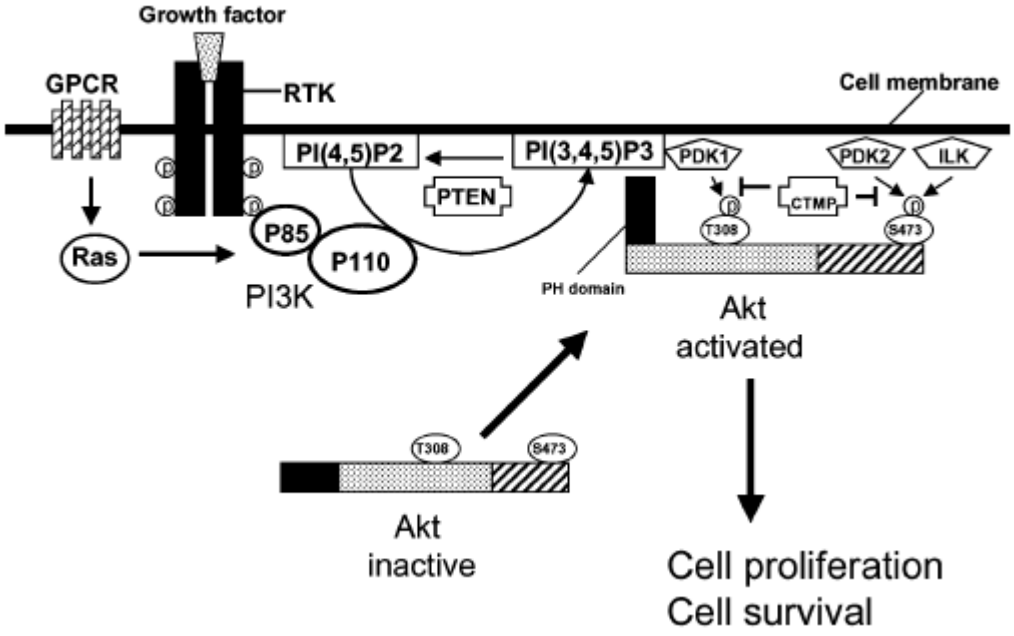


Figure 1-2 PI3K signaling pathway

Schematic of PI3K signaling pathway showing PTEN mediated negative regulation of the PI3K pathway by converting PI(3,4,5)P3 to PI(4,5)P2. PI3K is comprised of a regulatory p85 subunit and p110 catalytic subunit that mediates PI3K activity. AKT translocates to the cell membrane and interacts with PI(3,4,5)P3 via its PH domain and is phosphorylated at two critical residues (Thr308 and Ser473). (Osaki et al., 2004)

Genetic mutations	Cancer type	Percentage frequency*	References
PIK3CA (p110α)			
Mutations	Breast	26% (176/684)	78, 126–131
	Colon	26% (88/337)	78,132
	Glioma	8% (14/182)	78,133, 134
	Hepatocellular	36% (26/73)	128
	Ovarian	10% (35/365)	127,130
	Lung	2% (4/253)	78,128
	Gastric	7% (24/338)	78,128, 132,135
Amplifications	Head and neck	42% (54/128)	136,137
	Thyroid	9% (12/128)	138
	Lung:		139,140
	Squamous cell	66% (46/70)	
	Adenocarcinoma	5% (4/86)	
	Breast	9% (8/92)	126
	Gastric	36% (20/55)	141
	Oesophageal adenocarcinoma	6% (5/87)	142
	Cervical	69% (11/16)	143
PTEN			
Loss of heterozygosity	Glioblastoma	54% (98/180)	144–146
	Prostate	35% (88/250)	147–151
	Breast	23% (37/164)	152,153
	Melanoma	37% (53/143)	154–157
	Gastric	47% (14/30)	141
Mutations‡	Glioblastoma	28% (122/432)	144–146, 158–160
	Prostate	12% (26/218)	147–151, 161
	Breast	0% (0/164)	152,153
	Melanoma	8% (15/185)	154–157, 162
	Gastric	0% (0/30)	141

Table 1-1 Frequency of mutations in PTEN and PI3K in cancers

(Engelman et al., 2006)

Forkhead transcription factors

The forkhead transcription factors are DNA binding proteins that promote the expression of several apoptotic and cell cycle genes in the mammalian system (Engelman et al., 2006). The forkhead family of proteins contains about 100 members that are divided into various subclasses, of which members of the O subclass are direct substrates of AKT signaling pathway. The FoxO subfamily is known to mediate a diverse range of cellular functions involving differentiation, metabolism, proliferation and survival (Accili and Arden, 2004). One of the main mechanism by which FOXOs are regulated involves AKT phosphorylation of FOXO1 (FKHR), FOXO3a (FKHRL1), and FOXO4 (AFX) at three critical residues (i.e. T24, S256, S319 on FOXO1) enabling these transcription factors to be exported out of the nucleus, thus preventing target gene activation/repression (Manning and Cantley, 2007). Upon phosphorylation and nuclear exclusion, these FOXO proteins are sequestered by 14-3-3 scaffold proteins that lead to the inhibition of several cellular processes mediated by FOXO target genes (refer to Fig. 1-3). AKT blocks FOXO-mediated transcription of genes that result in apoptosis, induce cell-cycle arrest and regulate metabolism (Manning and Cantley, 2007). Among target genes that regulate apoptosis include the cytokine Fas ligand (FasL) and the proapoptotic BH3-only protein, BIM. FOXO target genes that regulate cell cycle include the cyclin dependent kinase inhibitor, $p27^{KIP1}$ and the retinoblastoma-like 2, RBL2 (Engelman et al., 2006). In PTEN-deficient cells, FOXO factors are constitutively phosphorylated by AKT and predominantly localized in the cytoplasm. In fact, FOXO1 has been linked as a tumor suppressor due to its ability to repress D-type cyclins that are involved in cell cycle progression (Ramaswamy et al., 2002). In this context, FOXO1 mediated repression of

D-type cyclins induced G1 arrest leading to the inhibition of cell proliferation and transformation (Ramaswamy et al., 2002). On the other hand, PTEN loss of function in prostate cancers showed increased apoptosis upon overexpression of FOXOs (Modur et al., 2002). This was due to FOXO mediated transcriptional activation of TRAIL, the proapoptotic member of the TNF family of death receptors (Modur et al., 2002). Moreover, microarray analysis showed a number of other genes that are differentially regulated upon induction of a constitutively active FOXO1 virus (FOXO-TM) in HUVEC cells as listed in Table 1-2 (Daly et al., 2004). Among them is survivin, a target gene that is predicted to be repressed upon FOXO-TM expression. FOXO transcription factors are one of the several AKT substrates that contribute to the activation of many cellular processes that are essential for cancer cell survival (refer to Fig. 1-3). Hence upon AKT activation, cancer cells are able to control the expression of several target genes in order to evade apoptosis and enhance cell proliferation, thereby promoting tumorigenesis. In this study we identified the molecular basis of FOXO mediated transcriptional regulation of survivin in the context of human cancers.

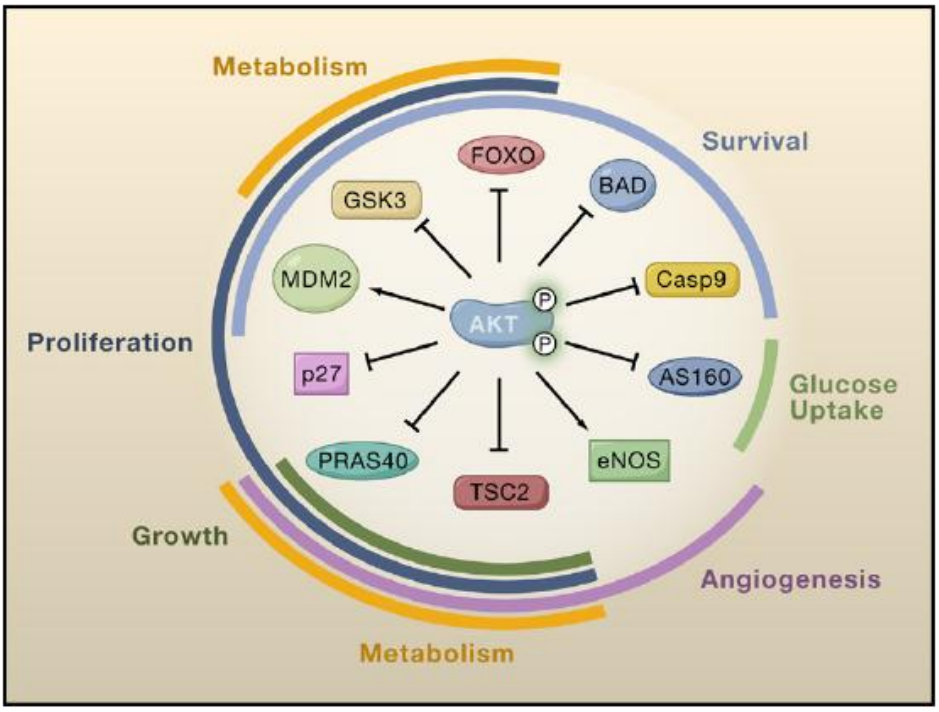


Figure 1-3 AKT substrates and their functions

(Manning and Cantley, 2007)

GenBank #	Genes induced by FKHR	Ratio FKHR/GFP 12 h	16 h	24 h	Functional class
BC005322	*decorin*	39.99	35.92	100.00	Matrix remodeling
NM_002423	*matrix metalloproteinase 7 (matrilysin)*	37.84	100.00	100.00	Matrix remodeling
AF020761	*ubiquitin-conjugating enzyme E2D 1*	8.31	13.28	9.28	Intracellular signaling
NM_024501	*homeo box D1*	7.74	5.32	4.92	Transcription
U40372	*phosphodiesterase 1C*	6.93	5.81	16.11	Intracellular signaling
AB009865	*angiopoietin 2*	6.89	17.01	14.33	Angiogenesis
AI679737	*EGF receptor pathway substrate 8 (eps8)*	6.55	4.99	4.76	Intracellular signaling
AF287272	*Kruppel-like factor 5 (KLF5)*	6.55	13.14	14.20	Transcription
AI299309	*Inhibitor of DNA binding 2 (Id2)*	6.33	8.78	13.48	Transcription
AW022060	*TNF superfamily, member 10 (TRAIL)*	6.13	3.72	2.76	Signaling/apoptosis
BG938585	*semaphorin 3C*	5.88	8.65	86.63	Axon guidance/angiogenesis
AB053450	*Fibrillin 3*	4.48	6.31	5.32	Matrix remodeling
AU137979	*Lumican*	3.14	7.69	26.83	Matrix remodeling
M69225	*Bullous pemphigold antigen 1 (230/240kD)*	2.54	7.27	11.97	Matrix remodeling
D45917	*TIMP-3*	1.94	3.46	6.35	Matrix remodeling
AL570066	*Fibulin 5 (DANCE; EVEC)*	1.82	3.92	11.15	Matrix remodeling
X14420	*Pro-α-1 type 3 collagen*	1.77	2.85	12.79	Matrix remodeling
M97252	*Kallmann syndrome 1 sequence (Anosmin)*	2.79	6.85	54.27	Matrix remodeling
AW263423	*Slit2*	2.60	3.55	5.77	Axon guidance/angiogenesis
U00115	*B-cell CLL/lymphoma 6 (BCL-6)*	2.58	5.80	6.41	Transcription/apoptosis
NM_006080	*Semaphorin III (sema 3A)*		8.16	10.52	Axon guidance/angiogenesis
NM_002019	*Fms related tyrosine kinase (Fit-1)*		6.88	11.30	Angiogenesis
U79716	*Reelin*		5.18	15.07	Cell migration
	Genes repressed by FKHR				
BF183143	*Serine/threonine kinase 12*	1.23	0.50	0.18	Cell cycle
BI091762	*RAB6 interacting, kinesin-like (rabkinesin 6)*	1.14	0.44	0.15	Cell cycle
AL571008	*Survivin*	1.06	0.52	0.19	Apoptosis/cell cycle
BC006510	*Similar to cyclin B1*	0.99	0.54	0.20	Cell cycle
NM_007317	*Kinesin-like 4*	0.89	0.38	0.19	Cell cycle
U73379	*Cyclin-selective ubiquitin carrier protein*	0.84	0.35	0.17	Cell cycle
U66838	*Cyclin A1*	0.69	0.44	0.20	Cell cycle
BG491883	*MCM2*	0.48	0.22	0.17	Cell cycle
X59798	*Cyclin D1*	0.16	0.12	0.31	Cell cycle
NM_004428	*Ephrin-A1*		0.32	0.19	Signaling
BC009426	*CDC20*		0.31	0.14	Cell cycle
AL560982	*Centromere protein A (17kD)*		0.29	0.17	Cell cycle
NM_002060	*Connexin 37*		0.13	0.09	Signaling
NM_005266	*Connexin 40*		0.19	0.10	Signaling
NM_005524	*Hairy* Drosophila *homolog (HES1)*		0.11	0.09	Transcription

HUVEC's were infected with adenoviruses encoding either GFP or FKHR-TM for 12, 16, or 24 h. Total RNA was isolated and subjected to microarray analysis. The table lists a subset of the genes whose RNA level changed by more than fivefold in FKHR-expressing cultures relative to GFP-expressing cultures. For a complete list of FKHR-regulated genes, see Supplementary Tables S1–S4.

Table 1-2 Genes regulated by FKHR in endothelial cells

(Daly et al., 2004)

Chapter 2. Caspase 2- Mediated Tumor Suppression Involves Survivin Gene Silencing

Minakshi Guha, Fang Xia, Christopher M. Raskett, and Dario C. Altieri

The work done in this chapter represents experiments, observations and analysis conducted primarily by myself. Fang Xia contributed by cloning truncated RIP1 constructs. Christopher Raskett helped conduct the xenograft studies in mice. Dario Altieri contributed to the design of the experiments and writing of the manuscript. Members of the lab helped me discuss and troubleshoot many of the experiments represented below. I would like to thank Drs. Bert Vogelstein (John Hopkins) for providing human colorectal $p53^{+/+}$ and $p53^{-/-}$ HCT 116 cells, Michelle Kelliher (UMass Medical School) for providing the RIP1 cDNA, and Neal Silverman (UMass Medical School) for providing NFκB p65 cDNA and p65 mutant (S529).

Abstract

Disabling cell survival mechanisms is a hallmark of tumor suppression, but the molecular circuitries of this process are not well understood. Here, we show that caspase 2, a death effector with largely unknown functions, represses survivin gene transcription, a general regulator of cell division and cytoprotection in tumors. This pathway involves the catalytic site of caspase 2 that induces the proteolytic cleavage of the NFκB activator, RIP1. In turn, loss of RIP1 abolishes transcription of NFκB target genes, including survivin, resulting in deregulated mitotic transitions, enhanced apoptosis, and suppression

of tumorigenicity *in vivo*. Therefore, caspase 2 functions as an endogenous inhibitor of NFκB-dependent cell survival, and this mechanism contributes to tumor suppression.

Introduction

The process of malignant transformation is almost always associated with heightened cell survival threshold (Luo et al., 2009), which contributes to disease progression, metastatic spread, and resistance to conventional or targeted therapy. Bypassing cell death under these conditions involves deregulated expression of anti-apoptotic mechanisms mediated by Bcl-2 or IAP family proteins, resulting in the inhibition of mitochondrial cell death (Cory and Adams, 2002), or antagonizing caspase function (Srinivasula and Ashwell, 2008), respectively. These cytoprotective processes are balanced by intrinsic pro-cell death signals, which silence the expression of anti-apoptotic protein(s) (Accili and Arden, 2004), antagonize their function (Du et al., 2000), or result in the activation of cell death effectors (Vogelstein et al., 2000). The contribution of these endogenous cell death pathways to cellular homeostasis is not entirely clear, but their integrity is likely to provide an important barrier against malignant transformation, as various tumor suppression mechanisms execute a pro-apoptotic program to remove an acquired cell survival advantage (Lowe et al., 2004). One of the effectors of this process in transformed cells is survivin (Altieri, 2008), a unique IAP protein with essential roles in the control of mitosis and protection from apoptosis. Survivin is also sharply differentially expressed in tumors, compared to normal tissues. In this context, strategies to mimic or (re)activate the endogenous cell death machinery are now actively pursued for novel cancer therapeutics (Fesik, 2005), and

targeting survivin may provide an attractive approach to lower a global anti-apoptotic and proliferative capacity in tumor cells (Mita et al., 2008).

Caspases are critical effectors of the intrinsic cell death machinery that has been linked to their ability to dismantle the cellular architecture (Shi, 2002). However, many of these molecules have been recently found intercalated in various signaling mechanisms of cell proliferation, migration and differentiation (Li and Yuan, 2008) that may also contribute to cell death regulation. There is correlative evidence that, at least in some cases, caspase signaling may be important to antagonize tumor growth, in vivo. In fact, loss or inactivation of a number of effector or upstream caspases, including caspase 3 (Soung et al., 2004), caspase 10 (Shin et al., 2002), or caspase 8 (Stupack et al., 2006) has been observed in several types of human tumors, and potentially associated with disease dissemination and unfavorable outcome. In this context, caspase 2 is an evolutionary conserved apical caspase (Krumschnabel et al., 2009), whose multiple signaling properties have been associated with endoplasmic reticulum stress (Upton et al., 2008), cytoskeletal disruption (Ho et al., 2008), mitotic catastrophe (Castedo et al., 2004), p53-dependent DNA damage (Baptiste-Okoh et al., 2008), and, more recently, endogenous tumor suppression, in vivo (Ho et al., 2009). In line with this conclusion, and despite the lack of an overt phenotype of caspase 2 knockout mice (Bergeron et al., 1998), recent data have shown that caspase $2^{-/-}$ fibroblasts are more easily prone to oncogenic transformation, resist apoptosis, and exhibit accelerated tumor growth in mice (Ho et al., 2009). However, the pathophysiological requirements of caspase 2 activation, in vivo (Krumschnabel et al., 2009) have remained largely elusive, and the mechanistic

underpinning of its potential function as a tumor suppressor (Ho et al., 2009) have not been identified.

In this study, we investigated mechanisms of cell death regulation in tumor cells. We found that caspase 2 tumor suppression function is centered on acute silencing of the survivin gene, and this pathway involves inhibition of NFκB signaling via caspase 2 proteolytic cleavage of the upstream NFκB activator, RIP1 (Lin et al., 1999).

Materials and Methods

Cells and culture conditions

Human colorectal $p53^{+/+}$ and $p53^{-/-}$ HCT116 cancer cells were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). For generation of stable clones, HCT116 $p53^{+/+}$ cells were transfected with wild type (WT) HA-tagged caspase 2 or caspase 9 cDNA, and selected in 1 mg/ml G418 (GIBCO). Colonies were picked after 2 weeks, and confirmed for expression of HA or caspase 9 by Western blotting. Breast adenocarcinoma MCF-7 cells stably transfected with survivin were described previously (Ghosh et al., 2008).

Plasmids and antibodies

A full length wild type caspase 2 cDNA (Invitrogen) was amplified by PCR with primers 5'-ATATACTCGAGTAAGCGGGAAATGGCGGCGCCG-3' (forward) and 5'-ATAGAGTCTAGATCATGTGGGAGGGTGTCCTGG-3' (reverse), digested with *Xba*I

and *Xho*I and inserted in HA-tagged pcDNA3.1. For recombinant protein expression, a full length caspase 2 cDNA was cloned into pGEX-4T vector (Amersham Biosciences) using *Sma*I and *Xho*I restriction sites. Primers used for amplification of recombinant caspase 2 (C2) were 5'-ATATACCCGGGTAAGCGGGAAATGGCGGCGCCG-3' (forward) and 5'-ATAGAGCTCGAGTCATGTGGGAGGGTGTCCTGG-3' (reverse). A constitutively active caspase 2 cDNA (Casp.2 152 Ac) was generated by PCR by removal of the prodomain to mimic the processed caspase using primers 5'-GCCTGTCGACAGATACTGTGGAACACTCC-3 (forward) and 5'-ATAGAGCTCGAGTCATGTGGGAGGGTGTCCTGG-3' (reverse). Full length or truncated caspase 2 mutants were generated by replacing the active site Cys320 to Ala (C320A) using site-directed mutagenesis (Stratagene). The catalytic activity of the various caspase 2 constructs was determined using a colorimetric assay kit (Calbiochem) in the presence of VDVAD-pNA as a substrate.

An 830 nt mouse *survivin* promoter construct fused upstream of GFP (ms-830-GFP) was characterized previously (Xia and Altieri, 2006). A putative NFκB consensus site at position -150 nt in ms-830-GFP was mutated using forward primer 5'-GGCGTGGGGC**ctGACTaTCC**CGGCTCG-3' (NFκBΔ). A RIP1 cDNA was the gift of Dr. Michelle Kelliher (University of Massachusetts Medical School). Wild type or mutant (S529A) p65 NFκB cDNA was the gift of Dr. Neil Silverman (University of Massachusetts Medical School). A truncated RIP1 NH_2 fragment (residues 1-350) was generated using primers 5'-GGATCCCCGGAATTCAGAATGCAACCAGACATG-3' (forward) and 5'-TTACTCCTCGAGAGGACCCTACCCAAGTCCCTG-3' (reverse). Similarly, a RIP1 –COOH terminus fragment (residues 351-672) was generated using

primers 5'-TCCCAGGAATTCGGGATGGGTCCTGTGGAGGAG-3' (forward) and 5'-CGGCCGCTCGAGTTAGTTCTGGCTGACGTAAAT-3' (reverse). Both RIP1 fragments were amplified and digested with *EcoR1* and *Xho*I and inserted into pcDNA3.1 vector. The antibodies against caspase 2, 3, 8 and 9 were from Cell Signaling. Antibodies to the p65 subunit of NFκB (Santa Cruz), or RIP1 (BD Biosciences) were used.

Protein and RNA analysis

Recombinant caspase 2 fused to GST was expressed in BL-21 *E.coli* strain, as described (Kang and Altieri, 2006). Caspase 2 conjugated to GST beads was used for RIP1 cleavage assay due to partial thrombin digestion of caspase 2 from GST beads. A RIP1 cDNA was transcribed and translated in vitro using T7, TNT coupled rabbit reticulocyte lysate system (Promega). Aliquots of ^{35}S methionine-labeled RIP1 was incubated with recombinant caspase 2 at 37ºC for 1 h, and analyzed by SDS gel electrophoresis. RIP1 cleavage products were viewed by autoradiography.

Changes in survivin mRNA were analyzed by semi-quantitative RT-PCR using primers for survivin, 5'- GCATGGGTGCCCCGACGTTG-3' (forward) and 5'-GCTCCGGCCAGAGGCCTCAA-3' (reverse) and GAPDH as described (Xia and Altieri, 2006), or real time PCR (QR-PCR), using fluorescent TaqMan and Applied Biosystem's gene expression assays Hs00153353_m1BIRC5 (survivin) and Hs9999905_m1GAPDH. Total RNA was extracted using RNeasy (Qiagen), and reverse

transcribed using first strand cDNA synthesis kit (Invitrogen). Analysis of gene expression was done using relative quantification *dd*Ct method.

Transfections and Reporter assays

β-galactosidase-normalized *survivin* promoter (pLuc-1430c, pLuc-649c, pLuc-441c, and pLuc-230c) luciferase activity was quantified as described (Li and Altieri, 1999). Differential ms-830-GFP expression in transfected cells was analyzed by fluorescence microscopy and Western blotting, as described (Xia and Altieri, 2006). Gene silencing experiments by small interfering RNA (siRNA) directed to Caspase 2/3/8 or RIP1 (Dharmacon) were carried out as described (Lee et al., 2008). A non-targeted siRNA characterized previously (Lee et al., 2008) was used as control.

Flow cytometry

Transfected HCT116 cells were treated with the apoptotic stimulus, staurosporine (STS, 0.8-1 μM), and analyzed by multiparametric flow cytometry using CaspaTag caspase 3 activity kit (Intergen). In some experiments, transfected cells were treated with STS and analyzed for nuclear morphology of apoptosis after 14-16 h, by fluorescence microscopy. Cell cycle analysis was carried out in thymidine-synchronized HCT116 cells, and quantified by propidium iodide and flow cytometry.

Electrophoretic mobility shift assay (EMSA)

Nuclear fractions were purified from TNFα- treated HCT116 cells using NucBuster kit (Novagen). DNA probes were synthesized using the survivin promoter sequence containing the NFκB site (5'-GTGGGGC**GGGACTTTCC**CGGCTC-3') and end-labeled with [γ-^{32}P] deoxyadenosine triphosphate, 1 μl T4 kinase, and 2.5 μl PNK for 15 min at 37°C and then 15 min at 65°C. The labeled probes were purified using nucleic acid purification columns (Bio-Rad), and incubated with 15 μg of nuclear extract as described (Lee et al., 2008). To determine binding specificity, 100-fold excess of unlabeled competitor, mutant competitor or antibody to p65 subunit of NFκB (Santa Cruz) was used as indicated. The reactions were resolved on a 5% non-denaturing polyacrylamide gel and visualized by autoradiography.

Analysis of tumorigenicity

Stably transfected HCT116 cells were cultured in soft agar for 14 days at 37°C, and colonies (>50 cells) were counted by light microscopy. All experiments involving animals were approved by an Institutional Animal Care and Use Committee at the University of Massachusetts Medical School. HCT116 transfectants were injected ($2x10^6$ in 100 μl of PBS) subcutaneously into the flanks of 6 to 8 week-old female CB17 severe combined immunodeficient (SCID)/beige mice (3 mice per group, 2 tumors per mouse, 2 independent experiments). Tumor growth was monitored every other day, and tumor size was calculated with a caliper according to the formula $L \times W^2/2$ (mm^3).

Statistical analysis

Data were analyzed using the unpaired t test on a GraphPad software package for Windows (Prism 4.0). A *p*-value of 0.05 or less was considered as statistically significant.

Results

Caspase 2 activity represses survivin gene expression in tumor cells

We began this study by testing the effect of anticancer agents on caspase activity, and focused on caspase 2 for its role at the interface between signaling and apoptosis (Troy and Shelanski, 2003). Treatment of HCT116 colorectal cancer cells with the small molecule Heat Shock Protein-90 (Hsp90) inhibitor, 17-allylaminogeldanamycin (17-AAG), or the DNA-damaging agent, cisplatin (CDDP), resulted in concentration-dependent increase in caspase 2 activity (Fig. 2-1A). To determine whether this response affected cell survival pathways, we next transfected HCT116 cells with a caspase 2 cDNA, and looked at a potential modulation of IAP family proteins (Srinivasula and Ashwell, 2008). Consistent with earlier observations (Troy and Shelanski, 2003), forced expression of caspase 2 in cells produced an active enzyme, potentially via autoproteolysis by induced proximity (Troy and Shelanski, 2003). In these studies, transfection of caspase 2 in HCT116 cells did not significantly reduce cell viability (see below). Conversely, caspase 2-expressing cells exhibited concentration-dependent loss

of endogenous survivin levels (Fig. 2-1B), whereas the expression of a related IAP, XIAP (Srinivasula and Ashwell, 2008), was not affected (data not shown).

We next asked whether caspase 2 modulation of survivin involved a transcriptional response, so we transfected HCT116 cells with the proximal 830 nt of the mouse *survivin* promoter fused to a GFP reporter gene (ms-830-GFP) (Xia and Altieri, 2006). In the presence of a control plasmid, or no plasmid, transfected cells expressed GFP under the control of the *survivin* promoter, by fluorescence microscopy (Fig. 2-1C). In contrast, transfection of caspase 2 abolished the GFP^+ population in HCT116 cells (Fig. 2-1C-D), suggesting that the *survivin* gene was transcriptionally repressed under these conditions. Consistent with this model, transfection of caspase 2 in HCT116 cells abolished transcription of several human *survivin* promoter constructs extending up to -1430 nt from the transcription start site(s), by luciferase reporter assay, as compared with control transfectants (Fig. 2-1E). We next asked whether this property was specific for caspase 2, or whether other caspase(s) also suppressed *survivin* gene expression. Consistent with the data above, transfection of caspase 2 abolished *survivin* promoter-luciferase activity in HCT116 cells, whereas expression of caspase 3 had no effect on either $p53^{+/+}$ or $p53^{-/-}$ HCT116 cells (Fig. 2-1F). Because p53 is a known repressor of the *survivin* gene (Hoffman et al., 2002; Mirza et al., 2002), we further characterized a potential participation of this pathway in caspase 2 gene regulation. In these experiments, caspase 2 induced silencing of the *survivin* gene indistinguishably in $p53^{+/+}$ or $p53^{-/-}$ HCT116 cells (Fig. 2-1D, F), thus ruling out a requirement of p53 in this response.

Caspase 2 targeting modulates endogenous survivin expression in tumor cells

To use a complementary approach, we next acutely silenced caspase 2 expression in HCT116 cells by small interfering RNA (siRNA), and examined potential changes in endogenous survivin levels. Transfection of these cells with a caspase 2-directed siRNA efficiently suppressed caspase 2 levels, as compared with control siRNA transfectants (Fig. 2-2A). Acute knockdown of caspase 2 under these conditions was associated with upregulation of endogenous survivin expression, by Western blotting (Fig. 2-2A), and semi-quantitative and quantitative PCR (Fig. 2-2B). Conversely, caspase 2 silencing did not affect the expression of XIAP (Srinivasula and Ashwell, 2008), in HCT116 cells (Fig. 2-2A). To further validate the specificity of caspase 2 in this response, we next silenced effector caspase 3 (Fig. 2-2C), or initiator caspase 8 (Fig. 2-2D), and looked at potential changes in survivin levels. Transfection of HCT116 cells with caspase 3 (Fig. 2-2C) or caspase 8 (Fig. 2-2D)-directed siRNA efficiently silenced the expression of the corresponding caspase, by Western blotting, but had no effect on endogenous survivin levels. As control, transfection of HCT116 cells with non-targeted siRNA was also ineffective (Fig. 2-2C, D).

Requirement of caspase 2 catalytic activity for survivin gene silencing

To test whether the enzymatic activity of caspase 2 was required for *survivin* gene regulation, we next generated caspase 2 variants lacking the prodomain, thus mimicking a

constitutively active enzyme (Casp.2 152 Ac) (Li et al., 1997a), or carrying an Ala substitution of the active site Cys320 (C320A), which produces a catalytically dead enzyme (Fig. 2-3A). Consistent with these predictions, $p53^{+/+}$ or $p53^{-/-}$ HCT116 cells transfected with full length caspase 2 exhibited a 2-fold increase in enzymatic activity, whereas prodomain-deleted caspase 2 was considerably more active, and a C320A caspase 2 mutant had no activity (Fig. 2-3B). Under these conditions, wild type caspase 2 or constitutively active caspase 2 suppressed survivin promoter-directed GFP expression in HCT116 cells, by fluorescence microscopy (Fig. 2-3C, D), and Western blotting (Fig. 2-3E). In contrast, active site caspase 2 mutant did not affect survivin promoter activity, as determined by GFP expression in HCT116 cells (Fig. 2-3C-E). In addition, transfection of HCT116 cells with active caspase 2 did not affect the expression of an unrelated plasmid, i.e. pEGFP (Fig. 2-3F), thus confirming the specificity of this response. Taken together, these data indicate that the catalytic activity of caspase 2 is required to suppress survivin gene transcription.

Caspase 2 targeting of NFκB activity

To identify the mechanisms(s) by which caspase 2 specifically antagonizes tumorigenicity via repression of survivin, we next focused on its signaling properties, and in particular the link to NFκB regulation (Tinel et al., 2007). In a first series of experiments, a radiolabeled DNA probe containing a consensus NFκB site in the proximal survivin promoter (Fig. 2-4A) bound nuclear extracts of tumor cells, in a reaction supershifted by an antibody to the p65 subunit of NFκB (Fig. 2-4B). Confirming

the specificity of this reaction, wild type, but not mutant unlabeled NFκB consensus sequence inhibited the formation of a survivin promoter-NFκB complex, by EMSA (Fig. 2-4B).

Next, we mutated one of the consensus NFκB sites in the survivin promoter used for the ms-830-GFP construct (Fig. 2-4A), and tested a potential effect on survivin gene expression. In these experiments, full length caspase 2 indistinguishably abolished the expression of GFP, whether driven by wild type or NFκB mutant ms-830-GFP, as determined by fluorescence microscopy of GFP^+ cells (Fig. 2-4C, D), and Western blotting of cellular GFP levels (Fig. 2-4E). Transfection of these cells with catalytically dead caspase 2 restored the expression of GFP driven by the wild type survivin promoter (Fig. 2-4C-E). In contrast, this mutant only partially rescued the activity of the NFκB mutant ms-830-GFP promoter (Fig. 2-4C-E). Taken together, these data suggest that caspase 2 functions as an upstream negative regulator of NFκB, in a pathway that requires at least one NFκB site in the proximal survivin promoter. Consistent with this model, active caspase 2 abolished NFκB reporter activity in TNFα-stimulated HCT116 cells, whereas catalytically dead caspase 2 had no effect (Fig. 2-4F).

Caspase 2 cleavage of RIP1

Because the catalytic activity of caspase 2 is required for modulation of survivin gene expression and NFκB activity, we next searched for upstream activator(s) of this pathway that may be potentially cleaved by caspase 2. We focused on RIP1 (Festjens et al., 2007) for its role in TNFα (Micheau and Tschopp, 2003)-, and DNA damage

(Janssens et al., 2005)-induced NFκB activation, as well as its interaction with caspase 2 in a large inducible protein complex (Lamkanfi et al., 2005). In these experiments, incubation of unconjugated or bead-conjugated recombinant caspase 2 with ^{35}S-labeled recombinant RIP1 in vitro resulted in the appearance of RIP1-derived proteolytic fragments of approximate molecular weight of 56 and 20 kDa, respectively (Fig. 2-5A). Addition of the caspase 2 inhibitor, z-VDVAD-fmk nearly completely prevented RIP1 cleavage by caspase 2, in vitro (Fig. 2-5A). Moreover, caspase 2 cleaved a ^{35}S-labeled–COOH terminus RIP1 fragment comprising residues 351-672, whereas a RIP1 NH_2 fragment comprising residues 1-350 was not cleaved by caspase 2 (Fig. 2-5B). To test whether a similar response occurred in vivo, we next looked for changes in RIP1 expression upon transfection of caspase 2 in HCT116 cells. In these experiments, transfection of constitutively active caspase 2 nearly completely abolished the expression of endogenous RIP1, whereas XIAP levels were not affected (Fig. 2-5C). In contrast, expression of catalytically dead caspase 2 had no effect on endogenous RIP1 levels in HCT116 cells (Fig. 2-5C), thus confirming the specificity of this response.

In parallel experiments, NFκB activation by TNFα, or transfection of HCT116 cells with the p65 subunit of NFκB (p65-NFκB), resulted in increased expression of endogenous survivin (Fig. 2-5D). Conversely, transfection of a S529A dominant negative mutant of p65-NFκB (Wang and Baldwin Jr, 1998) abolished endogenous survivin expression (Fig. 2-5D), thus confirming a role of NFκB in survivin gene expression. Therefore, we next tested a potential requirement of RIP1 in this pathway. Silencing of RIP1 by siRNA in HCT116 cells abolished the increase in endogenous survivin mediated by TNFα stimulation (Fig. 2-5D). However, this pathway is operative

only when stimulated with TNFα, as other pathways may compensate for survivin expression upon RIP1 knockdown in the absence of TNFα (Fig. 2-5D). In addition, under conditions of RIP1 silencing in TNFα treated cells, transfection of wild type p65-NFκB restored endogenous survivin levels in HCT116 cells, whereas expression of S529A p65-NFκB had no effect (Fig. 2-5E).

Mitotic defects and apoptosis induced by caspase 2 silencing of survivin

Previous studies have shown that survivin is required for tumor maintenance, and acute lowering of its levels in tumor cells results in cell cycle defects, inhibition of cell proliferation, and induction of apoptosis (Altieri, 2008). To test whether caspase 2 downregulation of survivin mirrored this phenotype, we next generated clones of HCT116 cells that stably express caspase 2, or a control plasmid. Generation of stable caspase 2 HCT116 transfectants was feasible (Fig. 2-6A), and resulted in significant repression of survivin gene transcription, by fluorescence microscopy of ms-830-GFP expression (Fig. 2-6B), and luciferase reporter assay (Fig. 2-6C). Cell cycle-synchronized HCT116 cells transfected with control plasmid exhibited a periodic increase in survivin expression, coinciding with entry into the G2/M phase of the cell cycle 8 h after thymidine release, and persisting throughout the completion of mitosis, 10 to 12 h after release (Fig. 2-6D, *top*) (Altieri, 2008). In contrast, HCT116 caspase 2 transfectants showed no increase in survivin expression at mitosis under the same conditions (Fig. 2-6D, *bottom*). Functionally, HCT116 cells expressing caspase 2

exhibited significantly slower mitotic transitions, with 21% and 40% of these cells re-entering G1, 10 and 12 h after thymidine release, respectively, as opposed to 30% and 51% of control transfectants (Fig. 2-6E).

To explore a potential link between caspase 2 and apoptosis, we next looked at a potential degree of spontaneous cell death in caspase 2 HCT116 transfectants. In the absence of cell death stimuli, expression of caspase 2 in synchronized cultures did not result in detectable apoptosis at any cell cycle phase tested, as compared with pcDNA transfectants, by hypodiploid DNA content and flow cytometry (Fig. 2-6E). Similarly, using an independent experimental approach, caspase 2 HCT116 transfectants were not associated with nuclear morphology of apoptosis, by DAPI staining and fluorescence microscopy (Fig. 2-7A), further ruling out a direct effect of caspase 2 on cell viability. In contrast, exposure of these cells to the broad cell death stimulus, staurosporine (STS), resulted in a two-fold increased sensitivity to apoptosis, as compared with control HCT116 transfectants (Fig. 2-7A). Cell death under these conditions was characterized by increased caspase 3 activity and loss of plasma membrane integrity, by multiparametric flow cytometry of DEVDase activity and propidium iodide staining (Fig. 2-7B). To test the specificity of this response, we first silenced caspase 2 in HCT116 cells by siRNA. Caspase 2 knockdown largely reversed STS-induced cell death, as compared with control siRNA (Fig. 2-7B), whereas the background level of cell death in the absence of STS was indistinguishable in control or caspase 2-silenced cultures (Fig. 2-7B). Next, we asked whether survivin could rescue the increased sensitivity to apoptosis mediated by caspase 2. In these experiments, acute expression of caspase 2 in breast adenocarcinoma MCF-7 cells resulted in increased cell death, as compared with

control transfectants, by fluorescence microscopy of nuclear morphology of apoptosis (Fig. 2-7C). Conversely, stable expression of survivin in MCF-7 cells completely reversed apoptosis induced by caspase 2 to background levels of control cultures (Fig. 2-7C).

Caspase 2 suppression of tumorigenesis

We next asked whether caspase 2 silencing of survivin, with the associated dual phenotype described above affected tumorigenicity, in vitro and in vivo. In a first series of experiments, HCT116 cells stably expressing caspase 2 exhibited significantly reduced cell proliferation over a 5-d interval, as compared with control cultures transfected with pcDNA (Fig. 2-8A). In addition, caspase 2 transfectants failed to form colonies in soft agar over a 2-week interval, indicating loss of anchorage-independent cell growth (Fig. 2-8B). In contrast, control HCT116 cells formed extensive colonies in soft agar under the same conditions (Fig. 2-8B). Lastly, we injected control or caspase 2 HCT116 transfectants in the flanks of immunocompromised mice, and examined the kinetics of tumor growth over a 4-week interval. Under these conditions, HCT116 cells transfected with pcDNA formed exponentially growing superficial tumors (Fig. 2-8C). In contrast, stable expression of caspase 2 in HCT116 cells completely abolished tumor growth, in vivo (Fig. 2-8C), further supporting a role of caspase 2 in endogenous tumor suppression. Moreover, when we looked at human tissue samples from colon cancer patients, we observed very little caspase 2 staining as compared to normal matched counterparts (data not shown). Thus, loss of caspase 2 may contribute to increased tumorigenesis, potentially due to elevated survivin levels.

Specificity of caspase 2 regulation of survivin expression and tumorigenicity

To validate the specificity of these findings, we next generated stable clones of HCT116 cells expressing comparable levels of caspase 9 (Fig. 2-9A). Similar to caspase 2, caspase 9 is a long prodomain-containing apical caspase (Shi, 2002), which can also be activated by induced proximity and overexpression, in vivo. Differently from caspase 2, however, HCT116 caspase 9 transfectants exhibited no modulation of endogenous survivin expression, by Western blotting (Fig. 2-9A), and no reduction in *survivin* promoter-dependent GFP expression, by fluorescence analysis of ms-830-GFP (Fig. 2-9B), or survivin promoter luciferase activity (Fig. 2-9C). Functionally, caspase 9-expressing HCT116 cells exhibited no defect in colony formation in soft agar (Fig. 2-9D), and their kinetics of cell proliferation was indistinguishable from that of pcDNA transfectants (Fig. 2-9E).

Discussion

In this study, we have shown that caspase 2, an apical caspase with still largely unknown functions (Krumschnabel et al., 2009), actively represses survivin gene transcription in tumor cells. In turn, acute loss of survivin causes mitotic defects, increased sensitivity to apoptosis and complete loss of tumorigenicity, *in vitro* and *in vivo*. Mechanistically, this pathway involves caspase 2 cleavage of RIP1, an activator of

NFκB (Festjens et al., 2007), which results in blocking the expression of NFκB-responsive antiapoptotic genes, including survivin (Kawakami et al., 2005).

Apart from a well-characterized mechanism of caspase 2 activation in response to DNA damage through a multimolecular PIDDosome complex (Tinel and Tschopp, 2004), other pathophysiological activation platforms for this caspase have been far less clear (Krumschnabel et al., 2009). Here, conventional (CDDP), or targeted (17-AAG) anticancer agents produced concentration- and time-dependent increase in caspase 2 activity, in agreement with the results obtained with docetaxel (Mhaidat et al., 2007), or bortezomib (Yeung et al., 2006). Caspase 2 activation under these conditions occurred at about the same kinetics as compared to caspase 3 activity (our unpublished observations), which can activate caspase 2 by removing its long prodomain (Van de Craen et al., 1999), suggesting that at least certain anticancer regimens may activate caspase 2 independent of effector caspase(s) (Yeung et al., 2006).

Identified here as one of the critical downstream targets of caspase 2, but not caspase 9, survivin is now viewed as a "nodal" IAP family protein (Srinivasula and Ashwell, 2008), intersecting multiple signaling pathways in the control of mitosis, inhibition of apoptosis, and modulation of the cellular stress response, especially in cancer (Altieri, 2008). In this context, a role of caspase 2 as a novel repressor of survivin gene expression adds to a broad array of signaling pathways that finely regulate survivin levels in tumor cells. These include transcriptional mechanisms by a host of activators and repressors (Altieri, 2008), mTOR regulation of survivin mRNA translation (Vaira et al., 2007), or post-transcriptional phosphorylation by mitotic kinases (O'Connor et al., 2000; Wheatley et al., 2007). Similar to the phenotype induced by antagonizing

transcriptional activators of survivin, such as Wnt/β-catenin (You et al., 2004), Stat3 (Zhou et al., 2009), or Notch (Lee et al., 2008), heightened caspase 2 activity resulted in acute loss of endogenous survivin levels, with ensuing mitotic defects, reduced cell proliferation, and sensitization to spontaneous or stimulus-induced apoptosis (Altieri, 2008). Mechanistically, this reflects the ability of survivin to control chromosomal segregation (Lens et al., 2006) and mitotic spindle formation (Xia et al., 2008), as well as antagonize mitochondrial cell death via regulated binding to cofactor molecules, such as XIAP (Dohi et al., 2007).

Functionally, caspase 2 silencing of survivin gene expression resulted in nearly complete ablation of tumorigenicity, with loss of colony formation in soft agar, and suppression of tumor formation in immunocompromised mice. This phenotype mirrors the effect of genetic deletion of caspase 2 (Ho et al., 2008), which caused delayed apoptosis in response to certain stimuli, increased rate of cell proliferation, enhanced cellular transformation, and accelerated tumor growth *in vivo* (Ho et al., 2009). Though there has been some speculation that caspase 2 has a role in cell cycle (Castedo et al., 2004), our study shows that caspase 2 overexpression can lead to deregulation of the cell cycle prior to stimulus-induced apoptosis. Taken together, these data fit well with a model of caspase 2 as a novel tumor suppressor, and identify acute survivin gene silencing as one of the pivotal effectors of this pathway. Moreover, this is consistent with correlative evidence linking increased expression of caspases to more favorable prognosis in cancer (Estrov et al., 1998; Faderl et al., 1999), or, conversely, loss of caspase to disease progression (Stupack et al., 2006).

Although the overexpression of survivin seen in most human cancer likely reflects activation of multiple oncogenic pathways converging on the survivin gene (Altieri, 2008), there is also evidence for a reciprocal process that aims at keeping the levels of survivin low in normal cells. Accordingly, pivotal tumor suppressor pathways, mediated by p53 (Hoffman et al., 2002; Mirza et al., 2002), Rb (Jiang et al., 2004), SIRT1 (Wang et al., 2008b), or PTEN (Guha et al., 2009) have been shown, similarly to caspase 2 (this study), to acutely silence the survivin gene, either directly, or via promoter regulation. Whether these mechanisms are responsible for the low to undetectable levels of survivin observed in most normal adult tissues remains to be elucidated (Altieri, 2008). However, it is possible that transcriptional silencing of the survivin gene provides a requisite mechanism for effective tumor suppression (Lowe et al., 2004), and, conversely, unrestrained transcriptional expression of survivin may play a role in the steps leading to the establishment of transformed clone(s).

The mechanistic requirements of how caspase 2 antagonizes tumor growth was not elucidated in a recent study (Ho et al., 2009). Here, we provide evidence that this pathway involves inhibition of NFκB signaling, via direct cleavage of the upstream NFκB activator, RIP1 (Festjens et al., 2007), in vitro and in vivo. Consistent with this model, the catalytic activity of caspase 2 was required for survivin gene silencing, and differential expression of RIP1 or NFκB was sufficient to modulate endogenous survivin levels in tumor cells. RIP1 has long been recognized as an ubiquitin-regulated component of a TNFα-induced multimolecular protein complex, which controls the activation of survival pathways via NFκB and MAPK signaling (Festjens et al., 2007). Whether caspase 2 also participates in these responses has been debated, and a non-

catalytic mechanism of caspase 2-mediated NFκB activation has been proposed (Lamkanfi et al., 2005). The findings presented here are at variance with this model (Lamkanfi et al., 2005), but in agreement with a prevailing consensus that caspase 2 functions as a pro-apoptotic effector antagonizing NFκB-mediated cell survival (Tinel et al., 2007). In this context, the possibility that caspase(s) may target RIP1 for degradation, and thus interrupt NFκB-mediated cell survival, has been postulated. Similar to the data presented here with caspase 2, caspase 8 cleavage of RIP1 at Asp324 has been implicated in suppressing NFκB-dependent survival in tumor cells (Lin et al., 1999), and during macrophage differentiation, in vivo (Rebe et al., 2007). Although caspase 2 cleavage of RIP1 was not observed in an earlier study (Lamkanfi et al., 2005), we have shown cleavage of the full length RIP1 protein that was largely reversed by the caspase 2 inhibitor, z-VDVAD-fmk (Krumschnabel et al., 2009), suggesting that an Asp-directed substrate recognition may also be involved in this cleavage reaction.

Functionally, inhibiting NFκB signaling by caspase 2 is expected to remove a broad cell survival mechanism, that involves transcriptional regulation of multiple antiapoptotic molecules, including survivin (Kawakami et al., 2005). Accordingly, an NFκB dependence of survivin gene expression has been demonstrated in various cell types (Anand et al., 2008; Makishi et al., 2008), and the identification of a functional NFκB consensus binding site in the proximal survivin promoter (this study), is consistent with these observations. The participation of other predicted NFκB consensus binding sites on the survivin promoter remain to be identified and their regulation by other potential pathways, in addition to caspase 2, remain to be elucidated. However, the loss-of-function phenotype, although partial, associated with mutagenesis of the single NFκB

site identified here suggests that, at least in certain tumor cell types, a basal level of survivin gene transcription is mediated by steady-state NFκB activity (Anand et al., 2008; Makishi et al., 2008).

In summary, we have found that caspase 2 functions in a broad tumor suppression network, lowering a general anti-apoptotic threshold via interruption of NFκB signaling (Karin, 2006), and abrupt silencing of survivin gene expression (Altieri, 2008). Among the portfolio of apoptosis modifiers, strategies to restore caspase activity in tumors are being pursued as viable therapeutic opportunities (Fesik, 2005). Based on the data presented here, restoring caspase 2 activity may be beneficial in tumors with elevated NFκB activity and high levels of survivin, thus potentially "addicted" to this general cytoprotective pathway (Karin, 2006).

Figures

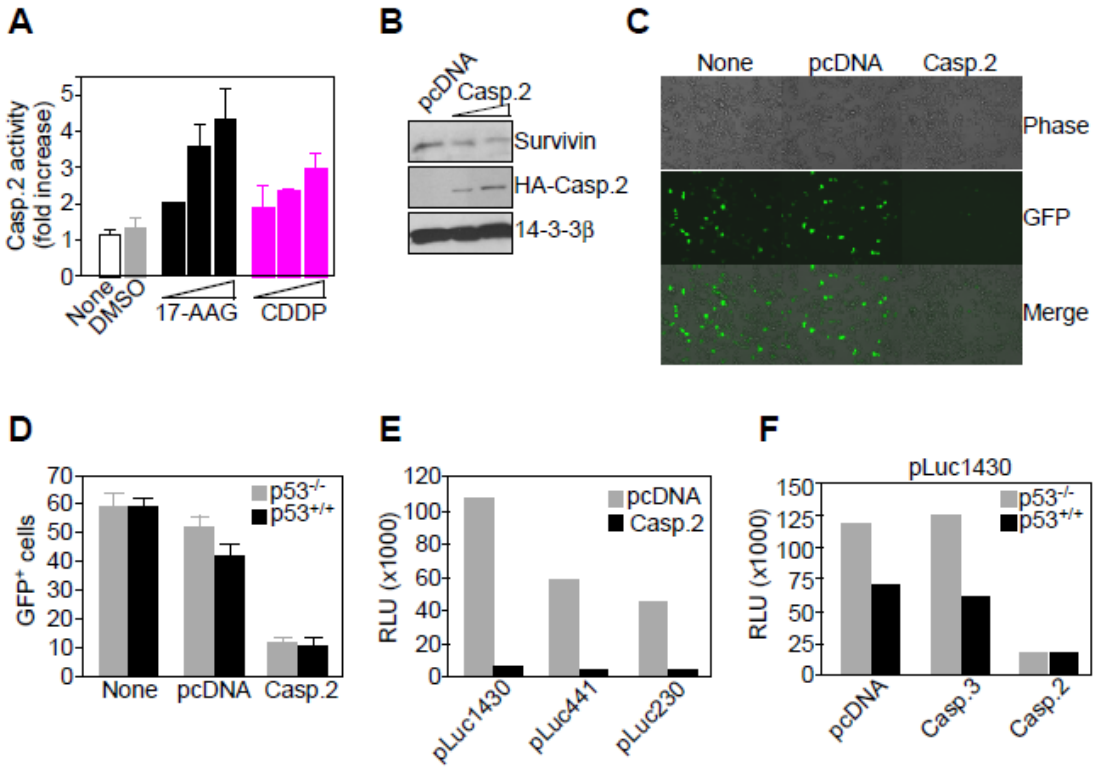


Figure 2-1 Caspase 2 represses survivin gene expression.

(A) Caspase 2 enzymatic activity in p53+/+ HCT116 cells treated with 17-AAG (0.1, 1 or 10 μM) or cisplatin (CDDP, 10, 30 μM or 50 μM) for 48 h. (B) Western blotting of HCT116 cells transfected with pcDNA or HA-tagged caspase 2. (C) Fluorescence microscopy of GFP expression in HCT116 cells transfected with ms-830-GFP plus pcDNA or caspase 2. Representative fields are shown. (D) The conditions are as in C, and the number of GFP+ cells were counted for p53+/+ or p53-/- HCT116 cells. (E) β-galactosidase-normalized survivin promoter (pLuc1430, pLuc441, pLuc230) luciferase activity in transfected HCT116 cells. (F) Analysis of survivin promoter luciferase activity in transfected p53+/+ or p53-/- HCT116 cells. RLU, relative luciferase units. For panels E and F, data are representative of at least two independent experiments.

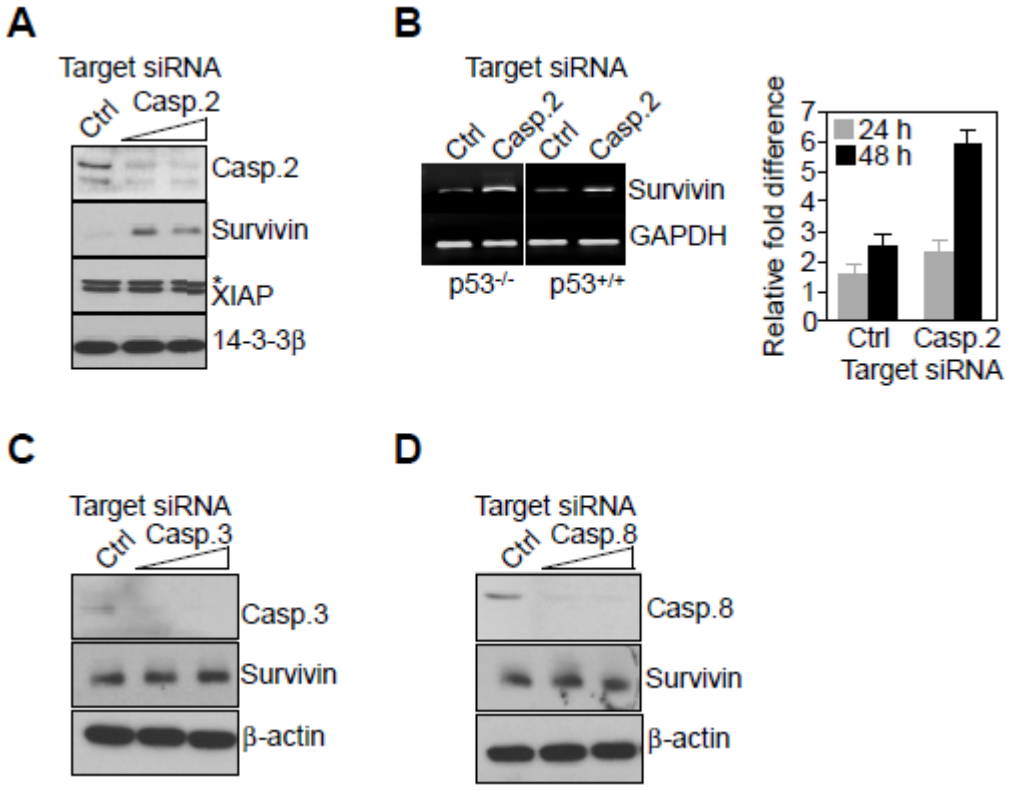


Figure 2-2 Transcriptional regulation of survivin by caspase 2.

(A) Western blotting of HCT116 cells transfected with control (Ctrl) or caspase 2-directed siRNA. *, non-specific. (B) Semi-quantitative (left), or quantitative (right) real time PCR amplification of survivin or GAPDH mRNA in transfected HCT116 cells. Right, GAPDH-normalized quantification of survivin mRNA expression. (C, D) Western blotting of HCT116 transfected with control (Ctrl), caspase-3 (C)- or caspase-8 (D)-directed siRNA.

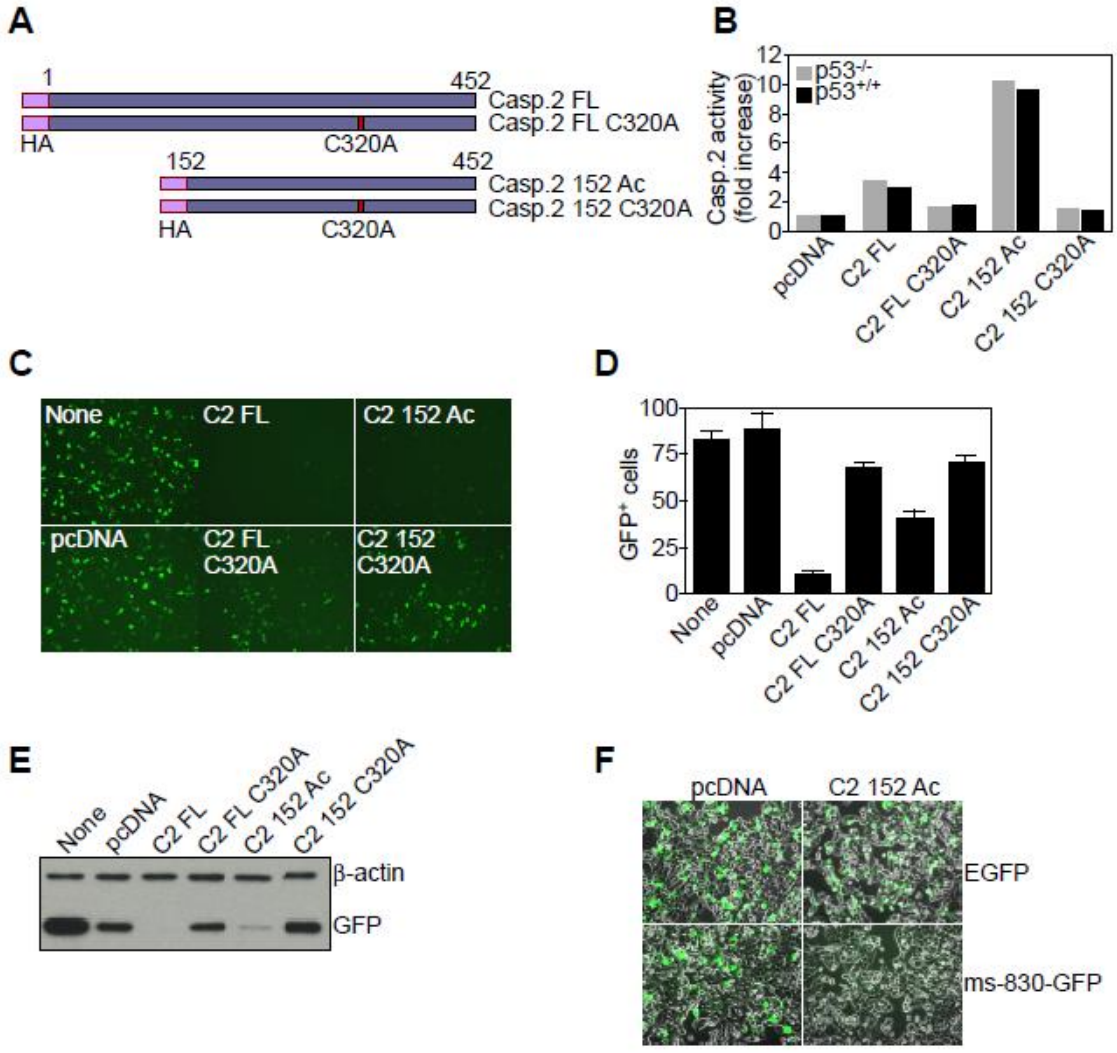


Figure 2-3 Requirement of caspase-2 catalytic activity for survivin gene repression.

(A) Schematic diagram of caspase 2 constructs used in this study. Casp.2 FL, full length; Casp.2 152 Ac, prodomain deleted, constitutively active caspase 2, Casp.2 C320A, catalytically inactive caspase 2. The position of an HA tag is indicated. (B) Analysis of caspase 2 activity in transfected $p53^{+/+}$ or $p53^{-/-}$ HCT116 cells using a caspase 2 colorimetric assay substrate. Data are representative of at least two independent experiments. (C) Fluorescence microscopy of ms-830-GFP expression in HCT116 cells transfected with the indicated caspase 2 constructs. Representative fields are shown. (D) Quantification of GFP^+ cells. The experimental conditions are as in C. Data are the mean±SD of two independent experiments. (E) Western blotting of GFP expression in transfected HCT116 cells. The experimental conditions are as in C. (F) Fluorescence

microscopy of GFP expression in HCT116 cells transfected with ms-830-GFP or pEGFP plus pcDNA or active caspase 2 (C2 152 Ac).

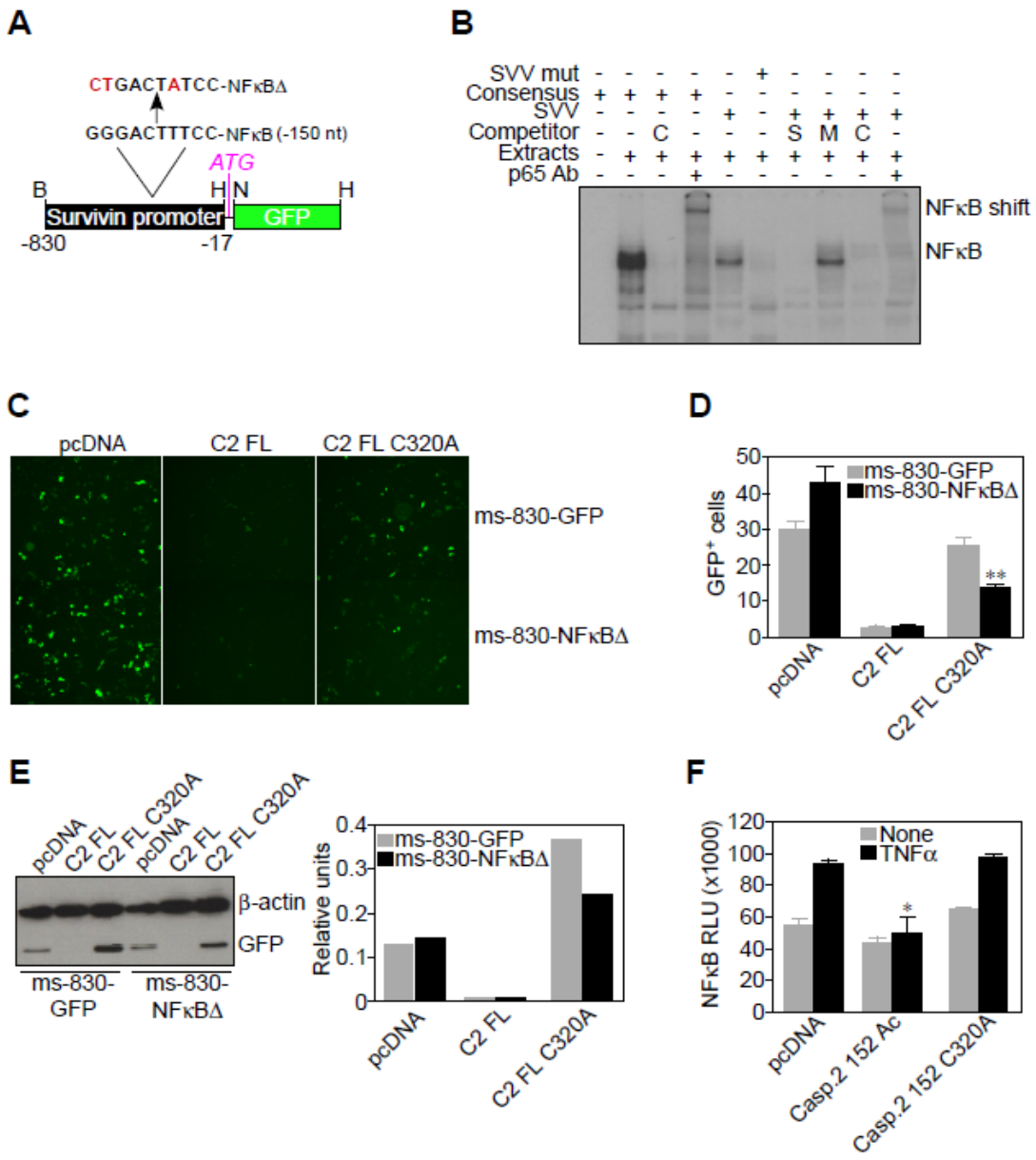


Figure 2-4 Caspase 2 targets NFκB-mediated survivin gene expression.

(A) Position and mutagenesis of an NFκB consensus binding site in the survivin promoter (ms-830-NFκBΔ). (B) EMSA of ^{32}P-labeled survivin (SVV) or NFκB consensus probes. Reaction mixtures contained unlabeled excess competitor of a generic NFκB consensus sequence (C), or wild type (S), or mutant (M) survivin. (C) Fluorescence microscopy of ms-830-GFP expression in transfected HCT116 cells. (D) Quantification of GFP$^+$ cells. The experimental conditions are as in C. **, p=0.0028. (E) Western blotting of transfected HCT116 cells. The experimental conditions are as in C. *Right*, densitometric quantification of protein bands. (F) NFκB luciferase reporter

activity in HCT116 transfectants in the presence or absence of TNFα. RLU, relative luciferase units, *, p=0.04.

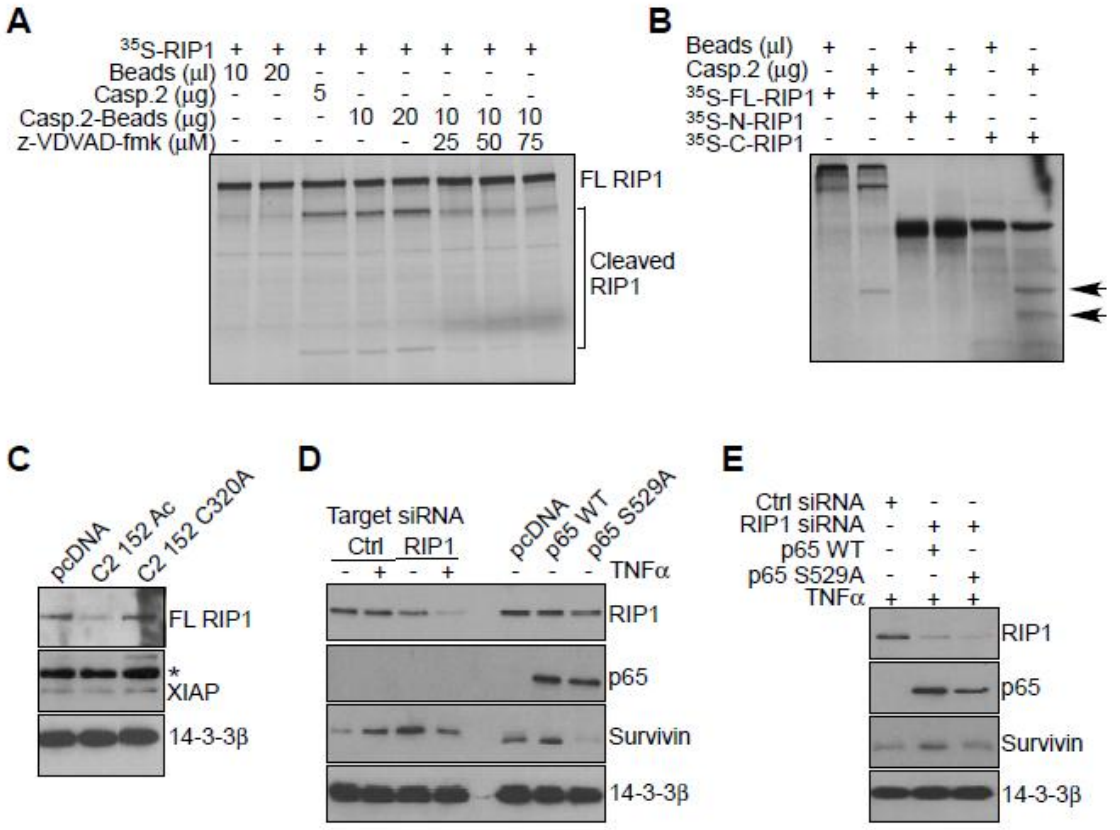


Figure 2-5 Caspase 2 cleavage of RIP1

(A) Autoradiography of caspase 2 cleavage of ^{35}S-labeled RIP1. z-VDVAD-fmk was used as a caspase 2 inhibitor. FL, full length. (B) Caspase 2 cleavage of ^{35}S-labeled RIP1 constructs, including full length (FL), NH_2-terminus (residues 1-350) or –COOH terminus (residues 351-672) fragments. *Arrows*, cleavage products visualized by autoradiography. (C) Western blotting of transfected HCT116 cells transfected with active (C2 152Ac), or mutant (C2 152 C320A) caspase 2. *, non specific. (D) Western blotting of HCT116 cells transfected with control (Ctrl) or RIP1-directed siRNA, or, alternatively, wild type or mutant (S529A) p65-NFκB in the presence or absence of TNFα. (E) Western blotting of TNFα-stimulated HCT116 cells transfected with control (Ctrl) or RIP1-directed siRNA, in the presence of wild type or mutant p65-NFκB.

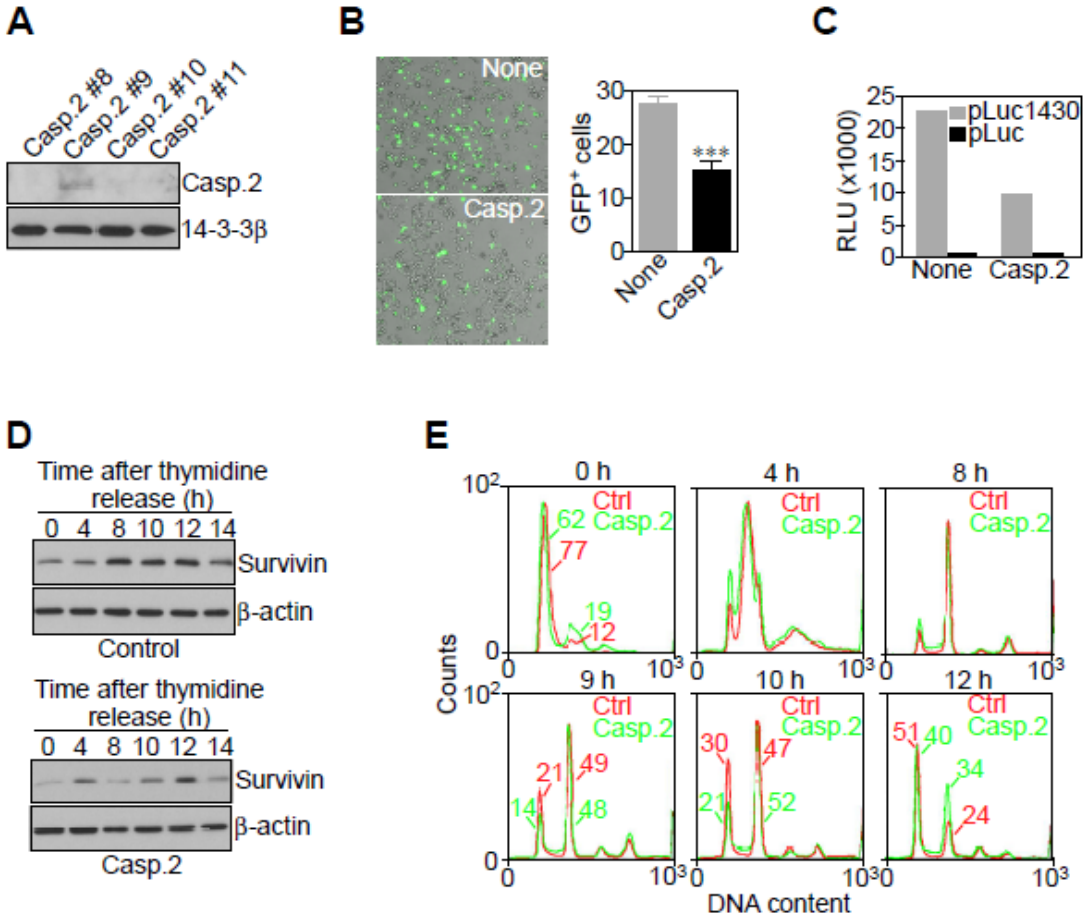


Figure 2-6 Characterization of caspase 2 HCT116 transfectants

(A) Western blotting of HCT116 stably transfected with caspase 2. Clone #9 was used in subsequent experiments. (B) Fluorescence microscopy of ms-830-GFP expression in stably transfected HCT116 cells. *Right*. Quantification of GFP^+ cells, ***p<0.0001. (C) β-galactosidase-normalized survivin promoter (pLuc 1430) luciferase activity in stably transfected HCT116 cells. Data are representative of at least two independent experiments. RLU, relative luciferase units. (D) Western blotting of cell cycle-synchronized HCT116 transfectants expressing pcDNA (*top*, control) or caspase 2 (*bottom*). (E) Cell cycle profile of synchronized HCT116 cells. The percentage of cells in the G1 or G2/M phase of the cell cycle is indicated per each clone. Ctrl, Control.

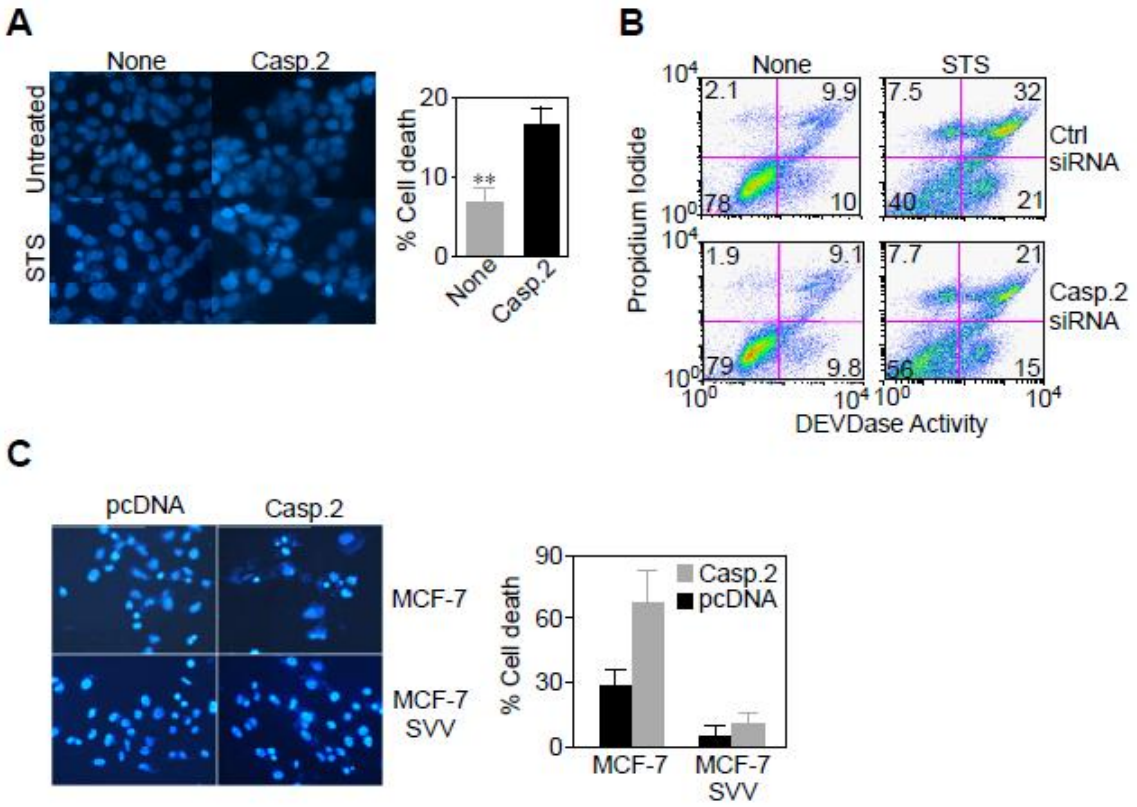


Figure 2-7 Regulation of caspase 2-induced apoptosis

(A) Fluorescence microscopy of nuclear apoptosis in HCT116 transfectants. Representative images are shown. STS, staurosporine. *Right*, quantification of apoptotic cells, **p=0.003. (B) Multiparametric flow cytometry analysis of DEVDase (caspase) activity and DNA content (propidium iodide) of transfected HCT116 cells treated with vehicle or staurosporine (STS). The percentage of cells in each quadrant is indicated. (C) Nuclear morphology of apoptosis in parental MCF-7 cells or MCF-7 cells stably expressing survivin (MCF-7 SVV) after transfection of caspase 2 or pcDNA. *Left*, DAPI staining of representative microscopy fields. Magnification, x200. *Right*, Quantification of cell death. Mean±SEM of replicates representative experiment of at least two independent experiments.

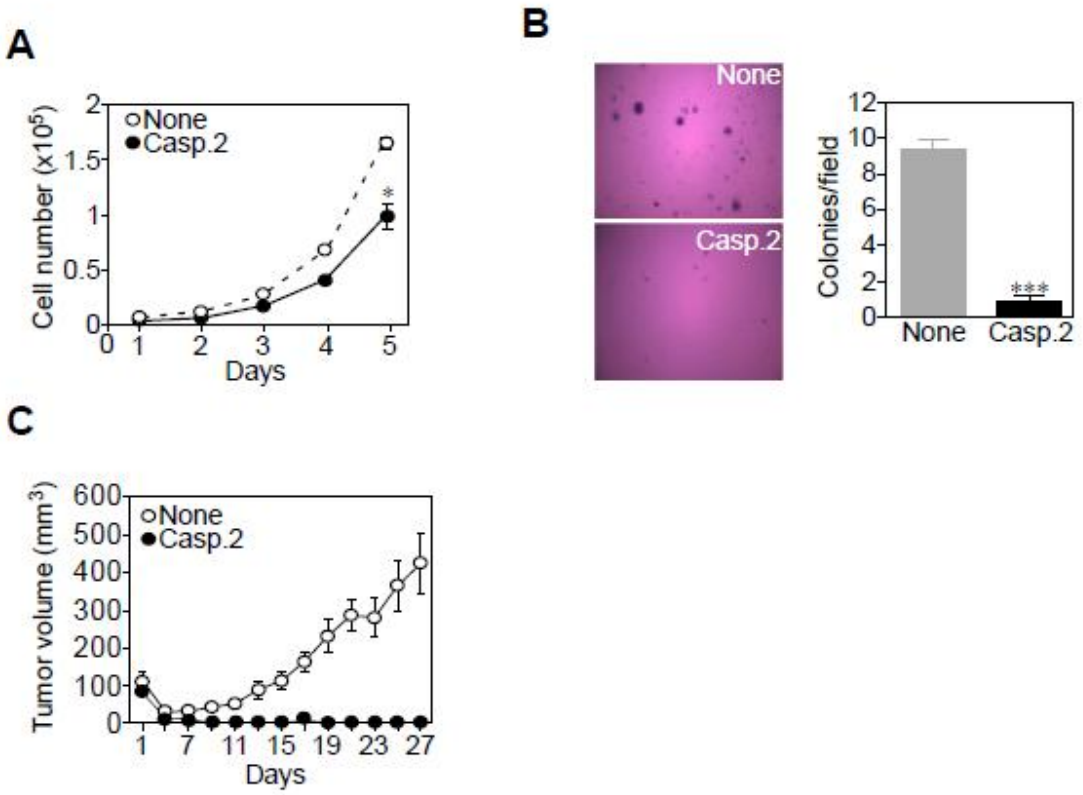


Figure 2-8 Caspase 2 suppression of tumorigenesis

(A) Proliferation of stable HCT116 transfectants, *, p=0.034. (B) Colony formation of HCT116 transfectants. *Left*, representative microscopy fields per cell type are shown. *Right*, quantification of colony formation, ***, p<0.0001. (C) Xenograft tumor growth of HCT116 transfectants. Mean tumor volume in mm^3 is shown for each time point. Representative of two independent experiments.

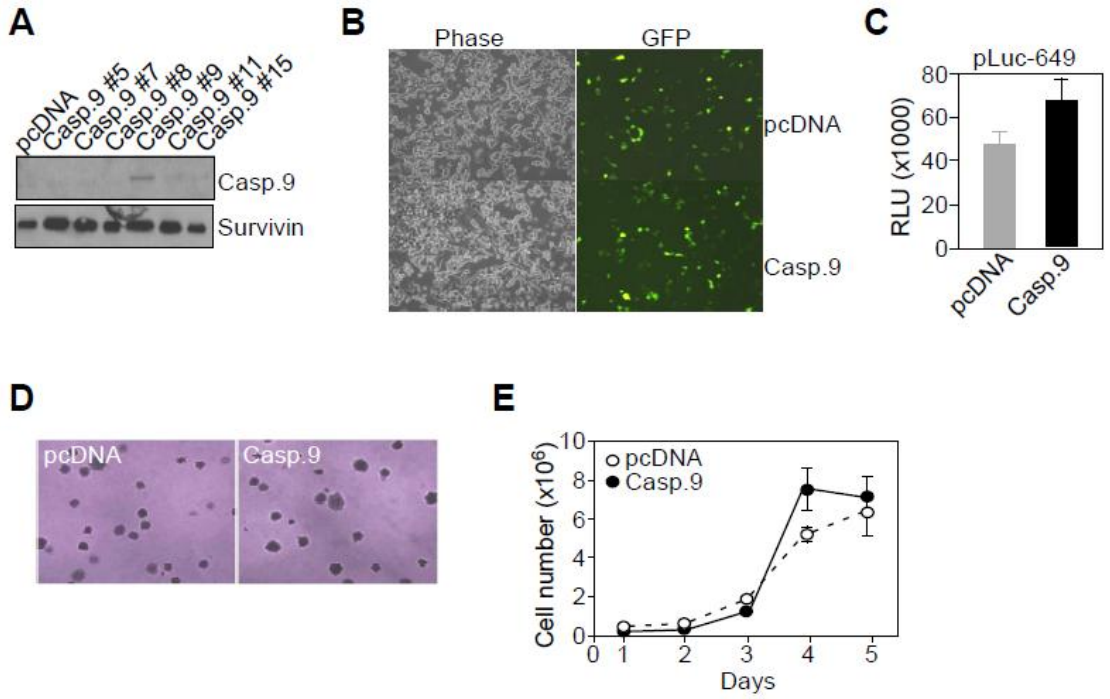


Figure 2-9 Characterization of HCT116 caspase 9 stable transfectants

(A) Western blotting of transfected HCT116 cells. Clone #9 was used in subsequent experiments. (B) Fluorescence microscopy of ms-830-GFP expression in HCT116 transfectants. (C) Analysis of survivin promoter luciferase activity in HCT116 transfectants. RLU, relative luciferase units. (D) Colony formation of HCT116 transfectants. (E) Cell proliferation of HCT116 transfectants. For panels C and E, data are the mean±SEM of replicates representative of at least two independent experiments.

Chapter 3. Endogeous Tumor Suppression Mediated by PTEN Involves Survivin Gene Silencing

Minakshi Guha, Janet Plescia, Irwin Leav, Jing Li, Lucia R. Languino, and Dario C. Altieri

This paper was accepted for publication in *Cancer Research* as a feature article on June 15th, 2009. Not all of the data represented in this chapter was included in the publication due to content limitations. Dario Altieri contributed to the design of the experiments and writing of the paper. Janet Plescia, the second author on this paper, contributed the immunohistochemistry staining of mouse prostate tissues as shown in Fig. 3-4, panel D. Irwin Leav, a veterinarian, helped interpret the IHC staining of the slides. Jing Li, a postdoc from Dr. Lucia Languino's lab was responsible for generating the prostate-specific PTEN conditional knockout mouse. I would also like to acknowledge and thank Drs. Bert Vogelstein (John Hopkins) for HCT116 cells, Alonzo Ross (UMass Medical School) for PTEN-GFP cDNA, Domenico Accili (Columbia) for FOXO1 adenoviral constructs, and Alex Toker (Harvard) for p85Δ cDNA.

Abstract

Endogenous tumor suppression provides a barrier against malignant transformation, but the molecular circuitries of this process are not well understood. Here, we show that the dual specificity phosphatase PTEN, a gene almost universally inactivated in human tumors, silences the expression of survivin, an essential regulator of cell division and apoptosis in cancer. This pathway is independent of p53, involves

active repression of survivin gene transcription, and is mediated by direct occupancy of the survivin promoter by FOXO1 and FOXO3a transcription factors. Conditional deletion of PTEN in the mouse prostate causes deregulated induction of survivin before full blown transformation, *in vivo*, whereas expression of survivin and PTEN is inversely correlated in cancer patients. Therefore, silencing the survivin gene is one of the essential requirements of PTEN mediated endogenous tumor suppression.

Introduction

The dual specificity phosphatase PTEN (Phosphatase and Tensin homolog deleted from chromosome Ten) functions as a nodal regulator of multiple signaling pathways that control cell proliferation, cell survival, and cell size (Keniry and Parsons, 2008). By removing the D3 phosphate from the lipid second messenger phosphatidylinositol triphosphate, PTEN shuts off growth factor receptor stimulation, and interrupts downstream signaling mediated by AKT and PDK1 kinases (Manning and Cantley, 2007). This pathway provides a broad network of tumor suppression, not only by antagonizing cell proliferation and promoting apoptosis, but also by maintaining chromosomal integrity (Shen et al., 2007), and directly cooperating with p53-dependent responses (Semba et al., 2006). In this context, the PTEN pathway is almost universally disabled in human cancer (Keniry and Parsons, 2008), often involving deletion of the PTEN gene in common malignancies of prostate, brain, breast, and colon (Li et al., 1997b). Although Forkhead transcription factors of the FOXO subfamily have been shown to contribute to PTEN signaling (Calnan and Brunet, 2008), the downstream gene

targets that cooperate to mediate tumor suppression have not been conclusively identified (Accili and Arden, 2004).

Survivin is a unique member of the Inhibitor of Apoptosis (IAP) gene family with essential roles in mitosis, the cellular stress response, and inhibition of cell death (Altieri, 2008). These properties are exploited in cancer, where survivin is universally over-expressed, and promotes unfavorable outcome (Altieri, 2008). Although several signaling pathways, including AKT (Wang et al., 2008a), have been associated with elevated survivin levels in cancer, the interplay between oncogenic mechanisms and tumor suppression networks in controlling survivin gene transcription has remained largely unclear.

In this study, we investigated the impact of the PTEN pathway on survivin gene expression. We show that loss of PTEN activates survivin gene transcription in a FOXO-dependent pathway. This involves active AKT to inhibit FOXO1 and FOXO3a binding to the survivin promoter region, thus turning on survivin promoter activity and gene transcription.

Materials and Methods

Cells and antibodies

Breast adenocarcinoma MCF-7 and MDA-MB-231, and prostate adenocarcinoma PC3 cells were obtained from the American Type Culture Collection (ATCC). $p53^{+/+}$ or $p53^{-/-}$ colorectal cancer HCT116 cells were kindly provided by Dr. Bert Vogelstein

(Johns Hopkins University, Baltimore, MD). MCF-7 cells stably expressing survivin (MCF-7 SVV) were described previously (Ghosh et al., 2008). MDA-MB-231 cells stably transfected with the proximal 830 nt of the mouse survivin promoter fused to GFP (ms-830-GFP) were as described (Xia and Altieri, 2006). A lentiviral plasmid encoding control pLKO or PTEN-directed short hairpin RNA (shRNA, Open Biosystems) was transfected into 293T packaging cells together with pCMVΔ8.9 and pMDG to generate lentiviruses. MCF-7 cells were infected with control or PTEN-directed lentivirus, and positive clones were selected in 1 μg/ml Puromycin (Sigma)-containing medium. Antibodies to PTEN, phospho-AKT (S473), cleaved caspase 3 (Cell Signaling), XIAP, GFP (BD Biosciences), FOXO1, FOXO3a, 14-3-3β (Santa Cruz Biotechnology), RNA Polymerase II, IgG (Active Motif), survivin (Novus Biologicals), or β-actin (Sigma) were used.

RNA and protein analysis

Total RNA was extracted from HCT 116 cells (Qiagen) and was reverse-transcribed using 1st strand CDNA synthesis by Oligo (dT) (Invitrogen). The cDNA was amplified with primers for survivin, 5'-GCATGGGTGCCCCGACGTTG-3' (forward) and 5'-GCTCCGGCCAGAGGCCTCAA-3' (reverse); GAPDH, 5'-ACGGATTTGGTCGTATTGGGCG-3' (forward) and 5'-CTCCTGGAAGATGGTGATGG-3' (reverse); FOXO1, 5'-AAGAGCGTGCCCTACTTCAA-3' (forward) and 5'-AGGCCATTTGGAAAACTGTG-3' (reverse); FOXO3a, 5'-

GCAAGCACAGAGTTGGATGA-3’ (forward) and 5’-CTGGCGTAGGGAGTTCAGAG-3’ (reverse). The amplified product was resolved on a 1% agarose gel by electrophoresis. Protein expression was analyzed by Western blotting.

Transfections

β-galactosidase-normalized survivin promoter luciferase activity (pLuc-3000, pLuc-1430, pLuc-649, and pLuc-441) was quantified as described (Li and Altieri, 1999). Cells were transfected with SMARTpool siRNA directed to PTEN, FOXO1 or FOXO3a (Dharmacon), or non-targeted siRNA (Ghosh et al., 2008). LY294002 (Calbiochem) was used as a PI3 kinase inhibitor. MDA-MB-231 cells stably transfected with ms-830-GFP were transduced with adenoviruses encoding FOXO variants at 30-50 multiplicity of infection (MOI) for 8 h, and harvested after 24-48 h.

Analysis of apoptosis

Cells transfected with pEGFP or PTEN-GFP were analyzed after 24 h by multiparametric flow cytometry using PE-Annexin V and 7-AAD staining (BD Biosciences) (Ghosh et al., 2008), or, alternatively, for DNA content by propidium iodide staining and flow cytometry.

Chromatin immunoprecipitation

Colorectal adenocarcinoma HCT116 cells ($4.7x10^7$) were fixed in 1% formaldehyde for 10 min at 22°C, lysed, and nuclear fractions were isolated prior to DNA shearing (fragment size of 200 to 600 nt) by sonication. Samples were incubated with protein G magnetic beads and various antibodies for 5 h at 4°C. The beads were pelleted, reverse-crosslinked, treated with proteinase K, and DNA was amplified by PCR using primers for regions in the human survivin promoter containing putative FOXO binding sites (-1428 nt): 5' -TGAGCTGAGATCATGCCACT-3' (forward), and 5'-CTGGTGCCTCCACTGTCTTT-3' (reverse), or devoid of FOXO binding sites (-2269 nt): 5'-TTGTTCCTTTCCTCCCTCCTGAG-3'(forward), and 5'-GTCAACTGGATTTGATAACTGCA-3'(reverse). Primers to amplify FOXO binding sites in the $p27^{Kip1}$ promoter (Li et al., 2008), or RNA polymerase II binding sites in the GAPDH promoter were used as control.

Oncomine Analysis

Oncomine data were reviewed for microarray analysis of differential gene expression in cancer *versus* matched normal tissues. Raw gene expression data were extracted for both PTEN and survivin from the same studies, and comparative analysis was performed using GraphPad Software (Prism 4.0). A *p* value was calculated using Spearman rank correlation test.

Histology

Five μm sections cut from prostate tissues of 20-24 week old prostate-specific PTEN conditional knockout mice (Wang et al., 2003) were stained with antibodies to survivin or control IgG by immunohistochemistry, as described (Xia and Altieri, 2006).

Analysis of tumorigenicity

Stably transfected MCF-7 cells with PTEN shRNA were cultured in soft agar for 14 days at 37°C, and colonies (>50 cells) were counted by light microscopy. All experiments involving animals were approved by an Institutional Animal Care and Use Committee at the University of Massachusetts Medical School. MCF-7 PTEN shRNA transfectants were injected ($2x10^6$ in 100 μl of PBS) subcutaneously into the flanks of 6 to 8 week-old female CB17 severe combined immunodeficient (SCID)/beige mice (3 mice per group, 2 tumors per mouse, 2 independent experiments). Tumor growth was monitored every other day, and tumor size was calculated with a caliper according to the formula $L \times W^2/2$ (mm^3).

Results

PTEN regulation of survivin gene expression

Transfection of breast adenocarcinoma MCF-7 cells with PTEN-directed siRNA efficiently suppressed PTEN expression, by Western blotting (Fig. 3-1A). This was associated with increased levels of endogenous survivin, as compared with cultures

transfected with non-targeted siRNA (Fig. 3-1A). XIAP, another IAP family protein stabilized by survivin (Altieri, 2008), was also increased in MCF-7 cells after PTEN knockdown, whereas the levels of 14-3-3β were unchanged (Fig. 3-1A). siRNA silencing of PTEN indistinguishably increased survivin expression in $p53^{+/+}$ or $p53^{-/-}$ HCT116 cells, indicating that p53 was not required for this response (Hoffman et al., 2002) (Fig. 3-1A). Consistent with a transcriptional mechanism, PTEN knockdown resulted in a two-fold increase in survivin mRNA, by RT-PCR (Fig. 3-1B), and increased survivin promoter activity, by luciferase reporter assay (Fig. 3-1C). Accordingly, MCF-7 cells transfected with the proximal 830 nt of the survivin promoter fused to GFP (ms-830-GFP) exhibited an increased number of GFP-expressing cells after PTEN silencing, by fluorescence microscopy (Fig. 3-1D). In contrast, PTEN siRNA did not affect the expression of control pEGFP, and a non-targeted siRNA was ineffective (Fig. 3-1D).

Transcriptional repression of survivin by PTEN signaling

In complementary experiments, transfection of a PTEN cDNA in PTEN-null breast adenocarcinoma MDA-MB-231 or prostate adenocarcinoma PC3 cells resulted in nearly complete suppression of Akt phosphorylation on Ser473 (Fig. 3-2A). This was associated with concentration-dependent reduction in endogenous survivin levels, by Western blotting (Fig. 3-2A), and repression of survivin gene transcription, by luciferase promoter assay (Fig. 3-2B). Similarly, downstream targeting of the PI3 kinase pathway with the pharmacologic inhibitor LY294002, or after transfection of a dominant negative (DN) mutant of the p85 regulatory subunit (p85Δ), suppressed survivin promoter-directed

GFP expression in HCT116 cells (Fig. 3-2C), and reduced endogenous survivin protein levels in MCF-7 cells (Fig. 3-2C, D), respectively.

FOXO silencing of survivin gene transcription

The FOXO family of Forkhead transcription factors function as potential effectors of PTEN signaling (Accili and Arden, 2004). Accordingly, transduction of MDA-MB-231 cells with an adenovirus encoding a FOXO1 DN mutant increased endogenous survivin levels, as compared with cultures transduced with wild type (WT) FOXO1 (Fig. 3-3A). Conversely, a constitutively active FOXO1 construct (ADA) slightly reduced survivin expression in MDA-MB-231 cells (Fig. 3-3A). In control experiments, expression of FOXO1 ADA induced spontaneous apoptosis in transduced cultures, whereas FOXO1 WT or DN mutant had no effect, by DNA content analysis and flow cytometry (Fig. 3-3A). In complementary experiments, siRNA silencing of FOXO1 or FOXO3a (Fig. 3-3B) increased the expression of endogenous survivin in HCT116 cells, by Western blotting (Fig. 3-3B), and enhanced survivin promoter luciferase activity (Fig. 3-3C), as compared with non-targeted siRNA. When analyzed in chromatin immunoprecipitation studies, FOXO1 and FOXO3a physically associated with a segment of the proximal survivin promoter (-1428 nt) containing putative FOXO binding sites, whereas an upstream region (-2269 nt) in the survivin gene was ineffective (Fig. 3-3D). In control experiments, FOXO1 and FOXO3a also bound to the $p27^{Kip1}$ promoter, which is regulated via FOXO-dependent transcription (Li et al., 2008), but not to an unrelated promoter, GAPDH (Fig. 3-3D). Finally, stable shRNA silencing of PTEN in MCF-7 cells resulted in increased survivin expression (not shown), and reduced formation of

FOXO3a complexes with the survivin promoter, whereas FOXO1 interactions were less prominently affected (Fig. 3-3E).

Survivin modulation of PTEN tumor suppression

Transfection of a PTEN cDNA in MCF-7 cells minimally affected cell viability, whereas the combination of PTEN expression plus the broad cell death stimulus, staurosporine (STS), caused approximately a 2-fold increase in apoptosis, by multiparametric flow cytometry (Fig. 3-4A). Stable expression of survivin in MCF-7 SVV cells reversed apoptosis induced by expression of PTEN plus STS to background levels of untreated cells (Fig. 3-4A), and reduced the extent of effector caspase 3 cleavage, by Western blotting (Fig. 3-4B). When analyzed *in vivo*, the expression of survivin and PTEN was inversely correlated in published microarray datasets of patients with glioblastoma (Sun et al., 2006) ($p<0.0001$), or colon cancer (Ki et al., 2007) ($p=0.0017$) (Fig. 3-4C). In addition, conditional deletion of PTEN in the mouse prostate resulted in a dramatic increase in survivin expression at the earliest stages of prostatic tumorigenesis, i.e. atypical hyperplasia, which persisted in more advanced disease phases of carcinoma in situ and invasive carcinoma, in vivo (Fig. 3-4D).

Pten regulation of tumorigenicity

To further characterize the MCF-7 cells stably expressing PTEN shRNA, we performed several *in vitro* and *in vivo* studies to determine the effects of PTEN silencing on tumorigenicity. Transfection of these cells with ms-830-GFP showed a remarkable

increase in the number of GFP-expressing cells (Fig. 3-5A, *left*). This correlated with the increase in phosphorylated AKT and survivin expression in the PTEN shRNA clone as compared to the empty vector control, plko (Fig. 3-5A, *right*). Similarly, MCF-7 shPTEN clone also showed an increase in survivin promoter-driven luciferase activity for both pluc 649 and pluc 441 as compared to control shGFP transfectants (Fig. 3-5B). To determine whether stable silencing of PTEN affects anchorage-independent growth, we performed soft agar colony formation assay over a period of 14 days. MCF-7 stably expressing PTEN shRNA showed a 10-fold increase in the number of colonies as compared to empty vector or GFP shRNA control transfectants (Fig. 3-5C). Moreover, the proliferative capacity of MCF-7 shPTEN cells exceeded that of MCF-7 shGFP cells by the 6^{th} day of cell counting (Fig. 3-5D). Lastly, we injected the stable transfectants into the flanks of immunocompromised mice and monitored tumor growth over a period of 3 weeks. Under these conditions, PTEN silencing in MCF-7 cells showed similar growth kinetics to the control group up to day 12 of tumor growth (Fig. 3-5E). However, MCF-7 shPTEN xenografts started to diverge from the control group on an increasing exponential after a latency period of about two weeks (Fig. 3-5D).

Discussion

The purpose of this study was to identify downstream gene targets of PTEN null tumors that contribute to tumorigenicity and resistance to therapy. Here we show that survivin, a member of the Inhibitor of Apoptosis (IAP) family, is a downstream effector of the PI3K/AKT pathway that is transcriptionally upregulated upon PTEN knockdown in both breast and colorectal cancer cells. Moreover, stable knockdown of PTEN by

lentiviral siRNA delivery in breast cancer cells results in increased survivin promoter activity, increased colony formation in soft agar and accelerated tumor growth in mice xenografts. Conversely, expression of a mutant form of PI3K (p85Δ), pharmacologic inhibition of PI3K by LY294002 or PTEN overexpression results in survivin downregulation and increased apoptosis. Reintroduction of survivin in PTEN-transfected cells partially rescues the apoptotic phenotype. In addition, we show by chromatin immunoprecipitation and luciferase reporter assays that forkhead transcription factors, FOXO1 and FOXO3a, which are excluded from the nucleus upon AKT phosphorylation, bind to the survivin promoter and negatively regulate its transcription. Accordingly, overexpression of a non-phosphorylable FOXO1 mutant (Foxo1ADA) that localizes to the nucleus promotes cell death via downregulation of survivin gene expression. Conversely, siRNA knockdown of both FOXO1 and FOXO3a results in increased survivin promoter activity and protein expression. These data point to a novel mechanism by which PTEN suppresses survivin levels via FOXO1 and FOXO3a-dependent transcriptional repression.

A funtional role for PTEN in tumorigenesis has been difficult to comprehend due to a cooperative role of the p53 tumor suppressor protein. Complete loss of PTEN in a mouse prostate cancer model resulted in senescence induced by p53 (Chen et al., 2005). Similarly, in our xenograft studies we did not observe a dramatic increase in tumor growth, perhaps due to the presence of p53 in the MCF-7 stable transfectants. Complete loss of PTEN *in vivo* does not lead to proliferative cell growth due to the role of p53 in promoting cellular senescence. Therefore, tumor growth kinetics may largely depend on the p53 status of PTEN-null tumors. On the occasion both tumor suppressor functions are

lost, tumor proliferation and carcinogenesis can proceed without having to deal with endogenous barrier mechanisms.

Tumor suppression mediated by PTEN may contribute to the differential expression of survivin in many disparate types of cancer (Altieri, 2008), given that defects in the PTEN pathway, or deregulated Akt signaling, occur in nearly every human tumor (Keniry and Parsons, 2008). Similar to PTEN (this study), other pivotal tumor suppressors, including p53 (Hoffman et al., 2002; Mirza et al., 2002), APC (Zhang et al., 2001), and BRCA1 (Wang et al., 2008b) have been shown to acutely silence the survivin gene, by different mechanisms. This suggests that maintaining low to undetectable levels of survivin is a general mechanism of an endogenous tumor suppression network, and provides a requisite to effectively antagonize neoplastic transformation. Moreover survivin-based therapeutics currently available in the clinic may be advantageous to restore apoptosis and inhibit cell proliferation in PTEN-null human tumors.

Figures

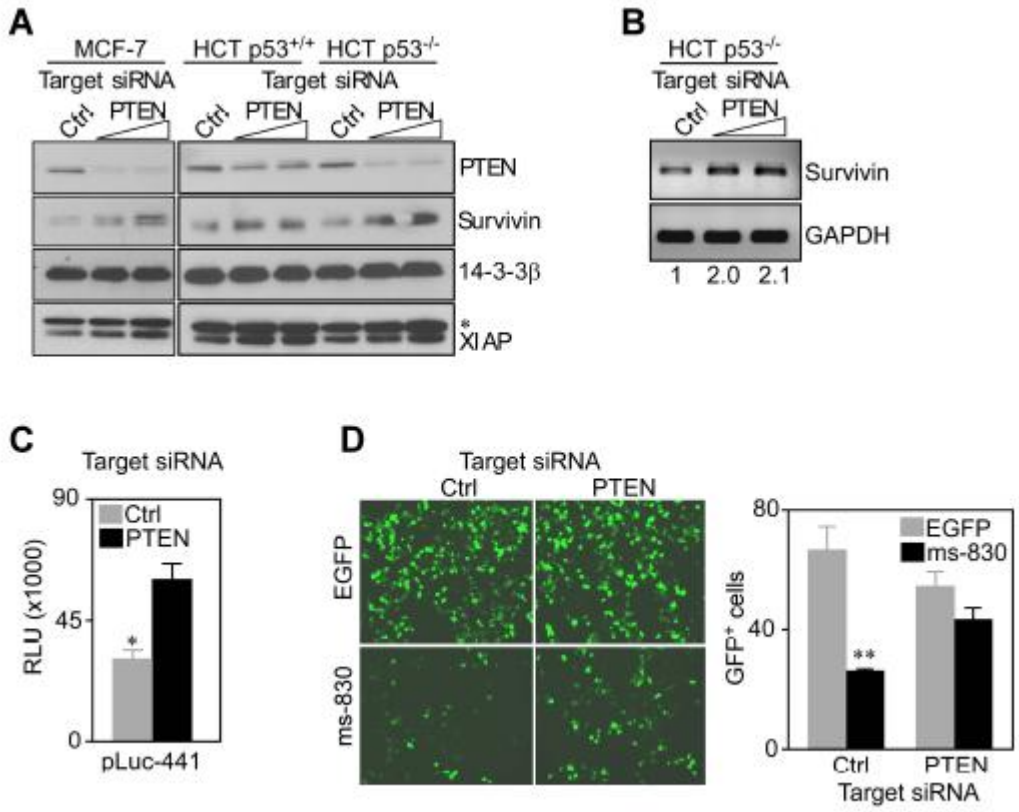


Figure 3-1 PTEN regulation of *survivin* gene expression.

(A) The indicated cell types were transfected with control (Ctrl) or PTEN-directed siRNA, and analyzed by Western blotting. *, non specific. (B) siRNA-transfected HCT116 cells were analyzed by RT-PCR. Numbers correspond to densitometric quantification of mRNA bands. (C) PTEN siRNA-silenced HCT116 cells were transfected with a survivin promoter luciferase construct (pLuc-441), and analyzed for luciferase activity. RLU, relative luciferase units. *, p=0.025. (D) HCT116 cells expressing pEGFP or ms-830-GFP were transfected with the indicated siRNA, and analyzed by fluorescence microscopy. *Right*, quantification of GFP-expressing cells. **, p=0.0061. For panels *C* and *D* (*right*), data are the mean±SEM of replicates from a representative experiment out of at least two independent determinations.

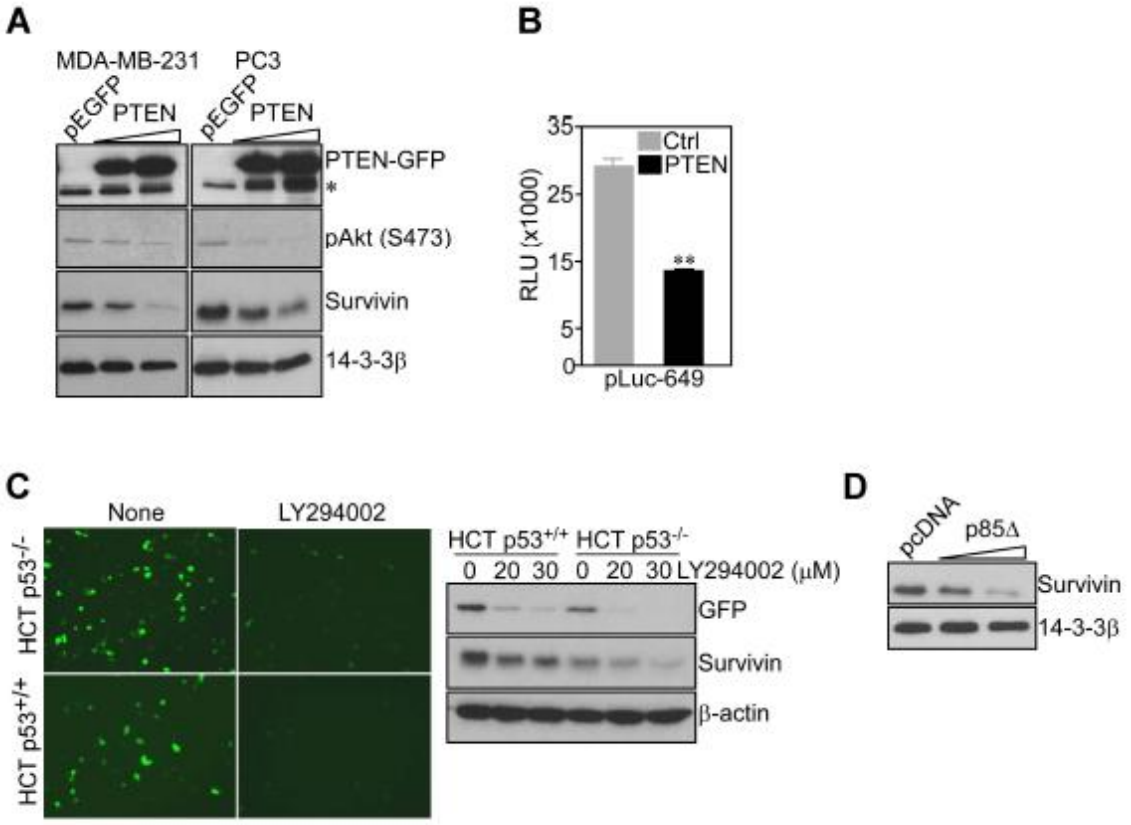


Figure 3-2 Transcriptional repression of survivin by PTEN

(A) The indicated cell types were transfected with pEGFP or increasing concentrations of PTEN cDNA, and analyzed by Western blotting. *, non specific. (B) HCT116 cells expressing pEGFP or PTEN cDNA were transfected with a *survivin* promoter luciferase construct (pLuc-649), and analyzed for luciferase activity. RLU, relative luciferase units. **, p=0.0048. Data are the mean±SEM of replicates of a representative experiment out of at least two independent determinations. (C) HCT116 cells were transfected with ms-830-GFP, treated with vehicle or LY294002 (30 μM), and analyzed by fluorescence microscopy. *Right*, Western blotting. (D) MCF-7 cells were transfected with pcDNA or PI3 kinase p85Δ DN mutant and analyzed by Western blotting.

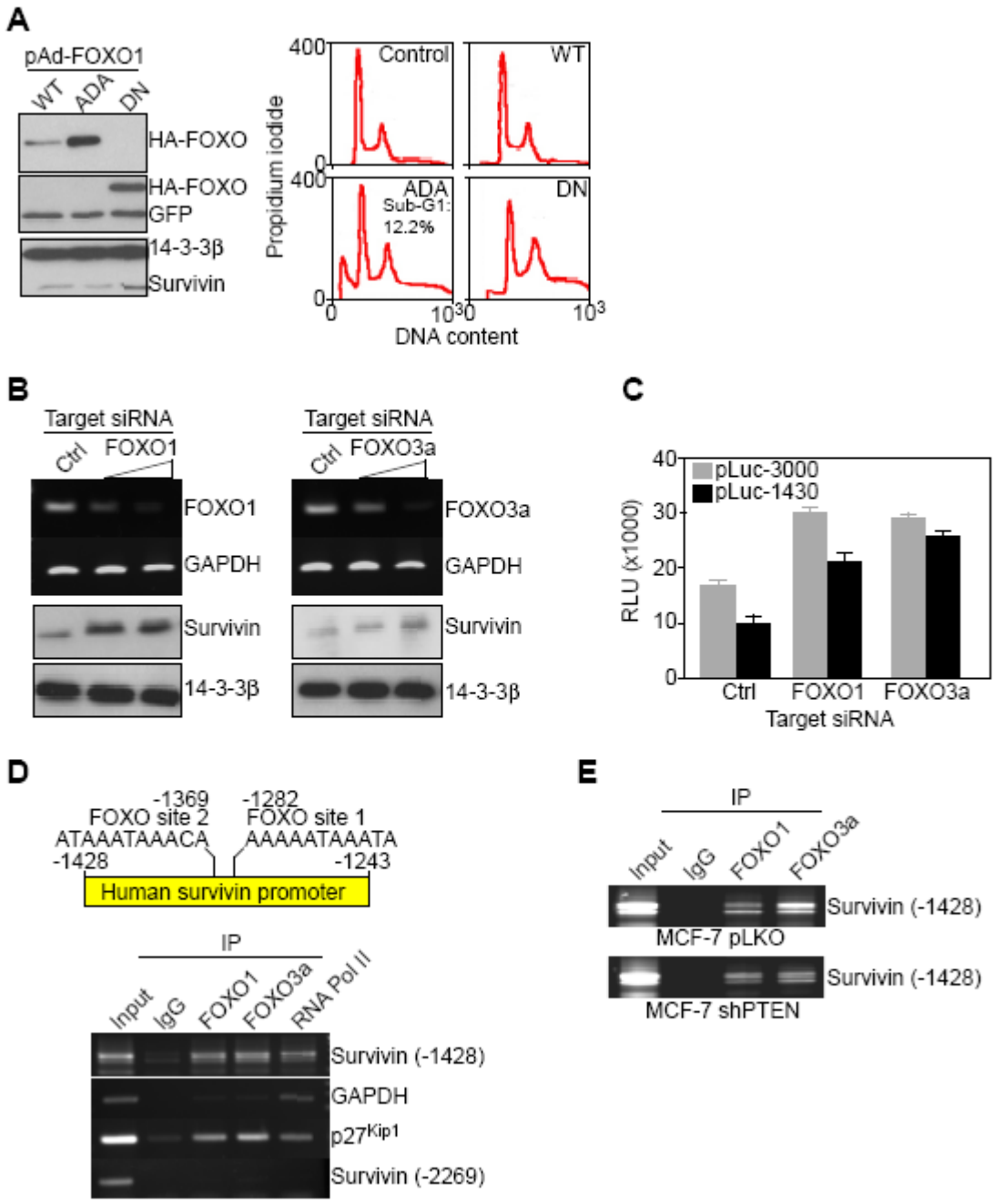


Figure 3-3 FOXO regulation of survivin gene transcription

(A) MDA-MB-231 cells stably expressing ms-830-GFP were transduced with the indicated FOXO1 adenoviruses (pAd), and analyzed by Western blotting. WT, wild type; ADA, constitutively active; DN, dominant negative. *Right*, DNA content analysis of transduced cells. The percentage of cells with sub-G1 DNA content is indicated. (B) HCT116 cells transfected with control (Ctrl) or FOXO1- (*left*) or FOXO3a (*right*)-

directed siRNA were analyzed by RT-PCR (*top*) or Western blotting (*bottom*). (C) HCT116 cells silenced for FOXO1 or FOXO3a were transfected with survivin promoter luciferase constructs (pLuc-1430 or pLuc-3000) and analyzed for luciferase activity. RLU, relative luciferase units. Data are the mean±SEM of replicates of a representative experiment out of at least two independent determinations. (D) Nuclear extracts of HCT116 cells were immunoprecipitated (IP) with the indicated antibodies, and the immune complexes were amplified with primers corresponding to *survivin*, $p27^{Kip1}$ or *GAPDH* promoter sequences. *Top*, position of putative FOXO sites in the survivin promoter. (E). Nuclear extracts of MCF-7 cells stably expressing control pLKO or PTEN-directed shRNA were immunoprecipitated with the indicated antibodies and amplified with primers corresponding to survivin.

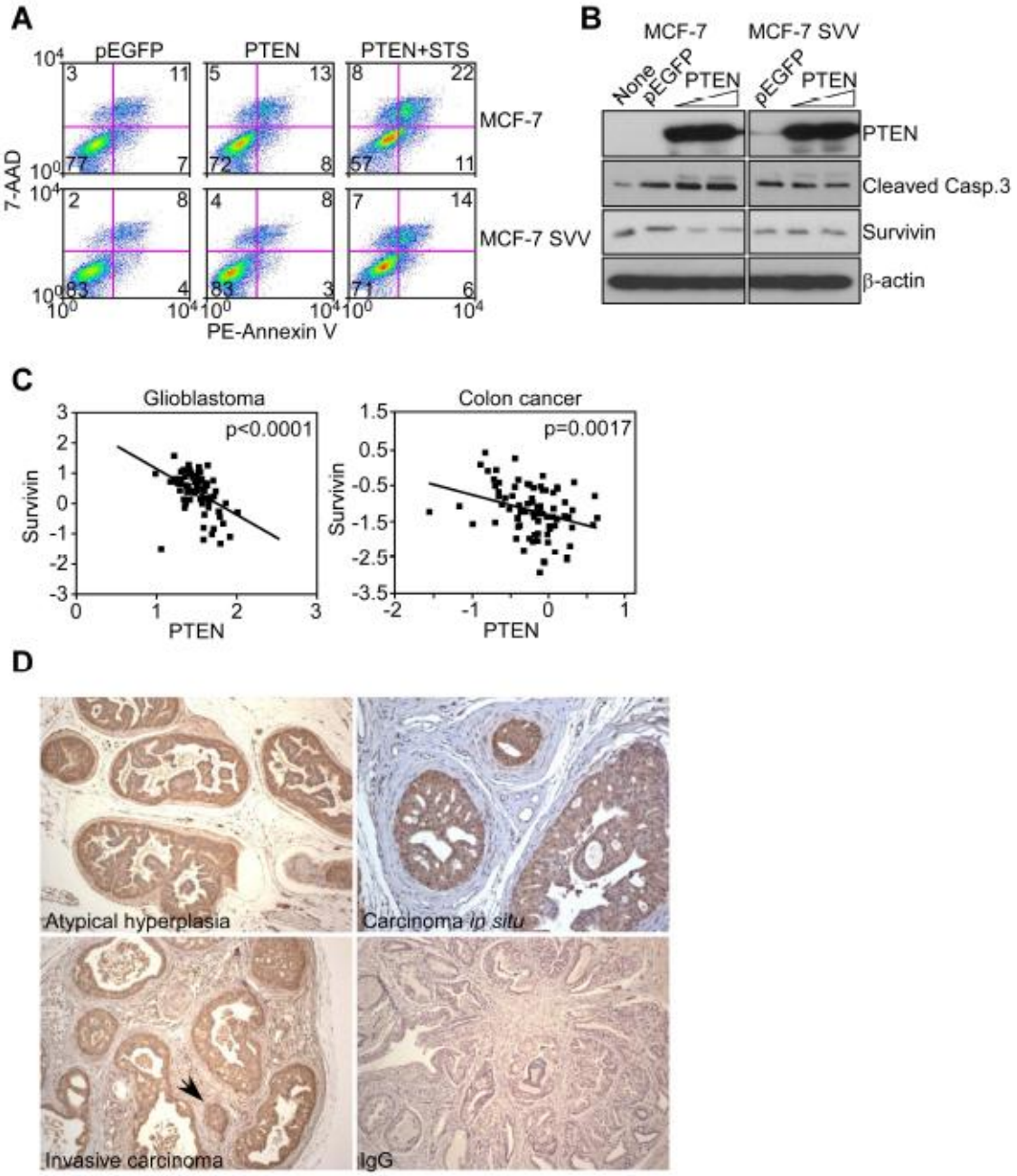


Figure 3-4 Regulation of PTEN tumor suppression by survivin

(A) MCF-7 or MCF-7 SVV cells were transfected with pEGFP or PTEN with our without STS (1 μM), and analyzed by multiparametric flow cytometry. The percentage of cells in each quadrant is indicated. (B) Transfected MCF-7 or MCF-7 SVV cells were

analyzed by Western blotting. (C) Microarray datasets of patients with glioblastoma (77) or colon cancer (81) were examined for expression of PTEN or survivin by linear regression analysis. (D) Prostate tissues from 20-24 week-old prostate-specific PTEN conditional knockout mice were stained with IgG or antibodies to survivin. *Arrow*, area of local invasion. Magnification, x400.

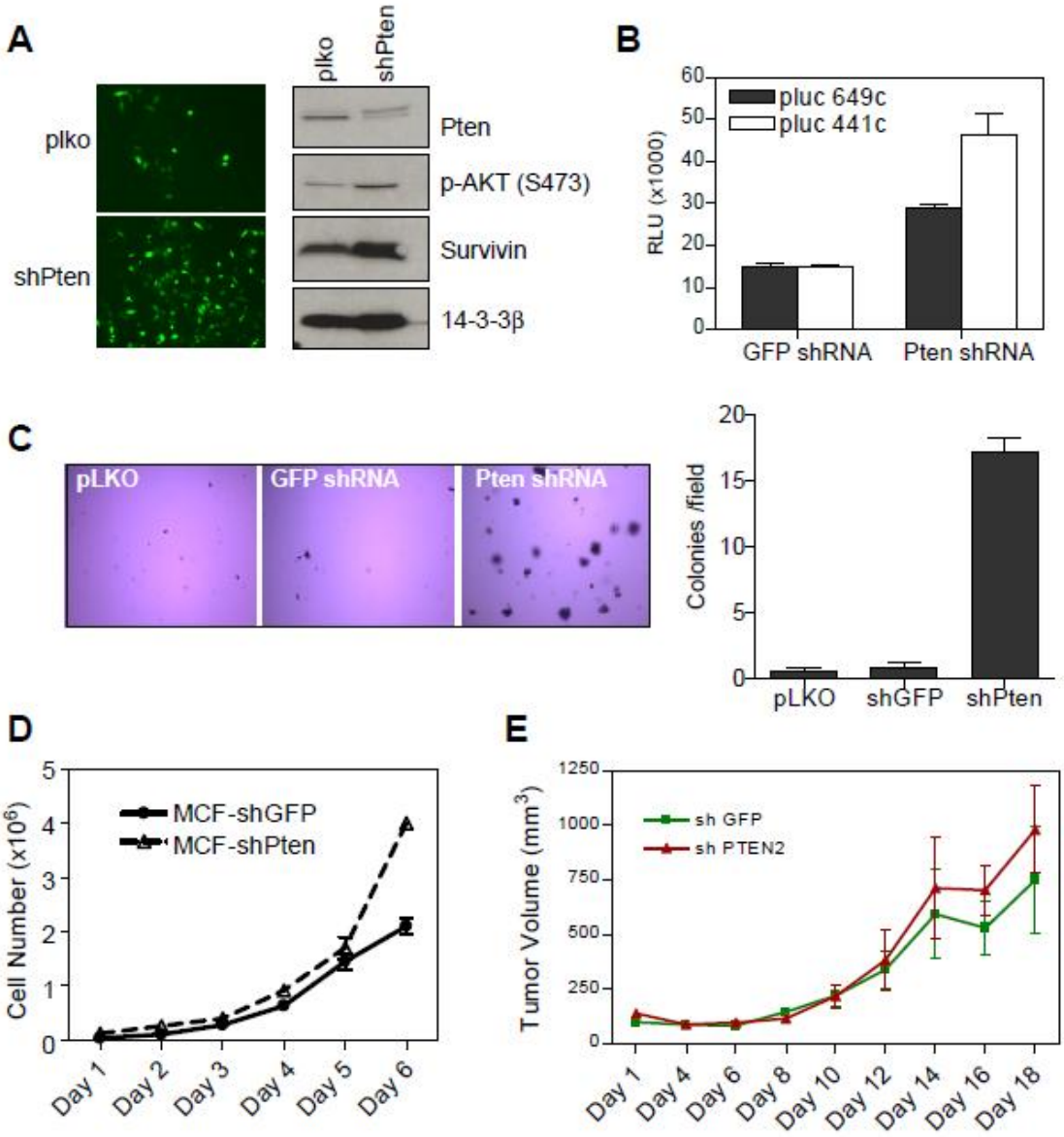


Figure 3-5 Characterization of stable MCF-7 cells expressing PTEN shRNA

(A) MCF-7 stable cells expressing empty vector (plko) or PTEN shRNA (shPten) were transfected with ms-830-GFP and analyzed by fluorescence microscopy *(left)* or western blotting *(right).* (B) MCF-7 stable cells were transfected with a *survivin* promoter luciferase construct (pLuc-649 and pLuc 441), and analyzed for luciferase activity. RLU, relative luciferase units. (C) Colony formation of MCF-7 transfectants. *Left*, representative microscopy fields per cell type are shown. *Right*, quantification of colony formation. (D) Cell proliferation assay of MCF-7 transfectants over a period of 6 days.

(E) Xenograft tumor growth of MCF-7 transfectants. Mean tumor volume in mm^3 is shown for each time point. Representative of two independent experiments.

Chapter 4. Final Thoughts and Future Directions

Since the discovery of survivin in 1997, there have been about 2,600 pubmed articles on survivin to date. The evolving interest in survivin biology among scientists all over the world is the very indication that this protein encompasses essential functions in tumor progression and maintenance. In order to translate the burgeoning research into promising therapeutic potential, it is crucial to have a complete understanding of the functional implications of survivin. Although much is known about survivin's role as a regulator of cell division and an inhibitor of apoptosis, there is still more to the function of survivin that has yet to be discovered. Recent evidence from our lab points to survivin's role as a transcriptional regulator of fibronectin gene expression that facilitates tumor invasion and metastasis. In addition to survivin's multiple functional roles, it is equally important to understand upstream regulators that control survivin gene expression in cancer cells as opposed to normal cells. Our study provides evidence for two novel endogenous tumor suppressors that regulate survivin gene transcription, namely caspase-2 and PTEN. The loss of function of either of the two proteins results in deregulated expression of survivin leading to uncontrolled cancer cell survival and proliferation.

Despite caspase 2 being one of the first caspases to be discovered, there still remains controversial evidence on the exact function of caspase 2 in the cell death cascade. Conventionally, caspase 2 plays a role in regulating the intrinsic cell death pathway by acting as an initiator caspase involved in mitochondrial permeabilization upon DNA damage (Kumar and Vaux, 2002). In our study we provide evidence to suggest a novel role of caspase 2 in regulating survivin gene transcription via the NFκB

singaling pathway. Upon activation of caspase 2 following DNA damage or chemotherapy, caspase 2 cleaves RIP1 thereby suppressing NFκB transcription factor from nuclear translocation (Fig. 4-1). In turn, NFκB is unable to initiate target gene transcription involved in apoptosis inhibition, including survivin. This leads to consequent mitotic arrest and cell death in those cancer cells that are dependent on NFκB-mediated survival signaling. Alternatively, TNFα is able to stimulate NFκB activity by phosphorylation of IκBα inhibitory protein by the IKK complex, which leads to subsequent ubiquitination and degradation of IκBα by the proteasomal pathway. Overexpression of active caspase 2 interferes with this pathway by preventing NFκB from initiating its transcriptional activity via the intermediary cleavage of RIP1 activator kinase as illustrated in the model figure (Fig. 4-1).

The data represented above gives a new perspective into understanding cancer as a disease with multiple pathways that intricately intersect resulting in deregulated gene expression, abnormal cell proliferation and resistance to cell death. Previously, colorectal cancer was known to expand from colonic crypts to malignant transformation by a process involving a complex of the transcription factors T-Cell Factor (TCF-4)/β-catenin. Deregulation and stabilization of TCF-4/β-catenin would result in abnormal target gene expression, including survivin (Kim et al., 2003). Although, there is some data supporting the TCF-4/ β-catenin pathway in colorectal cancer progression, it is important to identify parallel pathways that may significantly contribute to disease dissemination. Caspase 2 regulation of NFκB pathway provides one such perspective that would allow clinicians to better predict disease outcome and allow for combination therapy. Another possibility is

to target survivin as a nodal protein in patients with multiple pathway mutations that may be a better strategy as opposed to targeting any single deregulated pathway.

In chapter 3 we have described a comprehensive mechanism showing PTEN regulation of survivin via FOXO transcription factor binding. In this study we concluded that PTEN negatively regulates survivin transcription by shutting off the PI3K/AKT pathway and allowing FOXO1- and FOXO3a-mediated survivin gene repression (Fig. 4-2). Since PTEN regulates the PI3K/AKT pathway, it is important to note that survivin has also been linked to mTOR signaling, which is also a target of AKT. A previous study done in our lab showed IGF-1 stimulation of mTOR signaling increased a translational pool of survivin mRNA (Vaira et al., 2007). Translation of survivin was directly regulated by p70S6K , a downstream substrate of mTOR that is involved in protein synthesis. Thus, survivin is regulated by the PI3K/AKT pathway not only via a FOXO-dependent transcriptional mechanism, but also a pathway that controls survivin translation via p70S6K. In this study we have uncovered novel survivin transcriptional mechanisms that add to the list of existing transcriptional and translational regulation of survivin, suggesting that a single mechanism is probably not sufficient for maintaining survivin levels in cancer cells. Although p53 is a valid transcriptional repressor of survivin (Hoffman et al., 2002), we have presented two p53-independent mechanisms for survivin gene regulation. Nevertheless, it is important to keep in mind these alternative pathways when comparing cancer subtypes and their p53-status. As with the PTEN story, we have shown that survivin is regulated independently of p53 status in vitro. However, since there are several reports of crosstalk between PTEN and p53, we cannot neglect this possibility. Since we didn't observe a difference in tumor growth of PTEN-

null xenografts as compared to controls, one must speculate that there are alternate mechanisms at play. This could be true especially in an in vivo setting, as several other p53-driven tumor suppression mechanisms may be turned on that counteract the increase in survivin levels upon PTEN silencing. Perhaps, it would be interesting to find a link to p53 by using mutant p53 cell lines to conduct some of the PTEN knockdown studies *in vivo*. In this context, it may also be beneficial to study the effects of senescence in response to these tumor suppressors. Although there is no literature linking survivin to senescence, it would be worthwhile to investigate a potential role for survivin in regulating senescence.

The intricate network of signaling molecules in tumor progression can only be understood when all the members involved have been identified and accounted for. In our study, we have established part of the tumor suppressor network in controlling survivin gene transcription (Fig. 4-3). Although disparate molecules converge on survivin to either suppress or activate survivin gene expression, it may be interesting to establish a relationship among the various transcription factors that bind to the survivin promoter. In other words, it would be worthwhile to determine possible overlap/cooperation of the different promoter binding sites. For example, upon AKT activation, FOXO transcription factors are excluded from the nucleus, and are unable to initiate their role as a transcriptional repressor of survivin gene expression. In this context, it may be possible that NFκB transcription factors cooperate with the PI3K/AKT pathway to promote survivin transcription in the absence of FOXO factors. Moreover, we could potentially determine epigenetic factors such as chromatin modifications involving (de)acetylation of the survivin gene locus by histone (de)acetylases (HDAC/HAC). Similarly, we could also

investigate CpG island methylation status of the survivin promoter region in controlling gene expression. These are just a few ideas that come to mind when studying survivin transcriptional regulation in cancer cells.

Several pharmacological antagonists have been developed to inhibit signaling pathways in cancer that are constitutively activated. Though there is ample literature describing the mechanism of targeted therapies, it is equally important to determine downstream effectors of these canonical pathways. These pathways include PI3K/AKT or EGFR signaling that are hyper-activated in most cancers. Drugs that target these pathways include LY294002 (PI3K inhibitor) and gefitinib (EGFR inhibitor) that are currently undergoing testing for cancer therapy (Vlahos et al., 1994). However, it is important to keep in mind that PI3K pathway is also an important regulator of several normal cellular functions. Thus targeting this pathway may lead to several side-effects. In fact, PI3K pathway has been shown to be involved in insulin signaling and metabolism (Cho et al., 2001). Therefore targeting this pathway may lead to reduced insulin sensitivity and increased risk for diabetes. However, as opposed to normal cells, tumor cells are addicted to PI3K signaling such that the pathway is in a hyperactive state. Thus using drugs to normalize PI3K activity in tumor cells can potentially be of therapeutic benefit. To validate the efficacy of PI3K inhibitors in eradicating cancer cells and not normal cells, one could imagine looking at survivin levels as a potential biomarker. Lowering survivin levels in cancer cells together with other inhibitors of apoptosis proteins may help validate drug targets. Thus, it is crucial to have a complete understanding of the PI3K/AKT signaling pathway in tumors as opposed to normal cells in order to design better targeting strategies for anti-cancer therapy.

The PTEN/ PI3K/AKT pathway has become an attractive target for drug development since these agents induce cell cycle arrest, increase apoptosis and decrease resistance to cytotoxic therapy. Inhibitors of kinases involved in the PI3K/AKT signaling pathway have been aggressively pursued, and some have also entered clinical trials. These drugs include BKM120, BGT226, and BEZ235 that are currently being pursued by Novartis, a pharmaceutical company that has invested in phase I/II clinical trials for these compounds (NCT00620594; ClinicalTrials.gov). Among these drugs, BEZ235 seems to be of particular interest due to its recent popularity among academic scientists and industrial professionals at the American Association of Cancer Research (AACR) annual conference. This competitive inhibitor was shown to inhibit PI3K and mTOR activity and block AKT phosphorylation and activation. Moreover, MCF-7 breast cancer cells were sensitive to this drug by inducing caspase 3 and PARP cleavage (known indicators of apoptosis). They also noticed that full length caspase 2 was greatly reduced after BEZ235 treatment, but were unsure of the importance of this finding. Our study provides some clues as to what is happening and how this is relevant to understanding the drug's mechanistic and functional features. As we have learned, caspase 2 is activated and undergoes autocatalytic cleavage that would reduce full length caspase 2 and generate cleaved fragments. Active caspase 2 can suppress the NFκB signaling pathway by cleaving RIP1 and subsequently decreasing survivin gene transcription. Thus, BEZ235 may act as an upstream activator of caspase 2, that ultimately leads to sensitization of tumor cells by lowering an anti-apoptotic threshold by targeting survivin, an IAP protein. In addition, investigators at Novartis also found that breast adenocarcinoma MDA-MB231 and colorectal HCT116 cell lines in particular were insensitive to this drug.

Although, we know that many tumors are addicted to PI3K signaling, there are alternative pathways that maybe triggered as a compensatory mechanism. In a recent talk given by Dr. Lewis Cantley of Harvard Medical School showed that Bez235 does not decrease Ras-driven tumors in mice, suggesting that these tumors rely on an alternative survival pathway, namely the MEK/Erk signaling pathway downstream of Ras. Perhaps, this is true of the MDA-MB231 and HCT116 cells that were not sensitive to BEZ235 treatment as they may have high levels of phosphorylated Erk. Therefore a better strategy for these particular tumors would be to use combination therapy that targets both PI3K and MEK, or Bez235 of Novartis combined with ARRY-886 of Array Biopharma.

Several studies have indicated an activated NFκB gene signature by microarray analysis of multiple myeloma patients that make this pathway an attractive drug target (Mulligan et al., 2007). Similar to this, two recent studies have pointed to a significant role of NFκB in promoting tumorigenesis by the inactivation of a negative regulator of NFκB, also known as A20 (Compagno et al., 2009; Kato et al., 2009). This protein encodes a ubiquitin-modifying enzyme that is induced upon TNFα stimulation. Genome-wide analysis showed that A20 was a common genetic lesion in B-cell lymphomas with up to 30% of patients displaying somatic mutations of this gene (Kato et al., 2009). The second study identified multiple genetic mutations in more than 50% of B-cell lymphomas involving genes that converge on the NFκB signaling pathway, ultimately leading to its activation (Compagno et al., 2009). Once again, A20 was found to be the most commonly mutated gene in these studies, thus characterizing A20 as a tumor suppressor involved in negatively regulating NFκB activity. Both studies showed that re-expressing A20 in a lymphoma-derived cell line with mutant form of the gene resulted in

suppression of cell growth and initiation of apoptosis (Compagno et al., 2009; Kato et al., 2009). Caspase 2 similar to A20 act as upstream regulators of NFκB signaling by suppressing its transcription factor activity. However, further studies need to be pursued to determine if caspase 2 is also perhaps mutated or downregulated in lymphomas similar to A20. These studies may help provide a rational for targeting tumor-types with activated NFκB signaling pathway that encompasses several target genes involved in proliferation and survival, including survivin. Targeting tumors with high levels of NFκB activity could potentially be a better alternative to conventional cancer therapy. Clinical evidence suggests that Bortezomib (Velcade/PS-341; Millenium Pharmaceuticals), a reversible 26S proteasome inhibitor, showed efficacy in Phase III clinical trials for multiple myeloma patients (Baud and Karin, 2009). Although Bortezomib is not a direct inhibitor of NFkB activity, studies indicate that the efficacy of this drug may in part be due to inhibiton of the NFκB pathway (Hideshima et al., 2001).

In chapter 2, we identified caspase 2 as a negative regulator of NFκB activity and performed comprehensive studies to show that caspase 2 acts as a tumor suppressor gene. Once we established the role of caspase 2, the obvious question was to figure out whether this gene was mutated in human tumor specimens. For this, we extracted total RNA from frozen tissue sections of 3 colon cancer patients at various grades of the disease (Grades1-3). Sequencing analysis showed no relevant mutations of caspase 2 among tumor and normal matched tissue specimens. However, sequencing was done for an 800 nucleotide region spanning the active subunit, based on the hypothesis that the catalytic site regulates survivin gene transcription. Although there weren't any mutations in this region, the prodomain may play a role in tumor suppression mechanism that we have not

investigated in this study. In addition, caspase 2 function maybe suppressed due to epigenetic modifications and not somatic mutations. To test for such modifications, DNA sequencing would not be sufficient to determine the functional role of caspase 2 in these tumors. More importantly, it would be necessary to get a significant sample size of patient specimens to make any relevant conclusions. The patient sample size for the B-cell lymphoma study consisted of 238 cases from which they concluded that A20 is a relevant mutated tumor suppressor gene in about 30% of these patients (Kato et al., 2009). Therefore, we were limited with the number of patients available to us (3), and perhaps the technique we used for genotyping. A better method would have been to do single nucleotide polymorphism (SNP) genotyping microarray analysis that allows for a larger sample size analysis and thus more statistically significant results.

Similar to the lymphoma studies done for A20, Oncomine database has published 8 different studies of lymphoma cases that show a significant population with downregulation of caspase 2 gene expression when compared to normal specimens (Table 4-1A). This potentially supports the tumor suppressor role attributed to caspase 2 from our study which is quite similar to A20 being a negative regulator of NFκB. One might speculate that downregulation of caspase 2 in these lymphoma cases may point to a less active enzyme that may support a steady-state NFκB activity and support transcription of NFκB target genes. Suprisingly however, only lymphomas are reported to have a significant downregulation of caspase 2 expression when compared to other cancer types reported on the Oncomine database (Table 4-1A). One of the caveats of Oncomine microarray analysis is that it fails to account for tumor variability. In other words, these tumors are from multiple patients at different stages of diagnosis as well as

treatment. Therefore, gene expression profiles may fluctuate depending on the treatment that is received by the individual patient. In fact, upon chemotherapy, we have observed a dose-dependent increase in caspase 2 activity as described in Fig. 2-1A. Perhaps, the upregulated caspase 2 gene expression profile in most other human tumors is indicative of the effects of chemotherapy. Nevertheless, we should not neglect the data represented for lymphomas as this particular cancer type may be resistant to conventional chemotherapy due to inactivation of A20 and/or downregulation of caspase 2.

The overall theme from the studies presented here points to a general tumor suppression network that regulates survivin gene expression, primarily through a transcriptional network (Fig. 4-3). Together with past regulators of tumor suppression that target survivin gene transcription, we have identified two novel pathways that converge on survivin to suppress its gene expression. Perhaps, these endogenous tumor suppression mechanisms are essential in preventing cells from succumbing to the effects of DNA damage or harmful mutations by inducing apoptosis or senescence. Inactivation of these tumor suppressors either by mutation or loss of heterozygozity enables malignant clones to be established that rely on anti-apoptotic mediators to sustain their survival. In fact, deregulation of the cell death program leads to unchecked growth of tumors that develop resistance to conventional chemotherapy (Fesik, 2005). Therefore, re-engaging the apoptotic program in these cancer cells by novel targeted therapies would enable for selective killing of tumor cells. Currently, there are agonist antibodies that target TRAIL receptors and TRAIL ligands that are in phase I/II clinical trials for anti-cancer therapy (Fesik, 2005). Moreover, Bcl-2 antisense oligonucleotides are in phase III clinical trials, and a small molecule Bcl-2 inhibitor, also known as ABT-737 is in early phase I clinical

trial (Fesik, 2005). Recently, several small molecule IAP inhibitors have also been developed that are currently going through preclinical testing (Fesik, 2005). Since survivin is an IAP protein that is differentially expressed in cancer cells as opposed to normal cells, targeting pathways that regulate survivin expression may be beneficial in future drug discovery endeavors. In parallel to targeting these antiapoptotic regulators, there also remains the potential to activate certain tumor suppressors by re-instating their enzymatic activity. However, these remain to be explored as one can imagine the caveats of developing a drug that may increase PTEN phosphatase activity or mimic caspase activity. Although, there may be compensatory mechanisms that may overcome the effects of a single drug, there is some hope with new anticancer-therapy that targets mutated tumor suppressors in transformed cells. Two such small molecules recently discovered include PRIMA1 and RITA that help re-activate the p53 tumor suppressor pathway in cancer cells (Fig. 4-3) (Grinkevich et al., 2009; Lambert et al., 2009). Although these studies are preliminary, there still remains potential for further research and discovery of novel drugs that re-activate tumor suppressor pathways that may have been turned off in cancer cells.

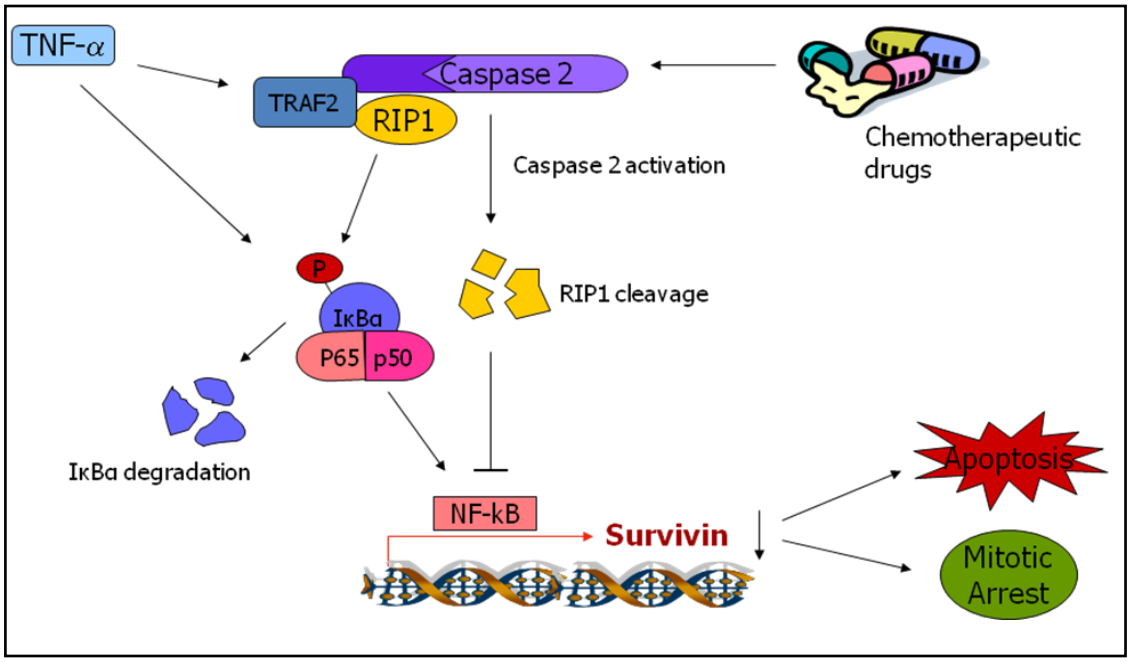


Figure 4-1 Model for caspase 2 regulation of survivin

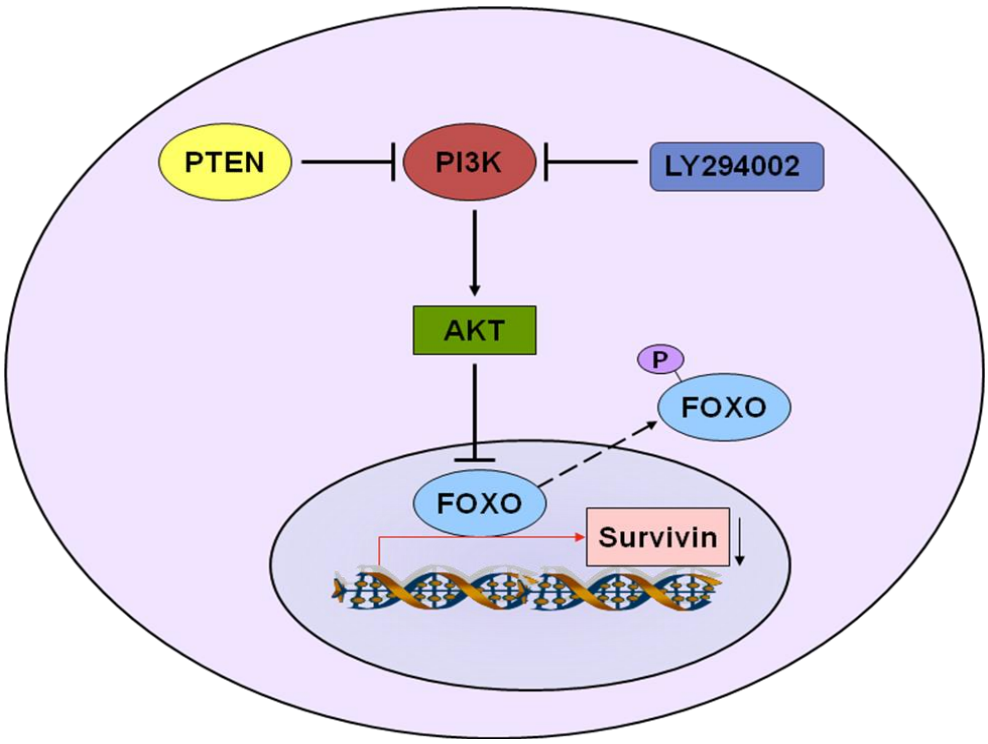
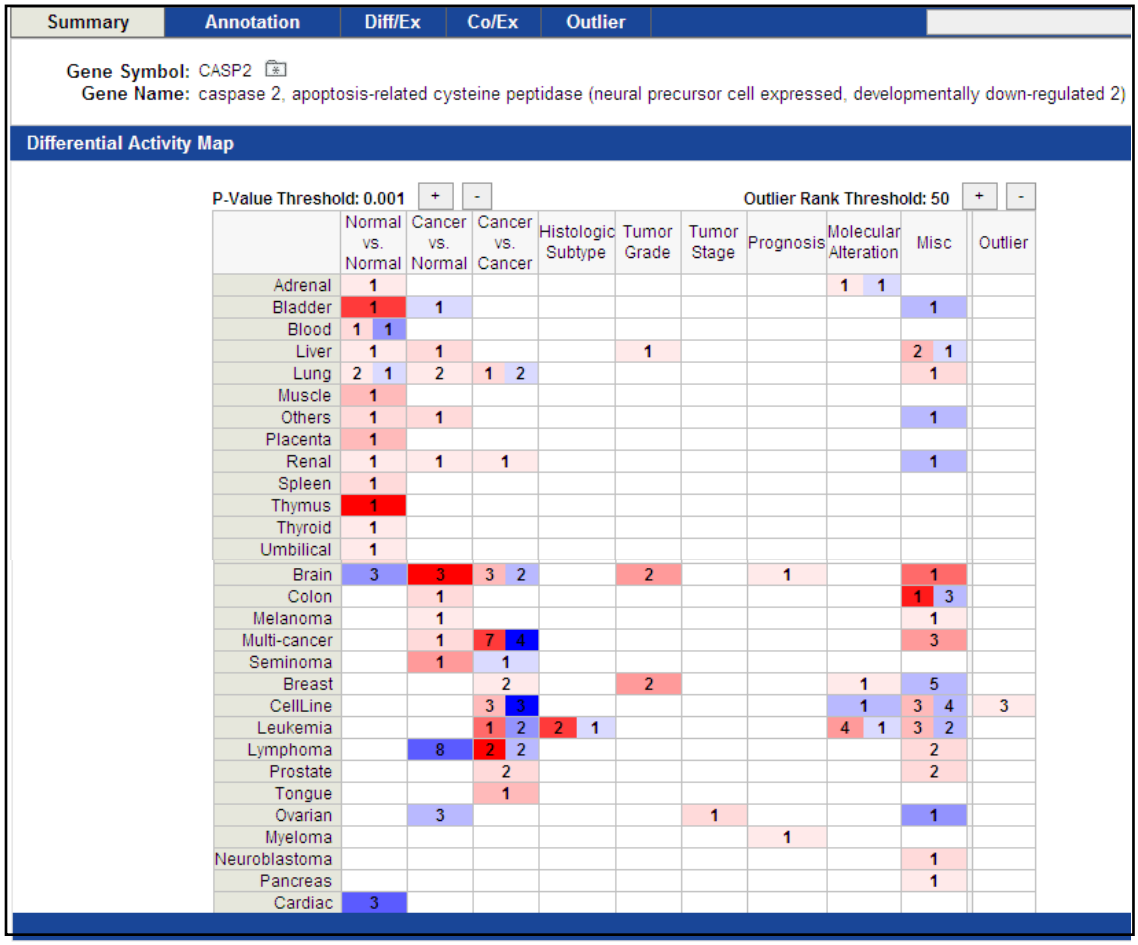


Figure 4-2 Model for PTEN regulation of survivin



	Normal vs. Normal	Cancer vs. Normal	Cancer vs. Cancer	Histologic Subtype	Tumor Grade	Tumor Stage	Prognosis	Molecular Alteration	Misc	Outlier
Adrenal	1							1 1		
Bladder	1	1							1	
Blood	1 1									
Liver	1	1			1				2 1	
Lung	2 1	2	1 2						1	
Muscle	1									
Others	1	1							1	
Placenta	1									
Renal	1	1	1						1	
Spleen	1									
Thymus	1									
Thyroid	1									
Umbilical	1									
Brain	3	3	3 2		2		1		1	
Colon		1							1 3	
Melanoma		1							1	
Multi-cancer		1	7 4						3	
Seminoma		1	1							
Breast			2		2			1	5	
CellLine			3 3					1	3 4	3
Leukemia			1 2	2 1				4 1	3 2	
Lymphoma		8	2 2						2	
Prostate			2						2	
Tongue			1							
Ovarian		3				1			1	
Myeloma							1			
Neuroblastoma									1	
Pancreas									1	
Cardiac	3									

Table 4-1 Oncomine differential activity map for caspase 2

Oncomine data summary showing caspase 2 expression analysis in various normal and cancer tissue types. Red indicates upregulation of gene expression and blue indicates downregulation of gene expression. Numbers correspond to the number of independent microarray analysis.

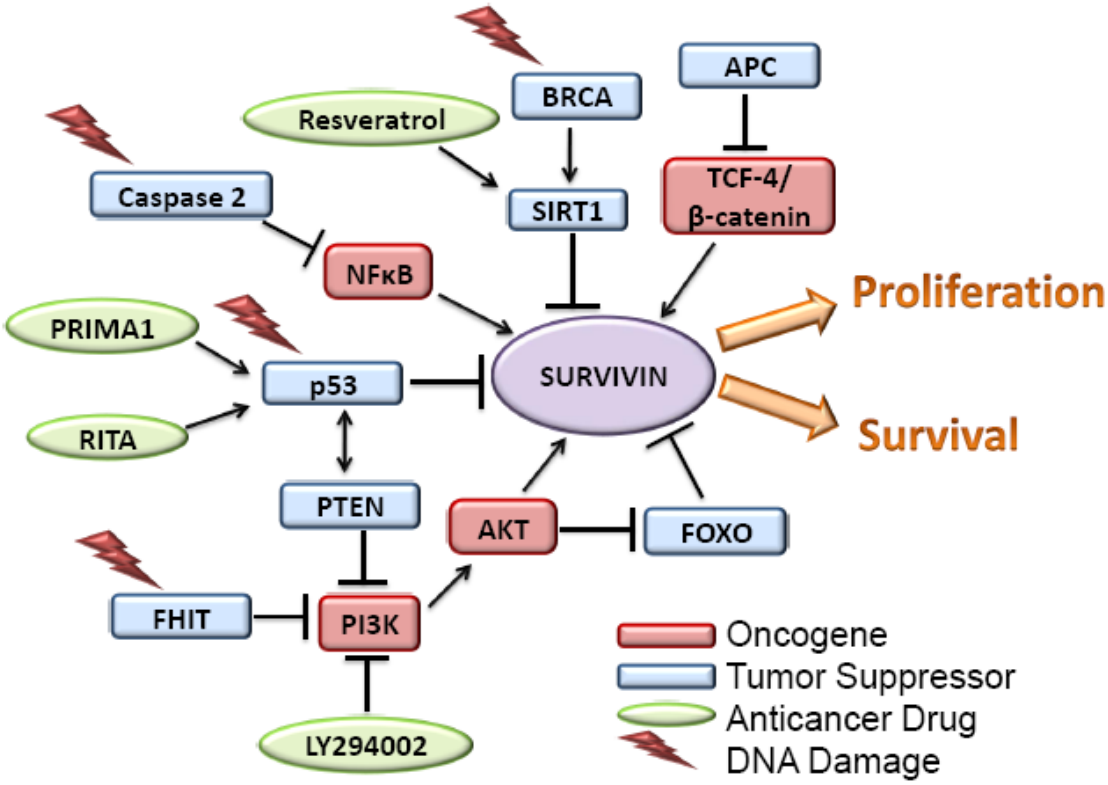


Figure 4-3 Tumor Suppression Network Targeting Survivin Gene Expression

Known regulators of the tumor suppression network that mediate survivin gene expression.

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