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P38 Phoshporylates RB On SER567 By A Novel, Cell Cycle-Independent Mechanism That Triggers RB-HDM2 Interaction And Apoptosis

Rachel Delston
Washington University in St. Louis

WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biological and Biomedical Sciences

Molecular and Cellular Biology

Dissertation Examination Committee:
J. William Harbour (chair)
David Beebe
Gregory Longmore
Anthony Muslin
Helen Piwnica-Worms
Reid Townsend
Jason Weber

P38 PHOSHPORYLATES RB ON SER567 BY A NOVEL, CELL CYCLE-INDEPENDENT MECHANISM THAT TRIGGERS RB-HDM2 INTERACTION AND APOPTOSIS

by

Rachel Baker Delston

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ABSTRACT OF THE DISSERTATION

p38 phosphorylates Rb on Ser567 by a novel, cell cycle-independent mechanism that triggers Rb-Hdm2 interaction and apoptosis

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Rachel Baker Delston

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Professor J. William Harbour, Chair

The retinoblastoma protein (Rb) inhibits both cell division and apoptosis, but the mechanism by which Rb alternatively regulates these divergent outcomes remains poorly understood. Cyclin dependent kinases (Cdks) promote cell division by phosphorylating and reversibly inactivating Rb by a hierarchical series of phosphorylation events and sequential conformational changes. The stress-regulated mitogen activated protein kinase (MAPK) p38 also phosphorylates Rb, but it does so in a cell cycle-independent manner that is associated with apoptosis rather than with cell division. Here, we show that p38 phosphorylates Rb by a novel mechanism that is distinct from that of Cdks. p38 bypasses the cell cycle-associated hierarchical phosphorylation and directly phosphorylates Rb on Ser567, which is not phosphorylated during the normal cell cycle. Phosphorylation by p38, but not Cdks, triggers an interaction between Rb and the human homologue of murine double minute 2 (Hdm2), leading to degradation of Rb, release of E2F1 and cell death. These findings provide a mechanistic explanation for how Rb regulates cell division and apoptosis through different kinases, and reveal how Hdm2 may functionally link the tumor suppressors Rb and p53.

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This dissertation is dedicated to my greatest accomplishment to date—my son Andrew Delston Byron. Andy, you are the light of my life!

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Chapter 1

Introduction

1.1 Introduction to the dissertation

This thesis addresses a central question in cell signaling field—how do proteins control the fate of the cell? More specifically, we investigated how the retinoblastoma protein (Rb) regulates two central cellular processes—cell division and cell death. This work sheds light on both basic research on how cells choose to divide versus die and also on translational research as the Rb pathway is disrupted in virtually all cancers. Understanding the intricacies of Rb signaling could lead to advances in treating cancer. Using melanoma cells as a model system we found that p38, a MAPK member, phosphorylates Rb on residue Ser567 during genotoxic stress. In this chapter, the major proteins and cellular processes discussed in the dissertation are introduced.

1.2 The retinoblastoma protein and the pocket protein family

This dissertation centers around the tumor suppressor Rb. Located on chromosome 13q14, the RB gene encodes the ubiquitously expressed 110 kDa Rb protein. A nuclear phosphoprotein that regulates the G_1/S cell cycle checkpoint, Rb also plays a critical role in differentiation, chromosomal stability, and cell survival (Fung et al., 1987; Halaban, 2005; Harbour and Dean, 2000b; Knudsen et al., 1999; Lee et al., 1987; Zhang et al., 2000; Zheng and Lee, 2002). Rb has 16 potential Cdk serine/threonine-proline phosphoacceptor sites located throughout the protein (**Figure 1.1**). Rb consists of an N-terminus, a pocket domain, and a C-terminus. The N-terminus is not required for tumor suppressor ability and for many years was thought to have no function. More recently, it has been shown to be similar in structure to the pocket domain and to be capable of binding the pocket domain (Hassler et al., 2007). The pocket domain, so called because it forms a pocket-like structure, consists of an A box, a spacer, and a B box. The B box contains a leucine-x-cysteine-x-glutamate (LxCxE) consensus sequence that allows for binding to chromatin remodeling enzymes and viral oncoproteins such as Adenovirus E1A, SV40 Large T antigen and Papillomavirus E7 (Chow et al., 1996; DeCaprio, 2009; Dyson et al., 1989; Kaelin et al., 1992; Weintraub et al., 1992). The pocket domain is essential for tumor suppressor ability and is highly conserved across species. The A and B boxes are similar in structure to cyclins and may have originated from duplication of cyclins (Claudio et al., 2002). The E2F transcription

factor (E2F) binding site spans the B box and the C-terminus and is distinct from the LxCxE site allowing Rb to simultaneously bind both E2Fs and chromatin remodeling enzymes, creating a repressor complex (Harbour and Dean, 2000a). The C-terminus of Rb is progressively phosphorylated during the cell cycle and also binds several proteins including c-Abl (a pro-apoptotic tyrosine kinase proto-oncoprotein) and the human homologue of murine double minute 2 (Hdm2) (Borges et al., 2007; DeCaprio et al., 1992; Uchida et al., 2005).

Rb is a member of the pocket protein family which also includes p107 (Retinoblastoma-like protein 1) and p130 (Retinoblastoma-like protein 2). As the name suggests, the family shares homology in the pocket domain. Like Rb, p107 and p130 halt the cell cycle by recruiting chromatin remodeling complexes and repressing E2Fs, are phosphorylated by cyclin-Cdk complexes, and lose tumor suppressor ability when bound by LxCxE motif containing DNA tumor viruses (DeCaprio, 2009; Sun et al., 2007). The pocket protein family members have varying expression levels in the cell. Rb remains constant throughout the cell cycle, p130 is high in G_0 and differentiated cells and low in proliferating cells, while p107 has just the opposite expression pattern (Genovese et al., 2006). Interestingly, recent work revealed that Rb, p107, and p130 can all bind the RB promoter and regulate Rb transcription (Burkhart et al., 2010). While compensation for Rb does not appear to occur in humans, p107 can compensate for loss of Rb in the mouse. Rb related proteins with conserved pocket domains have been found in a variety of species ranging from Drosophila and C. elegans to plants such as maize and *Arabidopsis.* The alga *Chlamydomonas* has a protein with a region homologous to the pocket, including the LxCxE domain and the spacer, however the protein does not play a role in G_1 arrest (Claudio et al., 2002; Genovese et al., 2006). And while they have no sequence similarities, Rb does have a functional homolog in yeast, Whi5 (Cooper, 2006). This dissertation focuses on Rb, as it is the primary family member disrupted in cancer (Ewen et al., 1991; Yeung et al., 1993).

1.3 Rb is a tumor suppressor

Cancer is a term for many diseases which can be grouped together as all having uncontrolled growth of damaged cells. Cancer is second most common cause of death in the United States (ACS, 2010). There were over 1.5 million new cancer cases and over half a million people are expected to die of cancer in the Unites States in 2010 (ACS, 2010). Many factors contribute to cancer such as genetic predisposition, aging, physical carcinogens (such as ultraviolet and ionizing radiation), chemical carcinogens (such as tobacco smoke and asbestos) and infections (viruses and bacteria). During carcinogenesis a cell with damaged DNA replicates out of control due to inherited or progressive genetic changes involving overexpression of oncogenes and inactivation of tumor suppressor genes. Hanahan and Weinberg have suggested 6 hallmarks of cancer: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000).

One such type of cancer is retinoblastoma, the most common form of eye cancer in children. One patient per 20,000 live births is affected and there are about 300 cases of retinoblastomas a year in the United States (ACS, 2010; Harbour, 2006). The disease typically affects children under two years of age and when treated properly the survival rate is greater than 90%. One way in which retinoblastoma is diagnosed is through a child's eye appearing white in a photograph due to the tumor preventing the light from reaching the retina (**Figure 1.2**). Treatment options include enucleation (removal of the eye), plaque radiation, laser therapy, and chemotherapy. When left untreated retinoblastoma can metastasize to the lungs, bone, brain, and other sites (Harbour et al., 1994).

The study of retinoblastoma the disease and the Rb protein have led to major advances in the understanding of cancer. In 1971 Knudson proposed the two hit hypothesis, that cancer is caused by two mutational events, based on retinoblastoma cases, creating a paradigm shift in the field (Knudson, 1971). He hypothesized that either a germline mutation was present at birth and a second somatic mutation occurs (hereditary) or two somatic cell mutations occur (non-hereditary). Hereditary retinoblastoma makes up 40% of cases and is usually diagnosed

between six and twelve months of age. Tumors are most often present in both eyes, but primary tumors can occur in other locations such as the skin and bone. In non-hereditary retinoblastoma, which make up 60% of cases, the tumor is unilateral and there is no risk of tumor formation in the other eye. Non-hereditary retinoblastoma is usually diagnosed later, between one and two years of age.

Following up on Knudson's finding, Friend and colleagues cloned RB in 1986 and classified it as the first tumor suppressor (Friend et al., 1986). Further work revealed that the RB gene is mutated in additional cancers such as small cell lung cancer and osteosarcomas (Harbour, 1998; Harbour et al., 1988; Wadayama et al., 1994). In virtually all other types of tumors the Rb protein is functionally inactivated through hyperphosphorylation, by over-expression of cyclin D (regulatory subunit) which activates its catalytic counterpart cyclin dependent kinase (Cdk) 4/6, or through inactivation of the Cdk4/6 inhibitor $p16^{Ink4a}$ (Okamoto et al., 1994; Rogoff and Kowalik, 2004; Sherr, 1996).

1.4 Mouse models of Rb

There is 91% amino acid identity and 87% nucleotide identity between human and mouse Rb, thereby making the mouse an excellent system in which to study Rb. The first knockout mouse of a tumor suppressor, the RB knockout mouse, was created by Tyler Jacks in 1992 (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Deleting RB is embryonic lethal between days E12-15. The mice have defects in neurogenesis and erythropoiesis, and massive apoptosis and excessive proliferation occurs. Chimeric mice composed of Rb-deficient and wildtype cells are viable and rapidly develop pituitary tumors and thyroid adenomas but do not develop retinoblastomas (Maandag et al., 1994; Williams et al., 1994). Heterozygous $Rb^{+/-}$ mutant mice primarily develop tumors of the pituitary gland and thyroid hyperplasia, but no retinoblastomas develop in these mice (Jacks et al., 1992). In order to create an in vivo model that recapitulates human retinoblastoma, p107 or TP53 must be knocked out in addition to RB (Zhang et al., 2004). Knocking out E2F partially rescues the RB null phenotype (Tsai et al., 1998).

A decade after the first RB knockout mouse was made, Wu et al published a landmark paper revealing that a wildtype placenta largely rescues the RB null phenotype (Wu et al., 2003). Pups were able to reach term, although they died shortly after birth. These studies revealed that many of the features of the RB knockout phenotype were due to excessive trophoblast (placenta precursor cells) proliferation rather than cell autonomous effects of Rb loss per se.

1.5 Rb arrests cell cycle at the G_1 checkpoint through interaction with E2Fs

The cell cycle, the process in which one cell divides and replicates into two daughter cells, can be broken up into several phases (Coller, 2007). G_1 (a gap phase during which the cell grows in size and a checkpoint to confirm the cell is free of DNA damage), S (the synthesis phase in which DNA replication occurs), and G_2 (a second growth and checkpoint gap), all make up the Interphase portion of the cell cycle. The final phase is Mitosis in which the chromosomes divide and cytokinesis occurs. G_0 is a resting state in which cells have arrested and exited the cell cycle. Cells can reenter the cell cycle from this quiescent state or can remain in G_0 indefinitely as is the case for fully differentiated cells.

Rb plays a critical role in the cell by arresting damaged cells at the G_1 checkpoint. The ability of Rb to arrest cells in G_1 is intricately linked to its ability to regulate E2Fs (Coller, 2007). The E2F family includes E2Fs 1, 2, and 3a, activators which promote transcription of cell cycle promoters in late G_1 and S phases, and E2Fs 3b, 4, and 5, repressors which abrogate transcription of cell cycle promoters during G_0 and G_1 phases. E2Fs 6-8 are repressors thought to recruit chromatin remodeling complexes to DNA (van den Heuvel and Dyson, 2008). Rb binds the C-terminal activation domain of E2Fs 1-5. p130 and p107 also bind E2F3b, 4, and 5. E2Fs 1-6 heterodimerize with the differentiation-regulated transcription factor-1 polypeptide (DP) family. Rb represses transcription by at least two basic mechanisms. First, Rb binds and blocks the E2F transactivation domain (Flemington et al., 1993; Helin et al., 1993). Second, when Rb is brought to promoters through interaction with E2Fs, it actively represses transcription. Rb recruits

chromatin remodeling proteins to promoters where they alter local chromatin structure and inhibit access of the transcriptional machinery (Frolov and Dyson, 2004; Harbour and Dean, 2000a; Weintraub et al., 1992). Several major classes of chromatin remodeling proteins have been shown to interact with Rb, including histone deacetylases (e.g., HDAC1-3) which promote nucleosome formation, SWI/SNF ATP-dependent nucleosome assembly proteins (e.g., BRG1 and BRM), polycomb group proteins (e.g., HPC2 and Ring1), DNA methyl-transferases (e.g., DNMT1), and histone methyltransferases (e.g. SUV39h) (Brehm et al., 1998; Dahiya et al., 2001; Dunaief et al., 1994; Eden et al., 1998; Frolov and Dyson, 2004; Lai et al., 1999; Lu and Horvitz, 1998; Luo et al., 1998; Magnaghi et al., 1998; Nielsen et al., 2001; Panteleeva et al., 2004; Robertson et al., 2000; Schumacher and Magnuson, 1997; Trouche et al., 1997; Vandel et al., 2001).

1.6 Phosphorylation of Rb allows for cell cycle progression

Rb is progressively phosphorylated throughout the cell cycle (DeCaprio et al., 1992). Phosphorylation of Rb is catalyzed by cyclin D-Cdk4 in early G_1 (Kato et al., 1993), then by cyclin E-Cdk2 late in G_1, and later by cyclin A-Cdk2 in S phase (Sherr, 1996). These multiple phosphorylation events are necessary to inactivate Rb and allow for cell cycle progression (Lundberg and Weinberg, 1998). The progressive phosphorylation of Rb is hierarchical as the C-terminal sites are more readily phosphorylated than the pocket sites (Ma et al., 2003). Mechanistically, this can be understood as a stepwise series of increasingly energetically unfavorable phosphorylation events in which phosphorylation of an initial set of sites triggers intramolecular interactions that enable phosphorylation of the next set of sites (**Figure 1.3**) (Delston and Harbour, 2006; Harbour et al., 1999; Rubin et al., 2005). Similar sequential phosphorylation mechanisms have been shown for other proteins such as c-Fos (Mackeigan et al., 2005). Phosphorylation of Rb at sites in the C-terminus triggers an intramolecular conformational change in which the negatively charged C-terminus interacts with a positively charged lysine patch in the B box of the pocket domain (Harbour et al., 1999). Subsequent crystallographic studies confirmed this interaction between the C-terminus and pocket domain

(Lee et al., 2002; Rubin et al., 2005). The intramolecular interaction displaces LxCxE proteins such as HDACs from Rb, and this inactivation is sufficient to abrogate the ability of Rb to block the G_1-to-S transition (Zhang et al., 2000).

1.7 p53 is a tumor suppressor

There is significant crosstalk between the Rb and p53 pathways and therefore it is difficult to study one pathway without considering the other. E2F1 concomitantly transactivates ARF and ataxia telangiectasia mutated (ATM) which stabilize and activate p53 respectively (Berkovich and Ginsberg, 2003). In the following chapters, we investigate the role of p53 in genotoxic stress-induced apoptosis in melanoma cells.

p53 is the 393 amino acid product of the TP53 gene located on 17p13.1. While it migrates at 53 kDa as its name suggests, its actual mass is 43.7 kDa. p53 is a transcription factor that forms homotetramers . In 1991, experiments on myeloid leukaemic cells revealed that p53 acts as a tumor suppressor as addition of p53 caused these cancerous cells to die (Yonish-Rouach et al., 1991). p53 acts as a tumor suppressor by promoting apoptosis, senescence, or cell cycle arrest . p53 is mutated in over half of cancers and loss of TP53 in mice promotes tumors (Donehower et al., 1992; Hollstein et al., 1991; Jacks et al., 1994; Lowe et al., 1994; Yonish-Rouach et al., 1991). It is part of a family which includes p63 and p73 and while all three members can induce apoptosis, only p53 is commonly mutated in cancer (Dobbelstein and Roth, 1998; Marin et al., 1998). Interestingly, an ancestor of this family is present in organisms as evolutionally ancient as the sea anemone, where the protein promotes apoptosis in response to DNA damage (Belyi et al. 2010).

p53 is activated in response to a variety of stress signals such as oncogenic stimuli, hypoxia, and DNA damage (Levine, 1997). p53 is also activated and promotes apoptosis in response to disruption of the Rb pathway. Therefore, loss of Rb creates genomic instability and selective pressure for tumor cells to mutate p53 (Sherr, 2004). Rb plays a necessary role in the ability of

p53 to promote cell cycle arrest in response to DNA damage. Rb null/p53 wildtype cells undergo apoptosis, not arrest, in response to DNA damage. On the contrary, Rb can modulate the DNA damage response without the presence of p53 (Harrington et al., 1998; McClendon et al., 2010).

1.8 The INK4A/ARF locus

The INK4A/ARF locus is one of the most commonly targeted loci in cancers. Tumor cells delete the locus or methylate CpG-rich islands in the promoter regions, which represses transcription. Loss of INK4A and ARF disrupts the two major tumor suppressor pathways, Rb and p53, respectively. The INK4A/ARF locus encodes for two tumor suppressors in an unusually efficient use of transcript. Unique first exons are spliced into common exons 2 and 3 producing two unique proteins; the aptly named Inhibitor of Cdk4 ($p16^{Ink4a}$) and Alternate Reading Frame ($p14^{ARF}$). $p16^{Ink4a}$ leads to dephosphorylation and activation of Rb and cell cycle arrest. $p14^{ARF}$ resides in the nucleolus, a non-membrane-bound organelle in the nucleus where ribosomal biogenesis occurs, and is maintained at low levels in normal cells. Loss of ARF and INK4A in mice promotes tumorigenesis (Matheu et al., 2004; Sharpless et al., 2004).

DNA damage, Ras, Myc, or other pro-oncogenic signals leads to increased levels of $p14^{ARF}$. p53, which is normally kept at low levels by Hdm2-mediate degradation, is stabilized by $p14^{ARF}$. $p14^{ARF}$ stabilizes p53 by sequestering Hdm2 in the nucleolus so that it is unable to degrade p53. (Weber et al., 2000; Weber et al., 1999). In addition to Hdm2, $p14^{ARF}$ binds other proteins such as E2F1, Myc, and nucleophosmin (NPM), which is involved in ribosome biogenesis and in stabilization of $p14^{ARF}$ (Brady et al., 2004; Yu et al., 2006).

1.9 Rb and DNA damage

Genotoxic stress or DNA damage is an insult to the cell that damages DNA through double strand breaks, single strand breaks, thymine dimers, or other abnormal changes to base pairs of DNA. Genotoxic stress is caused by a variety of methods such as etoposide, gamma irradiation, UV exposure, and replication errors. In our experiments, we employed etoposide, a topoisomerase II

inhibitor used in chemotherapy, which induces double strand breaks in a similar manner to irradiation (Sun et al., 2005). Double strand breaks activate the DNA damage response pathway which is mediated by ATM and goes on to activate many downstream targets. One such target is nibrin, a component of the MRE11/RAD50 double-strand break repair complex. Nibrin is involved in non-homologous end joining and preventing S phase progression in cells with double stranded breaks (Wilda et al., 2000). p53, Rb, and E2F1 are also activated by ATM in order to promote cell cycle arrest, DNA repair, or death depending on the level of stress (Kastan et al., 1991; Rogoff and Kowalik, 2004; Sherr, 2004).

DNA damage promotes Rb-mediated cell cycle arrest through activation of cyclin-dependent kinase inhibitors (CKIs). CKIs prevent formation of cyclin-Cdks complexes rendering them unable to phosphorylate Rb. $p16^{Ink4a}$ inhibits cyclin D1 from activating Cdk4 and Cdk6. $p21^{Cip1}$ and $p27^{Kip1}$ inhibit cyclin E from activating Cdk2. In fact CKIs are currently being investigated for use in clinics to counteract the overexpression of cyclin-Cdks which occurs in many cancers (Galons et al., ; Malumbres et al., 2008; Sutherland and Musgrove, 2009).

E2F1 is activated during DNA damage by both acetylation and phosphorylation. Rb has a separate binding site contained in the C-terminus, rather than spanning the pocket domain and C-terminus, that binds E2F1, but not the other E2F family members. DNA damage activates PCAF/p300 which acetylates E2F1, resulting in loss of binding between E2F1 and this secondary C-terminal site on Rb, but leaving the other E2Fs unaffected. E2F1 is now released to transactivate pro-apoptotic target genes (Dick and Dyson, 2003; Engelmann and Putzer). E2F1 is phosphorylated on Ser31 and Ser364 in response to stress by ATM/ATR and Chk1/2. E2F1 also transcriptionally activates ATM/ATR and Chk1/2 creating a positive-feedback loop (Berkovich and Ginsberg, 2003; Lin et al., 2001; Powers et al., 2004; Stevens et al., 2003; Urist et al., 2004).

p53 levels are kept low by Hdm2-mediated degradation. In response to DNA damage, E2F1 transactivates $p14^{ARF}$ which inhibits Hdm2 from degrading p53. In parallel, ATM phosphorylates p53 on Ser15, stabilizing p53, which can then arrest cells through Rb by activating $p21^{Cip1}$ or promote apoptosis by acting as a transcription factor depending on the severity of the DNA damage signal (Sherr and Weber, 2000). Like E2F1, p53 is also phosphorylated by ATM and CHK1/2 and acetylated by PCAF/p300 (Polager and Ginsberg, 2009). These events promote p53-mediated apoptosis.

Cells must repair DNA damage before the cell replicates in order to maintain genomic stability. Rb plays a key role in the maintenance of the integrity of the genome. Loss of Rb leads to both aneuploidy and genomic instability (Knudsen and Wang, 2010 ; Zheng and Lee, 2002; Zhou and Elledge, 2000). The importance of Rb and E2F for the DNA damage response is well conserved. RBF and dE2F play an important role in responding to DNA damage in Drosophila (Du et al., 1996; Moon et al., 2008).

1.10 Rb is ubiquitinated by Hdm2 and degraded by the proteosome

The proteosome is the recycling center of the cell. Proteins are recognized by the 19S cap of the proteosome and translocated into the central pore of the 20S core where proteins are degraded into short peptides. The peptides are then used as building blocks for nascent proteins. A chain of ubiquitin groups attached to a lysine on the target protein marks the protein for the proteosome. A ubiquitin group is transferred from an E1 ubiquitin-activating enzyme to an E2 ubiquitin-conjugating enzyme, and finally to an E3 ubiquitin ligase in order to covalently link the ubiquitin group to the target protein in an ATP-dependent reaction.

Hdm2 is an E3 ubiquitin ligase for Rb, as well as for p53 and itself. The RING finger domain of Hdm2 possess ubiquitination activity (Fang et al., 2000; Sdek et al., 2004; Uchida et al., 2005; Xiao et al., 1995). Phosphorylation of Hdm2 at serine 269 impairs its interaction with the Rb protein. Hdm2 binds the Rb C-terminus on 792-928, leading to an increase in E2F activity. The

central acidic domain of Hdm2 (amino acids 254-264) is critical for the Hdm2-Rb interaction (Sdek et al., 2005). $p14^{ARF}$ is an Hdm2 agonist which is well known to inhibit Hdm2 ubiquitination of p53 by sequestering Hdm2 to the nucleus (Weber et al., 2005; Weber et al., 1999), but more recently has been shown to inhibit Hdm2-mediated ubiquitination of Rb (Uchida et al., 2005). The mechanism of Hdm2 ubiquitination of Rb is discussed in Chapter 3.

1.11 The Apoptotic response

Apoptosis, or programmed cell death, involves condensation of chromatin, fragmentation of DNA, blebbing of membranes, and shrinking and rounding of cells (Kerr et al., 1972). Apoptosis takes its name from Greek; *apo* meaning from and *ptosis* meaning falling, suggesting leaves falling from trees. Apoptosis is initiated by extracellular signals such as Fas and TNFR1 death receptors (extrinsic pathway) or intracellular signals such as release of cytochrome c from the mitochondria (intrinsic pathway). Commitment to the apoptotic response is determined by the Bcl2 family which consists of anti-apoptotic members such as Bcl2 and $Bclx_L$ and pro-apoptotic members such as Bad, Bax, and Bid (Gross et al., 1999). Cysteine proteases called caspases execute the apoptotic response. Inactive zymogens (pro-caspases) are cleaved and activated during the apoptotic response and go on to cleave target proteins. Initiator caspases (2, 8, 9, and 10) cleave and activate effector caspases (3,6, and 7) which promote transcription of pro-apoptotic genes. The extrinsic pathway signals through caspase 8 and the intrinsic through caspase 9 which both converge on caspase 3 to meditate the downstream response by cleaving cellular proteins such as poly(ADP-ribose) polymerase (PARP) which promotes DNA repair by activating the ATM pathway (Ranger et al., 2001).

1.12 p38 is a member of the MAPK family

p38 is a member of the mitogen-activated protein kinase (MAPK) family. The mechanism of p38-mediated phosphorylation of Rb will be discussed in the following chapters. MAPKs are a family of proteins conserved from yeast to humans that were discovered in the 1980s (Roux and Blenis, 2004; Sturgill and Ray, 1986; Widmann et al., 1999). There are four major arms of the MAPK

family; ERK1/2, ERK5/BMK1, SAPK/JNK, and p38 (**Figure 1.4**). They are activated by dual phosphorylation on Thr-X-Tyr motifs. MAPKs utilize a signaling cascade in which a biological response is elicited by a MAPK which is activated by a MAPK kinase (MAPKK), which in turn is activated by a MAPK kinase kinase (MAPKKK), which is activated by a variety of stimuli (Raman et al., 2007). The signaling cascade allows for many levels of regulation and amplification.

A variety of stimuli such as osmotic shock, inflammatory cytokines, lipopolysaccharides (LPS), UV light and growth factors, and Rho GTPases activate the p38 arm of the MAPK pathway. MAPKKKs; mixed lineage kinase 3 (MLK3), TGF-beta activated kinase 1 (TAK), delta-like-1 (DLK1), MEKK4, or Apoptosis Signal-Regulating Kinase 1 (ASK1) activates the MAPKKs; MKK3, MKK4 or MKK6, which activates the MAPK p38. MKK3 and MKK6 activate p38 exclusively while MKK4 also activates SAPK/JNK (Cuenda, 2000; Enslen et al., 1998). All three MKKs activate all 4 isoforms of p38 except for MKK3 which does not activate p38β. p38 activates many downstream targets to promote apoptosis (if there are high levels of p38) or arrest (if there are lower levels of p38) such as MEF2C, ATF1, CHOP, ELK-1, p53, p73, and MAPK2 (which activates Hdm2).

p38 was discovered in 1994 as a tyrosine phosphoprotein in extracts of cells treated with inflammatory cytokines. At the same time it was discovered as a target of a pyridinyl imidazole drug that blocked production of tumor necrosis factor (TNF) and as such was briefly called cytokine-suppressive anti-inflammatory drug binding protein or CSBP. In parallel, it was found in a screen as a kinase for the substrate MAPK2 (Han et al., 1994; Lee et al., 1994; Pearson et al., 2001). There are four isoforms of p38 which share 60% amino acid sequence homology. p38α and p38β are ubiquitous, p38γ is found in skeletal tissue, and p38δ is expressed in lung, kidney, pancreas, placenta, and testis. While p38α knockout mice are embryonic lethal, knockouts of the other isoforms are viable. p38 resides in both the cytoplasm and nucleus but does not have a nuclear localization signal (Ben-Levy et al., 1998). Wip1/PPMID is a phosphatase for p38 and is a downstream target of p53 and E2F1 (Takekawa et al., 2000). p38 is overexpressed in many cancers (Yu et al., 2007).

1.13 Melanoma cells as a model system to study the role of Rb during stress

In this dissertation, we use melanoma cells as a model system to study the Rb pathway. Melanocytes are neural crest derived cells located in the basal layer of the skin, the uvea of the eye, the inner ear, the meninges, bones, and in the heart (Bennett, 1993). Melanocytes are spindle-shaped cells that produce the pigment melanin. Melanoma, the cancer resulting from malignant transformation of melanocytes, occurs most often in the cutaneous form, but also can occur in the uveal tract of the eye, mucosal tissues, and other sites (Curtin et al., 2005; Harbour, 2003; Harbour, 2006; Rager et al., 2005; Sirsat, 1952).

Melanoma cells are a useful system to study Rb because they undergo apoptosis under Rb null conditions and therefore exemplify the central role of Rb in cell survival (Bennett and Medrano, 2002; Macleod et al., 1996). The Dowdy lab demonstrated that ablation of RB in melanocytes by cutaneous application of Tat-Cre in LoxP-Rb mice causes selective apoptotic loss of melanocytes (Yu et al., 2003). Acute inactivation of Rb in vivo by conditional mutagenesis in the skin resulted in the selective loss of melanocytes and severe depigmentation, indicating that Rb has a cell-autonomous role in melanocyte survival (Halaban, 1999; Yu et al., 2003). Indeed, $p16^{Ink4a}$ is frequently mutated in familial melanoma, resulting in hyperphosphorylation and inactivation of Rb, and individuals with RB gene mutations are at increased risk for melanoma (Begg et al., 2005; Halaban, 1999; Luca et al., 1995; Reed et al., 1995; Reymond and Brent, 1995). Virtually all mouse models of melanoma require inhibition of Rb, such as by overexpression of large T antigen or by inactivation of $p16^{Ink4a}$ (Castellano and Parmiani, 1999; Yu et al., 2003). Additionally, mutations in the RAS-RAF-MAPK pathway, which inhibit Rb through activation of cyclin D, are common in melanocytic tumors (Gupta et al., 2005).

Cutaneous melanoma is one of the most common and one of the most deadly types of cancer, spreading throughout the lymphatic system. It is estimated that there will be almost 70,000 new cases of melanoma in 2010 and almost 9,000 deaths (ACS, 2010). One of the most common

mutations found in melanoma is in BRAF. BRAF mutations occur in over 50% of cutaneous melanomas with 80% of these mutations being a V599E activating mutation (Davies et al., 2002). Raf is a part of the Ras-Raf-Mek-Erk-MAP kinase pathway and mutated BRAF is capable of transforming NIH3T3 cells. Over 25% of cutaneous melanomas harbor mutations in NRAS (Saldanha et al., 2006).

Uveal melanoma is the most common cancer of the eye and accounts for about 4% of melanomas (Harbour, 2006; Onken et al., 2004). The iris, ciliary body, and choroid make up the uveal tract of the eye (**Figure 1.5**). While uveal melanoma is rare, it is highly resistant to radiation and chemotherapy and is therefore very difficult to treat. Additionally, 50% of uveal melanoma cases metastasize hematogenously to the liver at which point the median survival is less than 6 months (Gragoudas et al., 1991). It is estimated that there will be over 200 deaths due to eye cancer and almost 2,500 new cases in 2010 in the United States (ACS, 2010). The common NRAS and BRAF mutations that occur in cutaneous melanoma do not occur in the uveal form of the disease (Cruz et al., 2003). While, no mutations have been found in the RAF/MEK/ERK pathway in uveal melanoma, mutations in GNAQ, an activator of this pathway occur in about 50% of uveal melanomas (Bauer et al., 2009; Gerami et al., ; Onken et al., 2008; Van Raamsdonk et al., 2009; Van Raamsdonk et al., 2004). Like NRAS and BRAF mutations in cutaneous melanoma, GNAQ mutations are most likely an early event as they do not correlate with survival (Onken et al., 2008).

1.14 Role of Rb in melanocyte differentiation

Rb plays a role in differentiation and the tumor suppressor function of Rb appears to be due, at least in part, to its ability to induce permanent cell cycle exit in association with senescence and differentiation (Dannenberg et al., 2000). The classic example of the role of Rb in differentiation is muscle cell differentiation in which Rb cooperates with the muscle-specific transcription factor MyoD, to activate genes involved in myocyte differentiation (Gu et al., 1993; Sellers et al., 1998). Our lab has shown that melanocyte differentiation is linked to cell cycle exit through activation of

Rb by the melanocyte differentiation factor microphthalmia-associated transcription factor (Mitf) (Loercher et al., 2005). Mitf interacts directly with and activates the INK4A gene, causing an accumulation of hypophosphorylated Rb and cell cycle arrest. This Mitf-Ink4a-Rb pathway is required for efficient cell cycle exit and melanocyte differentiation in cultured cells and in vivo. Rb may also interact directly with Mitf to co-activate other Cdk inhibitors such as $p21^{Cip1}$, which in turn re-enforce hypophosphorylation of Rb (Carreira et al., 2005). Rb is also important for maintenance of the differentiated state. Our lab found that cells induced to exit the cell cycle and to differentiate into melanocytes as a result of enforced Mitf expression occasionally escaped this growth inhibition, and these escape clones invariably exhibited methylation and inactivation of the INK4A promoter, resulting in hyperphosphorylation of Rb (Loercher et al., 2005). Thus, inhibition of Rb appears to be a key step in melanoma formation by allowing melanocytes to re-enter the cell cycle.

1.15 Dissertation goals

Throughout the years Rb has been portrayed as acting as a simple, binary "on-off" switch (Buchkovich et al., 1989; Chen et al., 1989; Coller, 2007; DeCaprio et al., 1989). When Rb is "on" it is active and hypophosphorylated, allowing it to bind and inhibit E2Fs that activate cell cycle genes. Conversely, Rb is inactivated, or "turned off" by hyperphosphorylation during every cell cycle, releasing E2Fs to activate cell cycle genes. There are several important weaknesses with this model. The release of E2Fs during cell cycle progression described in this model would also promote an apoptotic response, since free E2F promotes apoptosis (Phillips et al., 1997; Young and Longmore, 2004b). Indeed, recent work suggests that E2Fs are not completely released from Rb during the cell cycle (Young and Longmore, 2004b). Phosphorylated Rb and E2F co-IP in cycling cells (Ezhevsky et al., 1997). The Farnham lab has extensively demonstrated, using chromatin immunoprecipitation experiments (chIP), that pocket protein-E2F complexes persist at many E2F-responsive promoters well beyond the G_1/S transition (Ezhevsky et al., 2001; Ezhevsky et al., 1997; Wells et al., 2000; Wells et al., 2003). When Rb is partially phosphorylated by Cdk4 to allow cell cycle progression, it retains the ability to bind E2Fs and to repress

transcription of some genes (Ezhevsky et al., 2001; Ezhevsky et al., 1997; Wells et al., 2000; Wells et al., 2003).

Next, this model does not distinguish between the different phenotypes associated with Rb inactivation versus Rb deletion. There are several lines of evidence revealing that these states are functionally different. The apoptotic response that accompanies deletion or complete inactivation of Rb is not seen when Rb is functionally inhibited by phosphorylation (Harbour and Dean, 2000b, Shackney, 1999). For example, the massive apoptosis that occurs in RB-null mice does not occur in INK4A-null animals (Serrano et al., 1996), even though this genetic perturbation would be expected to maintain Rb in a hyperphosphorylated state. Similarly, RB-null cancer cells (such as retinoblastoma and small cell lung cancer) generally exhibit a high rate of spontaneous apoptosis, rapid cell division, and sensitivity to chemotherapy whereas RB-positive cancer cells that maintain Rb in a hyperphosphorylated state (such as melanomas) have a much lower rate of apoptosis, lower rate of proliferation, and are more resistant to chemotherapy (Shackney and Shankey, 1999).

Finally, this model does not explain how Rb regulates both the cell cycle and apoptosis. Indeed, a major unsolved question in the field has been how one protein, Rb, could control these opposing cell fates. Experiments by Jean Wang's lab suggest that the cell cycle and apoptotic functions of Rb may be distinct. Mice with a mutation in the Rb caspase cleavage site still retain tumor suppressor activity (Tan and Wang, 1998). Furthermore the Wang lab made a mutant in which Rb could not longer arrest the cell cycle but could regulate apoptosis (Chau et al., 2006).

In our work, we have addressed this question from a different perspective. The following chapters reveal a novel mechanism by which the cell cycle and apoptotic functions of Rb are differentially regulated (Delston et al., 2010). In Chapter 2, we investigate the p38-Rb signaling axis. Our data reveal that p38 phosphorylates Rb on Ser567, a pocket residue, through a novel mechanism leading to apoptosis in a p53- and E2F1-dependent manner. In Chapter 3, we

demonstrate the mechanism behind Rb-mediated apoptosis. We found that phosphorylation of Rb on Ser567 leads to Hdm2-mediated proteosomal degradation of Rb and cell death. In Chapter 4, the dissertation is summarized and future directions are proposed.

1.16 Figures

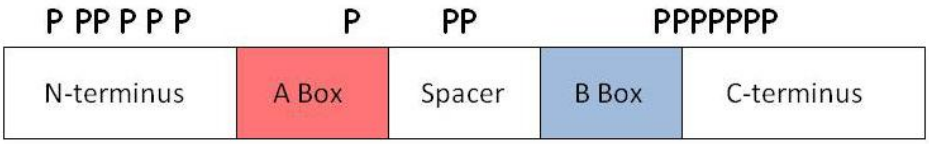

Figure 1.1 Phosphorylation sites on Rb

There are 16 Serine or Threonine phosphoacceptor sites located throughout the Rb protein: Thr5, Ser230, Ser249, Thr252, Thr356, Thr373, Ser567, Ser608, Ser612, Ser780, Ser788, Ser795, Ser807, Ser811, Thr821, and Thr826.

Figure 1.2 Picture of retinoblastoma tumor

The white eye seen in this photograph can be a symptom of retinoblastoma. The tumor blocks the light of the flash from reaching the retina. Image from J. William Harbour.

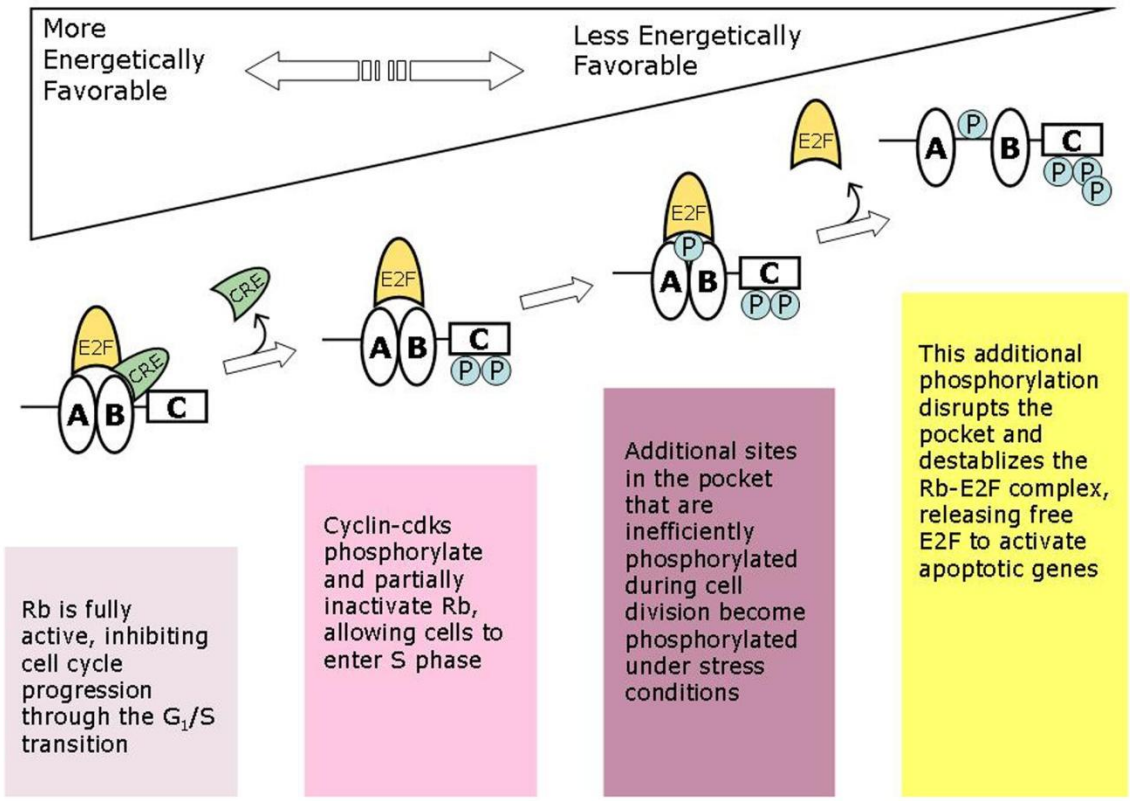

Figure 1.3 A model for sequential phosphorylation of Rb

Rb phosphorylation occurs in a stepwise and hierarchical fashion. Hypophosphorylated Rb arrests the cell cycle by forming multimeric complexes with E2F and chromatin remodeling enzymes (CRE). Partial phosphorylation by cyclin-Cdk complexes results in partial Rb inhibition to allow cell cycle progression. During abnormal stress conditions, further sites are phosphorylated, such as Ser567, which is efficiently phosphorylated only when Cdk activity is abnormally high or when stress kinases are activated. Phosphorylation of this site favors apoptosis over cell division by releasing free E2F to activate apoptotic genes. We hypothesize that this differential phosphorylation may allow Rb to buffer cells against apoptosis during normal cell division but to serve as a death checkpoint that triggers apoptosis in stress conditions. Adapted from (Delston and Harbour, 2006).

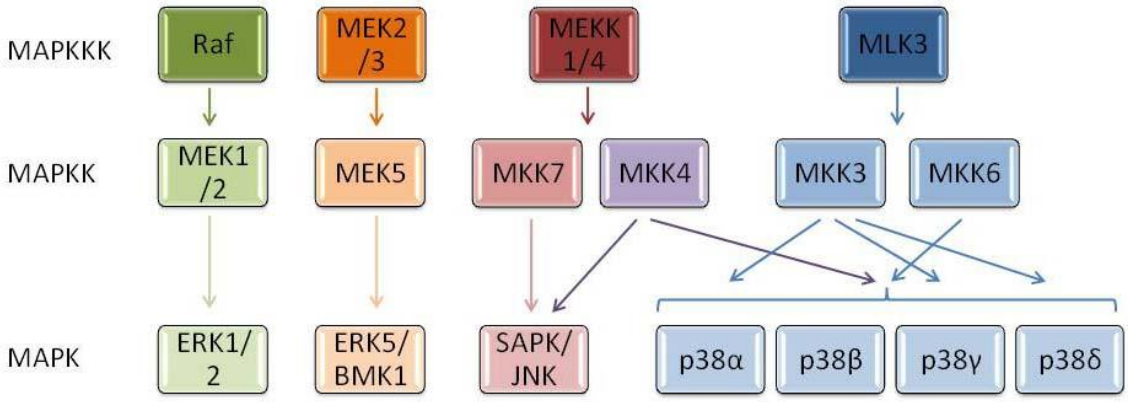

Figure 1.4 The MAPK pathway

There are four major arms of the MAPK family; ERK1/2, ERK5/BMK1, SAPK/JNK, and p38. MAPKs utilize a signaling cascade in which a biological response is elicited by a MAPK which is activated by a MAPK kinase (MAPKK), which in turn is activated by a MAPK kinase kinase (MAPKKK), which is activated by a variety of stimuli.

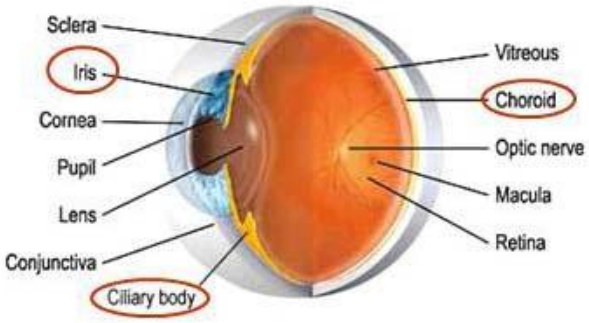

Figure 1.5 Diagram of the eye

Image from J. William Harbour. The iris, choroid, and ciliary body make up the uveal tract of the eye.

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Chapter 2

p38 phosphorylates Rb on Ser567 by a novel mechanism in response to genotoxic stress

2.1 Abstract

Rb is a tumor suppressor that regulates cell division, differentiation, and apoptosis. Rb was first identified as regulator of the G_1-to-S cell cycle transition; however, it later became evident that Rb also plays a role in blocking apoptosis. While the role of Rb in the cell cycle has been extensively studied, the role of Rb in apoptosis and the mechanism by which Rb coordinately regulates these two cellular outcomes remains poorly understood. Cyclin-Cdks phosphorylate Rb on the C-terminus, allowing for cell cycle progression however, the kinases and phosphorylation sites involved in apoptosis are less clear. In order to investigate the response of Rb to stress, we developed a model system in which we treated melanoma cells with a DNA damaging reagent. We found that p38 phosphorylates Rb on Ser567 in response to genotoxic stress, by a novel mechanism, distinct from Cdk phosphorylation of Rb. This phosphorylation event results in cell death through p53- and E2F1-dependent pathways. These findings provide a mechanistic explanation for how Rb differentially regulates cell division and apoptosis.

2.2 Introduction

The current model of Rb function focuses on the role of Rb in the cell cycle and does not address the role of Rb in regulating apoptosis. The existing model posits Rb as an "on-off" switch (Buchkovich et al., 1989; Chen et al., 1989; Coller, 2007; DeCaprio et al., 1989). This model asserts that when Rb is "on" it is completely unphosphorylated and bound to E2Fs in order to actively halt the cell cycle. When Rb is "off" it is fully phosphorylated and these events release its hold on E2Fs which are now free to transactivate cell cycle genes to promote cell division. This oversimplified model does not take a key piece of information into account; it is known that free E2F transactivates pro-apoptotic genes (Young and Longmore, 2004b). According to this model, pro-apoptotic genes would be transcribed with every round of the cell cycle leading to a very inefficient means of division. Additionally, although the current model asserts that phosphorylated Rb does not bind E2Fs, there is evidence to the contrary. Partially phosphorylated forms of Rb do indeed bind E2Fs (Ezhevsky et al., 2001; Ezhevsky et al., 1997), and Rb continues to co-localize with E2Fs at certain promoters in vivo beyond the G_1/S transition despite being sufficiently phosphorylated to allow for cell cycle progression (Wells et al., 2000; Wells et al., 2003).

To address these unresolved issues we propose an updated model which takes Rb's role in apoptosis into account. Instead of only having two states; phosphorylated or unphosphorylated, this model envisions Rb as existing with multiple phosphorylation states, that have differing abilities to bind E2Fs and other proteins, and to inhibit cell division and apoptosis (Lundberg and Weinberg, 1998; Ma et al., 2003). Work from our lab and others support the idea that different phosphorylated forms of Rb allow the cell to differentially control cell cycle genes and apoptotic genes, the former being derepressed by partial phosphorylation of Rb and the latter being activated by more complete phosphorylation of Rb (Young and Longmore, 2004a; Young et al., 2003; Zhang et al., 2000). Taken together, these observations indicate that the decision between proliferation and apoptosis is made, at least in part, by the manner in which Rb is phosphorylated.

Previously, Cdks were the only kinases known to phosphorylate and regulate Rb under physiologic conditions, but recently other kinases have been shown to phosphorylate Rb. In particular, p38 phosphorylates Rb in response to cellular stress, such as DNA damage and death receptor signaling, in multiple cell types, including endothelial cells, cerebellar neurons, Jurkat lymphocytic cells, colon cancer cells, and melanoma cells (Dasgupta et al., 2004; Kishore et al., 2003, Yeste-Velasco, 2009, Nath, 2003; Lee et al., 2003; Pillaire et al., 2000). Phosphorylation of Rb by Cdks, which occurs during normal cell division and in proliferating cancer cells, does not lead to apoptosis, at least in part, because Rb is only partially inactivated and can still repress pro-apoptotic E2Fs (Wells et al., 2003; Young et al., 2003). Cdk-mediated phosphorylation is reversible, allowing Rb to be dephosphorylated as cells complete the cell cycle. In contrast, we found that p38-mediated phosphorylation leads to degradation of Rb, release of E2Fs, and activation of pro-apoptotic genes.

Researchers have focused their attention on the phosphoacceptor sites on the C-terminus of Rb which play a role in cell cycle regulation. However, several lines of evidence point to Ser567 as playing an important role in Rb regulation. Ser567 is the only phosphoacceptor site on the A box of the pocket domain of Rb and is located at a critical location at the interface of the A and B

boxes and near an E2F binding site (**Figure 2.1**). The Ser567-Pro568 phospho-acceptor motif is highly conserved across species (Ma et al., 2003). Ser567 is the only phosphorylation site on Rb that is a target of naturally occurring mutations in human retinoblastoma, suggesting that it plays a different role than the other 15 phosphorylation sites (Templeton et al., 1991; Yilmaz et al., 1998). Strikingly, when Rb is phosphorylated on Ser567 the A and B boxes of the pocket domain dissociate (Harbour et al., 1999).

In order to investigate the role of Rb in a setting of genotoxic stress, we established a model system of etoposide-treated melanoma cells. Melanoma cells are an ideal model system as Rb is essential for differentiation and survival of melanocytes (Halaban, 1999; Yu et al., 2003). We used melanoma cells called Mel202s which have been extensively characterized. They are Rb and p53 wildtype and have a spindle morphology (Sun et al., 2005 1991). Mel202s were isolated from a uveal melanoma tumor and stop dividing at around 30 passages (Ksander et al., 1991). Since there was no satisfactory model in which to study the role of Rb during stress in melanoma cells, we decided to use etoposide, a DNA damaging reagent used in cancer treatments. Etoposide inhibits topoisomerase II, cuts double stranded DNA, activates the Cdk inhibitor $p21^{Cip1}$, and effects cells in a similar manner as ionizing radiation (Sun et al., 2005).

Loss of Rb can lead to p53-dependent or p53-independent apoptosis (Jacks et al., 1992; Lee et al., 1992; Macleod et al., 1996; Morgenbesser et al., 1994; Wu et al., 2003). Free E2F can stabilize p53 through $p14^{ARF}$ or ATM/CHK1/CHK2 allowing for activation of pro-apoptotic p53 target genes (Engelmann and Putzer, ; Wu and Levine, 1994). However, E2Fs can also promote p53-independent apoptosis through activation of p73 or BH3-only proteins such as Bid, Bim, and Puma (Engelmann and Putzer, ; Hsieh et al., 1997; Phillips et al., 1997). This finding was demonstrated both in vitro in Saos-2 cells which are p53 null and still underwent apoptosis (Phillips et al., 1997) and in vivo, as deletion of Rb in p53 null mice led to E2F1-induced apoptosis (Macleod et al., 1996). We investigated the roles of E2F1 and p53 in our system and found that apoptosis was E2F1- and p53-dependent.

We sought to determine mechanistically how phosphorylation of Rb by p38 differs from that of Cdks and why this results in such a dramatically different physiologic effect (apoptosis versus cell division, respectively). We show that residue Ser567 on Rb is preferentially phosphorylated by p38, but not Cdk2, in response to genotoxic stress. While C-terminal phosphorylation of Rb by Cdks results in cell cycle progression, we found that Ser567 phosphorylation of Rb by p38 leads to E2F1- and p53- mediated cell death.

2.3 Methods

Cell culture

Mel202 uveal melanoma cells were provided by B. Ksander (Harvard Medical School, Boston, MA, USA). Mel501 cutaneous melanoma cells were provided by L. Cornelius (Washington University, St. Louis, MO, USA). Mel202 and Mel501 cells were cultured in complete RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Sigma Aldrich, St. Louis, MO, USA), L-glutamine/antibiotics/anti-mycotics (Mediatech, Manassas, VA, USA) for no more than 30 passages.

Primary uveal melanocytes and melanoma cells were collected by J. William Harbour at the time of enucleation and maintained by O. Agapova. Samples were collected in HAM'S F-12 medium, incubated in trypsin and collagenase, and grown at 4% oxygen on collagen-covered tissue culture plates in HAM's F-12 (Invitrogen) supplemented with 10% BSA (Sigma), SITE supplement (Sigma), B27 supplement (Invitrogen), bFGF (PeproTech, Rocky Hill, NJ, USA), L-glutamine (Sigma), gentamicin (Sigma) and fungizon. Primary melanocytes and melanoma cells, Mel202s, and Mel501s were grown under 5% carbon dioxide and 4% oxygen.

U2OS osteosarcoma and C33A cervical cancer cells were grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Sigma), L-glutamine/antibiotics/anti-mycotics (Mediatech) under conditions of 5% carbon dioxide and 21% oxygen.

Inhibitors, constructs, and siRNA

When indicated, cells were treated with etoposide (Sigma), Cdk2 inhibitor (Roscovitine, Sigma), p38 inhibitor (SB 203580 Millipore, Billerica, MA, USA), and Cdk4 inhibitor (EMD, Darmstadt, Germany). Transfections of expression plasmids were performed using Effectene or Attractene (Qiagen, Germantown, MD, USA). Transfections of siRNA were performed using HiPerFect

(Qiagen). Validated siRNA against p38α, p38β, and E2F1 (Ambion, Austin, TX, USA), and p53 (Thermo Fisher Scientific, Waltham, MA, USA), were used. siRNA against Rb was designed using an algorithm previously described (Elbashir et al., 2001). A fluorescently tagged scrambled siRNA was used as a negative control (Allstars Neg siRNA AF488. Qiagen 1027284). SKTT was used as a control in several experiments. pCMV, pcDNA3, RC-CMV, RC-Cyclin D, RC-Cyclin E, p53, CD20, Rb, RbΔ567, RbΔ568, RbΔ780, RbΔ7, and E2F1, expression plasmids were used (Harbour et al., 1999; Ma et al., 2003). RSV2 and RSV2-MKK3 were provided by R. Davis (UMass Medical School, Worcester, MA, USA). The constitutively active p38 expression plasmid was provided by D. Engelberg (The Hebrew University of Jerusalem, Jerusalem, Israel) (Askari et al., 2009). Adenovirus expressing GFP (Ad-GFP) was provided by A. Samarel (The Cardiovascular Institute, Loyola University Chicago Stritch School of Medicine, Maywood, Illinois, USA) (Heidkamp et al., 2005). Adenovirus expressing LacZ (Ad-LacZ) was provided by T. Kuroki (Showa University, Hatanodai, Shinagawa-ku, Tokyo, Japan) (Ohba et al., 1998). Adenovirus expressing MKK3 (Ad-MKK3) was provided by Y. Wang (University of California, Los Angeles, Los Angeles, CA, USA) (Wang et al., 1998). Ad-cycD1 was provided by H. Piwnica-Worms (Washington University).

Antibodies

Immunoblot analysis, immunoprecipitations and immunofluorescence staining were performed as previously described (Ma et al., 2003). Bands were quantified using densitometry (Adobe Photoshop CS4 extended version 11.0). Antibodies against total Rb (IF8), E2F1, and total p53 were used (Santa Cruz Biotechnology, Inc Santa Cruz, CA, USA). Antibodies against Rb-phospho-567 were obtained from Santa Cruz and Bethyl Laboratories (Montgomery, TX, USA) as previously described (Ma et al, 2003). Antibody against GST was from Bethyl Laboratories. Antibodies against cleaved PARP, phospho-p38, p38 total, cyclin D1, Rb-phospho-807-811, Rb-phospho-780, Rb-phospho-795, and cleaved caspase 3 were from Cell Signaling Technology (Danvers, MA, USA). Antibody against α-tubulin was from Sigma Aldrich. α-tubulin was used as a loading control in immunoblots.

Cell viability assays

Cell viability assays were performed with methyltetrazolium sulfate (MTS) using the CellTiter 96 AQueous kit (Promega, Madison, WI, USA). Mel202 cells were plated into 96-well culture plates at 10^4 cells per well on the day prior to the assay. Dilute MTS was applied to each well and allowed to incubate at 37°C for 4 hours according to manufacturer's protocol (Bio-Rad, Hercules, CA, USA). Cell viability was determined by relative conversion of tetrazolium pigment, as detected by $Absorbance_{490}$ measured on an ELISA microplate reader, as compared to cells under control conditions.

Flow Cytometry

To determine the cell cycle profile, cells were collected by keeping the supernatant and combining with trypsinized cells. Cells were washed in phosphate buffered saline (PBS) and fixed in 70% ethanol overnight at 4 degrees. The next day cells were washed in PBS and incubated in DNA staining buffer (PBS pH 7.4 Triton X-100 0.1%, EDTA pH 7.4 0.5 mM, RNAse A 0.05 mg/ml) and propidium iodide 50 µg/ml for 30 min at room temperature in the dark. Cells were analyzed

by flow cytometry using a Beckman Coulter FC500 (Beckman Coulter Inc., Brea, CA, USA) and analyzed using CXP v2.1 software (Beckman Coulter Inc.). For cells transfected with CD20 which is expressed on the membrane and Rb constructs CD20 FITC (Abcam, Cambridge, MA, USA) was added to trypsinized cells and incubated for 30 min, before DNA staining buffer was added.

Luciferase assays

Luciferase assays were performed using the Luciferase Assay System (Promega, Madison, WI, USA) with an E2F luciferase reporter construct (Panomics, Fremont, CA, USA) or cyclin E promoter luciferase reporter construct (Ma et al., 2003) according to the manufacturer's instructions.

In Vitro Kinase Assays

In vitro kinases assays were performed using purified phospho-p38α kinase, purified cyclin E-Cdk2 kinase, or purified cyclin D1-Cdk4 kinase all from Cell Signaling. Kinase assays were also performed using endogenous immunoprecipitated kinase from cells using IgG antibody (Sigma Aldrich) or phospho-p38 antibody (Cell signaling). GST-tagged Rb small pocket domain with all sites mutated except for Ser567 (GST-Rb-SPΔ4) or the C-terminus of Rb (GST-Rb-C) were purified and used as substrates. 0.5 µg of purified full length Rb was also used (QED Bioscience Inc, San Diego, CA, USA). Kinase assays were performed by first pre-incubating the kinase with GST-Rb-C for 45 min and then adding in the GST-Rb- SPΔ4 for an additional 45 min in a 40 µl kinase buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 150 µM ATP, 100 µCi of γ ^{32}P-ATP). GST-beads (Sigma Aldrich) were added for 30 min, rotating at room temperature. Beads containing phosphorylated substrate were washed twice for 5 min each using incomplete kinase buffer.

TUNEL

TUNEL staining was performed using the In Situ Cell Death Detection Kit (Roche Diagnostics Corporation, Basel, Switzerland). Cells were plated on Permonox Lab-Tek Chamber Slides (Elextron Microscopy Sciences, Hatfield, PA, USA). To analyze the slides, wells were washed with PBS, fixed with 4% paraformaldehyde/PBS (pH 7.4) for 30 min at room temperature, washed with PBS, permeabilized with 0.1% Triton X–100, 0.1% sodium citrate) for 5 min on ice, washed with PBS, incubated with TUNEL reagent for 1 h at 37 degrees, washed with PBS, mounted with Vectashield Hardset with DAPI (Vector Laboratories, Inc, Burlingame, CA, USA), and a cover slip was applied. Pictures were taken with an Olympus Fluorescence BX51 microscope and data was analyzed using image J software.

2.4 Results

Loss of Rb triggers apoptosis in melanocytes

It is important to distinguish between inactivation of Rb by partial hyperphosphorylation and loss of Rb. RB is rarely deleted in cancers, yet Rb is hyperphosphorylated in virtually all tumors. Most melanomas overexpress cyclin D1, which functionally inactivates Rb without causing apoptosis. Interestingly, cancers in which RB is deleted such as retinoblastomas have large amounts of apoptosis (Harbour, 2006). Loss of Rb leads to apoptosis in many tissues, suggesting that there is an inherent pressure for organisms to eliminate aberrant cells that lack Rb (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992).

Functional and reversible inactivation of Rb by Cdk-mediated hyperphosphorylation, as occurs during normal cell division and in most proliferating cancer cells, does not induce cell death in melanoma cells (**Figure 2.2 A**). In contrast, complete and irreversible inactivation of Rb by RNAi-mediated depletion of the protein induced cell death both in Mel202 cells and in primary melanoma cells (**Figure 2.2 A-C**). This is consistent with previous work demonstrating that specifically knocking out Rb from cells such as melanocytes or IMR90 human fibroblasts led to cell death (Young and Longmore, 2004b; Yu et al., 2003).

Genotoxic stress induces apoptosis in melanoma cells

To explore why these mechanisms for inactivating Rb that have been assumed to be functionally equivalent result in such strikingly different phenotypic outcomes, we developed an experimental model system in which Rb degradation and apoptosis were induced by genotoxic stress in Mel202 cells, which have been extensively characterized and shown to be wildtype for Rb and p53 (Sun et al., 2005). At low concentrations, the DNA damaging agent etoposide resulted in stabilization of p53, *hypo*phosphorylation of Rb, and cell cycle arrest as expected (**Figure 2.**. We wondered if slightly higher concentrations of etoposide would continue to arrest cells or lead to cell death. Growth curves and BrdU assays revealed that over time etoposide halted proliferation (**Figures 2.4 and 2.5**). However, it was still unclear if the cells were arresting or dying. To address this question, we investigated markers of cell death. Cleavage of caspase 3, an indicator of initiation of apoptosis, was increased in etoposide-treated cells (**Figure 2.6 A**). Furthermore, PARP cleavage, a hallmark of apoptosis, was increased over time with etoposide treatment (**Figure 2.6 B**). MTS assays revealed that etoposide treatment led to death (**Figure 2.6 C**). Finally, the sub G_1 (the apoptotic) population of cells increased with etoposide treatment when propidium iodide was used to study the cell cycle (**Figure 2.6 D**). Similar results were obtained using low passage uveal melanocytes from two different patients (**Figure 2.7**) and U2OS osteosarcoma cells (**Figure 2.8**). Taken together, these findings reveal that while low levels of genotoxic stress result in arrest, higher levels induce apoptosis.

Genotoxic stress leads to hyperphosphorylation of Rb

Next, we investigated the phosphorylation status of Rb during etoposide treatment to determine if etoposide leads to hypophosphorylation or hyperphosphorylation of Rb. We initially hypothesized that etoposide would lead to hypophosphorylation of Rb because at low levels etoposide induces p53 phosphorylation, causing $p21^{Cip1}$ upregulation, which results in reversible hypophosphorylation of Rb and cell cycle arrest (Strobeck et al., 2000; Valentini et al., 2007). Using higher levels of etoposide, we found unexpectedly that apoptosis was accompanied not by

hypophosphorylation of Rb, but instead hyperphosphorylation of Rb in a dose-dependent manner (**Figure 2.9 A**). We confirmed that this hyperphosphorylation of Rb occurred not only in the Mel202 cells but also in uveal melanocytes cells (**Figure 2.9 B**). While low levels of genotoxic stress lead to hypophosphorylation of Rb and cell cycle arrest, we found that increased genotoxic promotes hyperphosphorylation of Rb.

Genotoxic stress triggers phosphorylation of Rb by p38

To determine which kinase catalyzes the phosphorylation of Rb in etoposide-treated melanoma cells we started by evaluating Cdks that phosphorylate Rb during cell division. Inhibitors of Cdk2 and Cdk4 did not block Rb hyperphosphorylation induced by etoposide (**Figure 2.10 A-B**). The Cdk2 inhibitor was able to inhibit serum starvation induced hyperphosphorylation confirming that the inhibitor was active (**Figure 2.10 C**). Additionally, kinase assays were performed to confirm that the Cdk2 and Cdk4 inhibitors were active (**Figure 2.10 D-E**). This led us to hypothesize that p38, a MAPK member which is activated by etoposide might mediate this phosphorylation (Olson and Hallahan, 2004; Pillaire et al., 2000). In recent years, p38 has been shown to phosphorylate Rb in response to stress and death receptor signaling in multiple cell types, for example in irradiated prostate cancer cells (Bowen et al., 2002), in sodium salicylate-treated colon cancer cells (Lee et al., 2003), in FasL-induced cerebellar granule neurons (Hou et al., 2002), in Jurkat lymphocytic cells (Wang et al., 1999) and in Fas-induced murine hepatocyte AML12 cells (Cho et al., 2010). First, we investigated whether or not p38 phosphorylated Rb in our system of etoposide-stressed melanoma cells. SB 203580 [4-(4´-fluorophenyl)-2-(4´-methylsulfinylphenyl)-5-(4´-pyridyl) imidazole], which acts as a competitive inhibitor of ATP binding, specifically inhibits p38α and p38β. It has no significant effects on ERKs, JNKs, p38γ or p38δ at the concentrations used (Goedert et al., 1997; Kuma et al., 2005). The interaction between SB 203580 and p38α and p38β is due to its specificity for residue Thr106 near the ATP-binding pocket which is replaced by a bulky methionine residue in p38γ and p38δ (Cuadrado and Nebreda). SB 203580 potently inhibited Rb phosphorylation suggesting that p38 is responsible for phosphorylating Rb during genotoxic stress (**Figure 2.11 A**)**.** We confirmed that the p38 inhibitor was working

(**Figure 2.10 C and Figure 2.11 B**). Since SB 203580 inhibits both p38α and p38β we used RNAi to determine which isoform of p38 was phosphorylating Rb in our system. siRNA-mediated knock down of p38α, but not p38β, inhibited hyperphosphorylation of Rb suggesting that p38α phosphorylates Rb in response to genotoxic stress (**Figure 2.11 C**). Consistent with these findings, p38 underwent activating phosphorylation during etoposide treatment (**Figure 2.11 D**). Importantly, chemical inhibition of p38 substantially reduced etoposide-induced apoptosis by flow cytometry (**Figure 2.12 A**) and by TUNEL (**Figure 2.12 B**). Taken together, these data suggest that during genotoxic stress p38 phosphorylates Rb, ultimately leading to apoptosis.

Activation of p38 by MKK3 leads to hyperphosphorylation of Rb and cell death

Etoposide induces global stress which activates many signaling pathways such as caspases, PI3K/Akt, myc, ERK1/2, ATM, and NF-kB (Benjamin et al., 1998; Fu et al., 2008b; Liu et al., 2006; Morotti et al., 2006). Since we determined the p38 pathway plays a role in Rb-mediated apoptosis, we next directly activated p38 in order to rule out any off-target effects that may be caused by etoposide treatment. MKK3, a MAPKK, is a highly specific direct upstream activator for p38α, p38γ, and p38δ, but not p38β (Cuadrado and Nebreda). MKK3 specifically activates p38 by dual phosphorylation of the Thr-X-Tyr motif. Adenoviral activation of endogenous p38 by Ad-MKK3 led to hyperphosphorylation of Rb, which was inhibited by a p38 inhibitor and was similar to the effect of etoposide treatment (**Figure 2.13 A-B**). Furthermore, MKK3-, but not cyclin D1--treated cells underwent increased apoptosis as shown by flow cytometry (**Figure 2.13 C**). Therefore, direct activation of endogenous p38 by expression of the upstream effector MKK3 triggers phosphorylation of Rb and induces apoptosis in a manner that is indistinguishable from etoposide treatment.

p38 phosphorylates Rb on Ser567 during genotoxic stress

We sought to determine which site on Rb was being phosphorylated during genotoxic and p38-mediated stress. Cyclin-Cdks were not responsible for phosphorylating Rb during stress and so we hypothesized their corresponding C-terminal phosphoacceptor sites would also not be

involved. Instead, we turned to a pocket site, Ser567, as it is the only site on Rb that is not phosphorylated during the normal cell cycle (Lees et al., 1991). An Rb Ser567-phospho-specific antibody showed that this site was phosphorylated in response to etoposide (**Figure 2.10 A**) and Ad-MKK3 (**Figure 2.13 A**). A p38 inhibitor (**Figures 2.11 A** and **2.13 A**), but not a Cdk2 inhibitor, (**Figure 2.10 A**) inhibited phosphorylation of Ser567. This suggests that p38, but not cyclin-Cdks, is involved in phosphorylation of Ser567 on Rb in response to genotoxic stress.

Ser567 on Rb plays a unique role during genotoxic stress-induced apoptosis

We wondered if phosphorylation of Rb on Ser567 played a role in genotoxic stress-induced apoptosis. Flow cytometry was performed using a mutant in which seven phosphoacceptor sites; Ser780, Ser788, Ser795, Ser807, Ser811, Thr821, and Thr825 were mutated to alanines (RbΔ7), leaving only three remaining functional phosphoacceptor sites; Ser567, Ser608, and Ser612. Etoposide treatment of RbΔ7 expressing cells led to apoptosis, evidence that the C-terminal sites are not important during etoposide-induced apoptosis (**Figure 2.14**). Next, we used a mutant that would block phosphorylation of Ser567. The Ser567 phosphoacceptor site mutant in which Ser567 is mutated to an alanine (RbΔ567) is unique in that it mimics, rather than blocks, Ser567 phosphorylation. This mutant could not be used to assess how blocking Ser567 phosphorylation affects Rb function. Instead, we used an Rb mutant in which proline568 was substituted with an alanine (RbΔ568), which remains partially active while blocking Ser567 phosphorylation (Harbour et al., 1999; Ma et al., 2003). Ectopic expression of RbΔ568, but not wildtype Rb or an Rb mutant in which Ser780, a C-terminal site, was substituted with an alanine preventing phosphorylation (RbΔ780), inhibited apoptosis in cells treated with etoposide (**Figure 2.15 A**) and cells treated with Ad-MKK3 (**Figure 2.15 B**). Phosphorylation of Ser567 on Rb is not only important to promote p38-induced death, but it is specifically required as inhibition of Ser567 phosphorylation inhibits apoptosis.

p38 phosphorylates Rb on Ser567 by a novel mechanism

While p38 has been shown to phosphorylate Rb (Wang et al., 1999), it remains unclear whether p38 phosphorylates Rb by the same mechanism previously shown for Cdks involving a hierarchical series of conformational changes or by a novel mechanism (Harbour et al., 1999). We performed in vitro kinase experiments to confirm the ability of p38 to phosphorylate Rb directly (**Figure 2.16 A**). Both recombinant p38α and endogenous p38, immunoprecipitated from melanoma cells treated with etoposide, resulted in robust phosphorylation of Rb in vitro. As our lab showed previously (Harbour et al., 1999), cyclin E-Cdk2 can phosphorylate Rb on Ser567 only in the presence of the phosphorylated Rb C-terminus. In contrast, p38 did not require the C-terminus for efficient phosphorylation of Ser567; instead, p38 phosphorylated this residue more efficiently in the absence of the C-terminus (**Figure 2.16 B-C**). This indicated that p38 phosphorylates Rb on Ser567 through a unique mechanism unlike that of Cdks. The mechanistic difference in which Cdks and p38 phosphorylate Rb sheds light on how these two kinases differentially regulate Rb.

Phosphorylation of Rb by p38 disrupts the E2F1-Rb interaction and triggers E2F1-mediated apoptosis

For many years, Rb was thought to be bound to E2F when arresting the cell cycle and released from E2F after being phosphorylated by cyclin-Cdks, allowing for cell cycle progression. While free E2F does promote cell division, it also transactivates pro-apoptotic genes making this model very inefficient. Recent work suggests that Rb continues to stay bound to E2Fs throughout the cell cycle (Ezhevsky et al., 2001; Ezhevsky et al., 1997; Wells et al., 2000; Wells et al., 2003). So, under what circumstances are E2Fs completely released from Rb? We reasoned that stress-related phosphorylation of Rb would be unique in its ability to release E2Fs that could then activate apoptotic genes, and that this could account for the apoptosis that occurs when p38 phosphorylates Rb. We chose to focus on E2F1, as it is the primary E2F family member involved in the apoptotic response and is upregulated in response to etoposide (Ginsberg, 2002). However, we do not rule out the possibility that other E2F family members may play a role (Dick and Dyson, 2003; Xu et al., 2007). To this end, we activated endogenous p38 in cells by

expressing MKK3, and used co-immunoprecipitation experiments to evaluate the interaction between E2F1 and ectopically expressed Rb. As expected, phosphorylation of Rb by p38 disrupted the interaction between Rb and E2F1 (**Figure 2.17 A**). The release of E2F from Rb repression by activated p38α allowed it to activate E2F-responsive promoters (**Figure 2.17 B**). We confirmed this finding by performing similar luciferase assays with MKK3, the direct upstream activator of p38 and found that in both Mel202s (**Figure 2.17 C**) and C33A cervical cancer cells, (**Figure 2.17 D**) MKK3 promotes release of E2F from Rb. RbΔ567, which mimics Ser567 phosphorylation, led to activation of E2F-responsive promoters, whereas the RbΔ568 construct, which blocks Ser567, phosphorylation repressed E2F (**Figure 2.17 E**). Taken together these data suggest that phosphorylation of Rb on Ser567 by p38 derepresses E2F1-mediated transactivation. Further, RNAi-mediated knock down of E2F1 inhibited Ad-MKK3-associated apoptosis by TUNEL, confirming that p38 induced apoptosis through release of E2F1 (**Figure 2.18 A-B**).

Phosphorylation of Rb during stress is p53-independent

We have shown that Mel202, and other melanoma cell lines, have wildtype, functionally active p53 which is induced by DNA damage (Sun et al., 2005). Recognizing that p53 is activated by etoposide as part of the DNA damage response (Lowe et al., 1993), we wished to determine whether p53 was required for p38-mediated Rb phosphorylation. Surprisingly, knock down of p53 by RNAi had no effect on the phosphorylation status of Rb induced by etoposide (**Figure 2.19 A**). Further, phosphorylation of Rb in response to Ad-MKK3 treatment was independent of p53 (**Figure 2.19 B**). These data suggest that p53 does not play a role in p38-mediated Rb hyperphosphorylation .

p38-mediated apoptosis is p53-dependent

Stress can lead to cell death in a p53-dependent or a p53-independent manner (Bacus et al., 2001; Fattman et al., 1998; Lemaire et al., 2005; Macleod et al., 1996; Wang et al., 2008). However, p53-dependent apoptosis tends to be due to genotoxic stress, while p53-independent

death usually results from growth factor deprivation (Peled et al., 1996). Recognizing that we were looking at a genotoxic stress setting and that both etoposide and p38 can activate p53 (Bulavin et al., 1999; Lowe et al., 1993), we wished to determine if p53 contributed to genotoxic stress-induced apoptosis. As hypothesized, knock down of p53 by RNAi did inhibit etoposide-induced cell death (**Figure 2.20 A**). Similar results were observed in Ad-MKK3-treated cells (**Figure 2.20 B**). These data indicate that p53 cooperates to promote apoptosis during genotoxic stress.

2.5 Discussion

In this study, we demonstrate that p38, a MAPK member, phosphorylates Rb on residue Ser567 in response to genotoxic stress. The ability of p38, but not Cdks, to efficiently phosphorylate Rb on Ser567 may provide a key to understanding the different effects that p38 versus Cdks have on Rb. Phosphorylation of Rb by Cdks occurs through a hierarchical series of phosphorylation events each triggering successive conformational changes that enable further phosphorylation in a stepwise manner (Brown et al., 1999; Harbour et al., 1999; Lundberg and Weinberg, 1998; Zhang et al., 2000). Previous work has demonstrated that Ser567 is the site least efficiently phosphorylated by Cdks (Harbour et al., 1999). Phosphorylation of Ser567 by Cdks requires prior phosphorylation of the C-terminus, which contains Cdk docking sites that bring cyclin-Cdk complexes into proximity of Ser567 through an intramolecular interaction between the C-terminus and the pocket (Harbour et al., 1999). Ser567 has not been reported to be phosphorylated in cycling cells (Lees et al., 1991), suggesting that it is not phosphorylated during normal cell cycle progression. Herein we show that p38 efficiently phosphorylates Ser567 under conditions favoring apoptosis. Consistent with this finding, Nath and colleagues determined that an Rb mutant in which all available phosphorylation sites except Ser567 were mutated to prevent their phosphorylation was unable to rescue p38-mediated inactivation of Rb (Nath et al., 2003). Similarly, von Willebrand and colleagues reveal that a phosphorylation-defective Rb mutant was not resistant to ubiquitination despite elimination of most of the phosphorylation sites; Ser567 was

one of the few sites that was not mutated (von Willebrand et al., 2003). Importantly, we found that phosphorylation on Ser567 by p38 does not depend on the intramolecular interactions and conformational changes associated with Cdk-mediated phosphorylation. This would allow p38 to bypass Cdk-mediated phosphorylation of the C-terminus and directly regulate Rb in a cell cycle-independent manner during apoptotic signaling. The ability of p38 to phosphorylate Ser567 may be due to its small size, allowing it to access Ser567, which is located in a recess at the interface of the A and B boxes of the pocket, where it would likely be inaccessible to the much larger cyclin-Cdk complexes under normal circumstances (Harbour et al., 1999; Lee et al., 2002). Our data demonstrate that Ser567 is necessary for stress-induced apoptosis, suggesting it is a critical node in the decision of cellular fate in response to an insult.

While the paradigm in the Rb field promotes that Rb is bound to E2Fs when active or hypophosphorylated and E2F is released when Rb is inactivated by phosphorylation (Coller, 2007), more recent work has shown that E2Fs indeed stay bound to Rb throughout the cell cycle (Ezhevsky et al., 2001; Ezhevsky et al., 1997; Wells et al., 2000; Wells et al., 2003) leading us to hypothesize that E2F1 is only released from Rb after Rb is degraded, not after inactivation. To this end, we looked to see if Rb was bound to E2F1 during genotoxic stress to determine if Rb was being inactivated or disrupted in this setting. Indeed phosphorylation of Rb by p38 disrupted the Rb-E2F1 interaction and derepressed E2F-mediated transactivation. Ser567 phosphorylation specifically was required for the release of E2Fs from Rb, further implicating this site as a major regulator of cell fate. This is supported by work revealing that Ser567 is the only phosphorylation site on Rb that, when phosphorylated or mutated, disrupts the A and B box interaction, destabilizes the pocket structure, and abrogates interaction with E2Fs (Harbour et al., 1999; Ma et al., 2003). Further, crystallographic studies have shown that Ser567 mediates critical contacts between the A and B boxes, and that phosphorylation of this site would be predicted to destabilize the pocket structure and eliminate the E2F binding site (Lee et al., 2002; Lee et al., 1998).

The p53 and Rb pathways are the two most commonly disrupted tumor suppressor pathways in cancer (Sherr, 2004; Sherr and McCormick, 2002). The two pathways are firmly intertwined as E2F activation of $p14^{ARF}$ and stabilizes p53 and p53 activates $p21^{Cip1}$ leading to hypophosphorylation and activation of Rb, making it difficult to study one tumor suppressor without considering the other. Furthermore p38 has been shown to directly phosphorylate p53 and promote p53-dependent apoptosis in response to UV radiation (Bulavin et al., 1999). Therefore, we investigated the role of p53 in etoposide-treated melanoma cells. While p53 did not contribute to the phosphorylation status of Rb, it did promote apoptosis in genotoxic-stressed melanoma cells. This is consistent with our data demonstrating that E2F1 is released from Rb in response to etoposide which would promote $p14^{ARF}$ transcription, stabilize p53, and allow for p53-mediated apoptosis.

Taken together, these findings suggest that p38 phosphorylates Rb through a novel, cell cycle-independent mechanism and illuminates the way in which Rb coordinately regulates the cell cycle versus apoptosis.

2.6 Figures

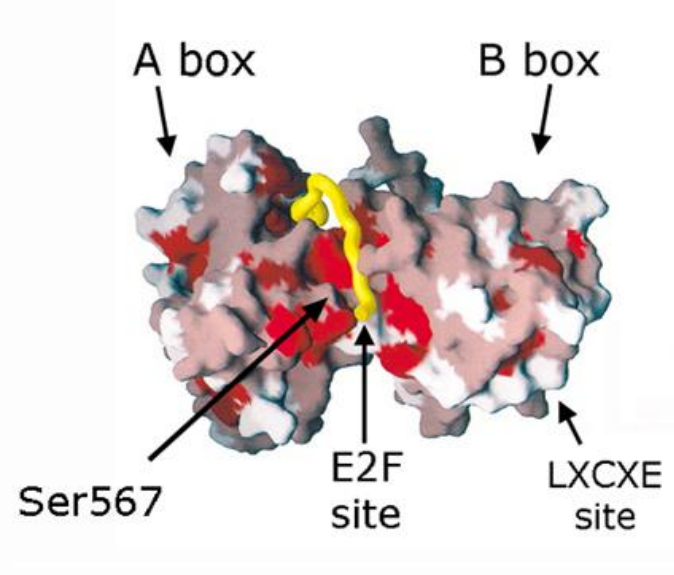

Figure 2.1 Representation of the crystal structure of the pocket domain of Rb

The A box, B box, Ser567 phosphoacceptor site, E2F binding site, and LxCxE site are shown in this illustration of the crystal structure of the Rb pocket domain. Adapted from (Lee et al., 2002).

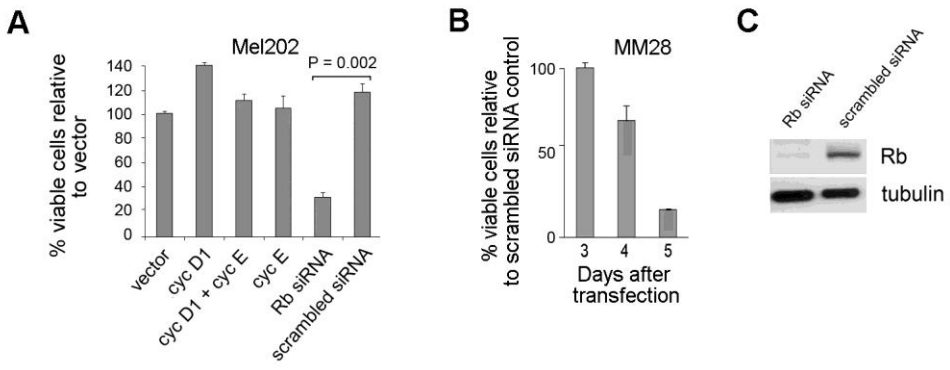

Figure 2.2 Loss of Rb leads to apoptosis

(**A**) Mel202 cells were transfected with scrambled siRNA or Rb siRNA, or with vectors expressing cyclin D1 and/or cyclin E to activate endogenous Cdks for 4 days and cell viability assays were performed. (**B**) Cell viability assays were performed in MM28 primary melanoma cells using scrambled siRNA or Rb siRNA. (**C**) Immunoblot analysis confirming effective knock down of Rb with siRNA in Mel202s after 4 days. Tubulin was used as a loading control in all immunoblot experiments.

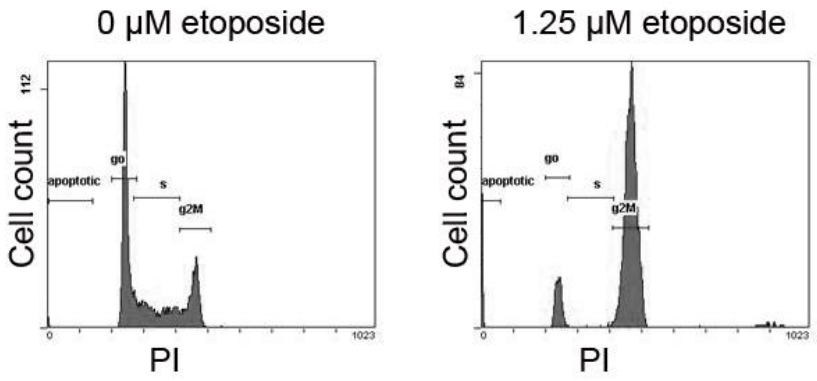

Figure 2.3 A low concentration of etoposide leads to G_2/M arrest

Mel202 cells were untreated or treated with 1.25 µM etoposide for 48 h and analyzed by flow cytometry.

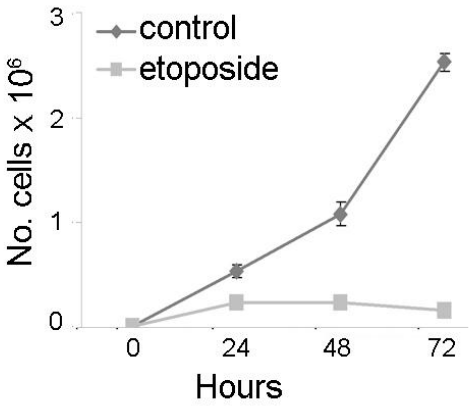

Figure 2.4 Genotoxic stress halts proliferation

12.5 µM of etoposide treatment halted cell growth in Mel202 cells as shown by growth curves.

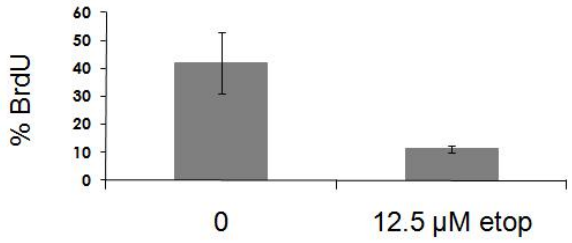

Figure 2.5 Genotoxic stress inhibits proliferation

12.5 µM of etoposide treatment inhibits cell proliferation in Mel202 cells as shown by BrdU experiments.

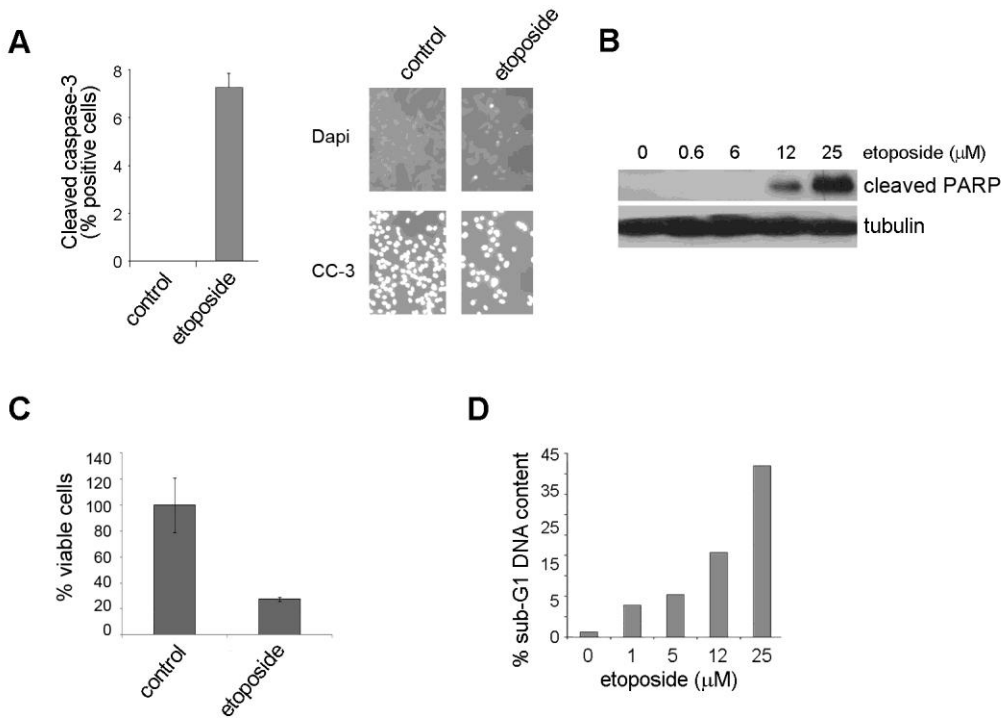

Figure 2.6 Genotoxic stress leads to cell death in Mel202 cells

(**A**) Cleaved caspase 3 staining of Mel202 cells treated with or without 12.5 μM of etoposide for 48 h. (**B**) Mel202 cells were treated with 0, 0.6, 6, 12, 25 μM etoposide for 48 h and immunoblotted for cleaved PARP. (**C**) Cell viability assays were performed in Mel202 cells that were untreated or treated with 12.5 μM of etoposide for 48 h. (**D**) Flow cytometry was performed using Mel202 cells treated with 0, 1, 5, 12, 25 μM etoposide for 48 h.

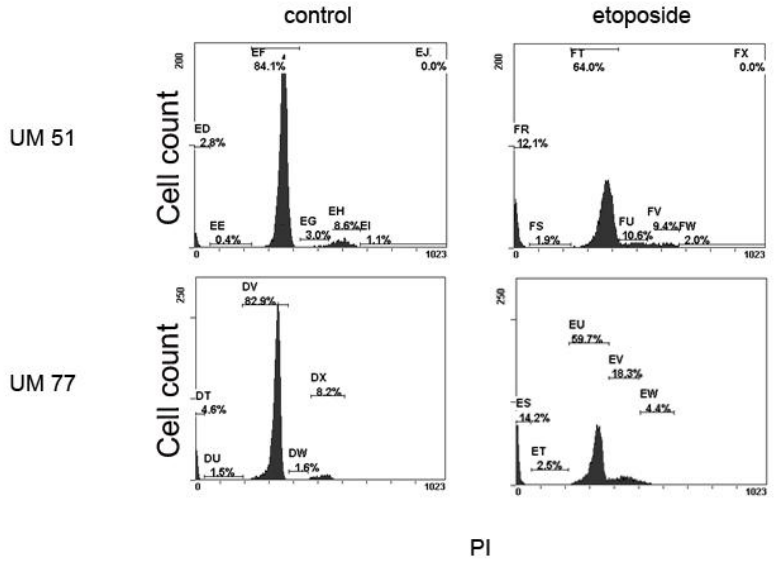

Figure 2.7 Genotoxic stress leads to cell death in primary human uveal melanocytes

Primary uveal melanocytes from two different patients were treated with or without 12.5 µM of etoposide for 48 h and analyzed by flow cytometry. Almost 6 times more death occurred in the treated cells.

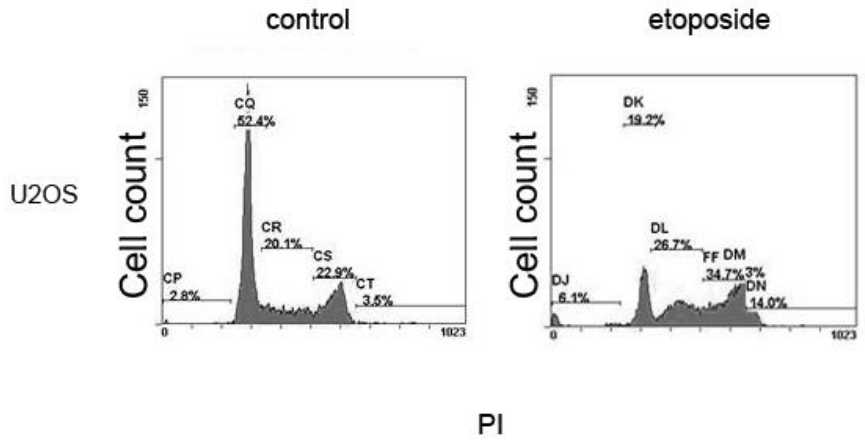

Figure 2.8 Genotoxic stress leads to cell death in osteosarcoma cells

U2OS osteosarcoma cells were treated with or without 12.5 µM of etoposide for 48 h and analyzed by flow cytometry. The amount of death doubled in the treated cells.

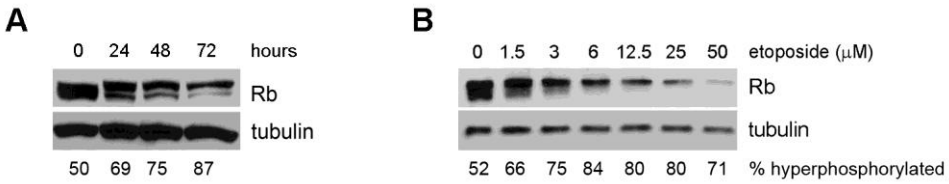

Figure 2.9 Genotoxic stress leads to hyperphosphorylation of Rb

(**A**) Mel202 cells were treated with 12.5 μM etoposide for 0, 24, 48, 72 h and immunoblotted for Rb. (**B**) Primary human uveal melanocytes were treated with 0, 1.5, 3, 6, 12.5, 25, 50 μM etoposide for 48 h and immunoblotted for Rb.

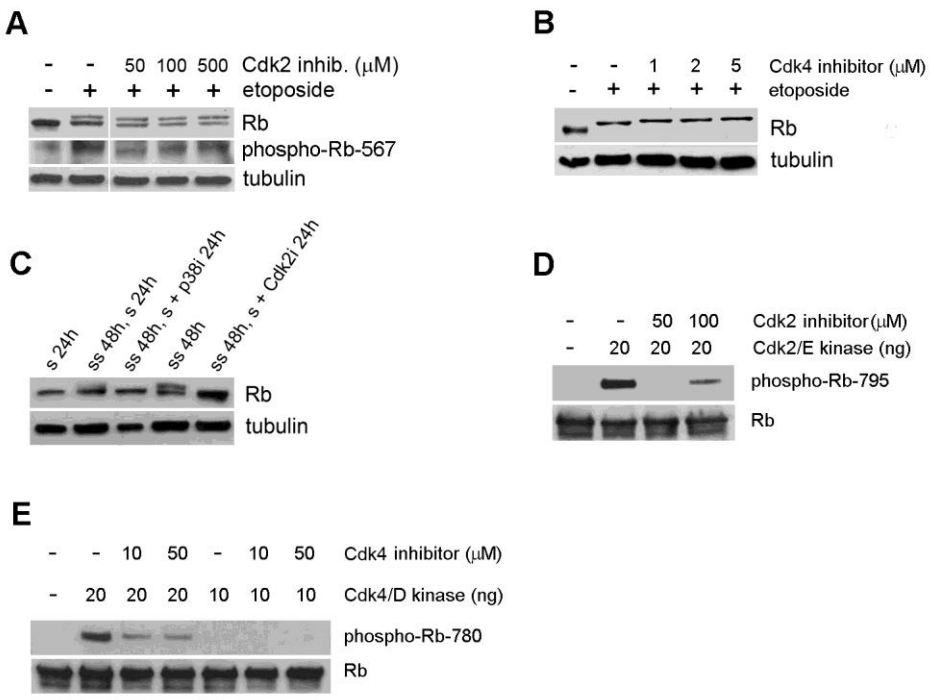

Figure 2.10 Cdks are not responsible for genotoxic stress-induced hyperphosphorylation of Rb

(**A**) Mel202 cells were treated with 12.5 μM of etoposide and 0, 50, 100, or 500 μM of the Cdk2 inhibitor, Roscovitine, for 48 h and then immunoblotted for total Rb and phospho-Rb-567. (**B**) Mel202 cells were treated with Cdk4 inhibitor and 12.5 μM etoposide for 48 h and immunoblotted for Rb. (**C**) Inhibition of serum starvation-induced hyperphosphorylation of Rb confirmed inhibitor activity in Mel202 cells. Lane 1, serum for 24 h. Lane 2, serum starved for 48 h, serum added back for 24 h. Lane 3, serum starved for 48 h, serum added back for 24 h with p38 inhibitor. Lane 4, serum starved for 48 h. Lane 5, serum starved for 48 h, serum added back for 24 h with Cdk2 inhibitor Roscovitine. (**D**) In vitro kinase assay confirmed that the Cdk2 inhibitor was active. Kinase assay was blotted with phospho-Rb-795, a site known to be phosphorylated by Cdk2. (**E**) In vitro kinase assay confirmed that the Cdk4 inhibitor was active. Kinase assay was blotted with phospho-Rb-780, a site known to be phosphorylated by Cdk4.

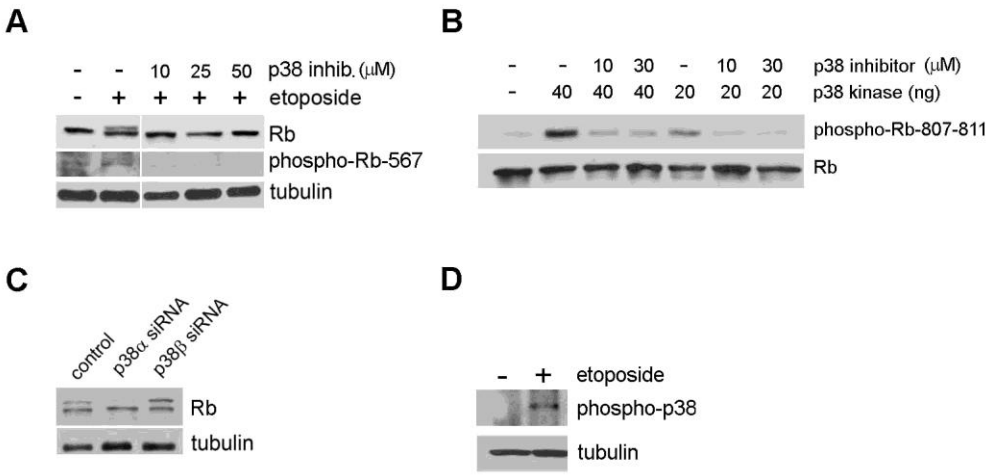

Figure 2.11 Genotoxic stress triggers phosphorylation of Rb on Ser567 by p38α

(**A**) Mel202 cells were treated with p38 inhibitor SB203580 for 48 h and immunoblotted for total Rb and phospho-Rb-567. (**B**) In vitro kinase assay confirmed that the p38 inhibitor was active. Kinase assay was blotted with phospho-Rb-807-811 and total Rb. (**C**) siRNA against p38α or p38β was transfected into Mel202 cells at a final concentration of 50 nM for 72 h, all samples were treated with 12.5 μM of etoposide for 48 h, and immunoblotted for Rb. (**D**) Mel202 cells were treated with or without 12.5 μM of etoposide for 48 h and immunoblotted for phospho-p38.

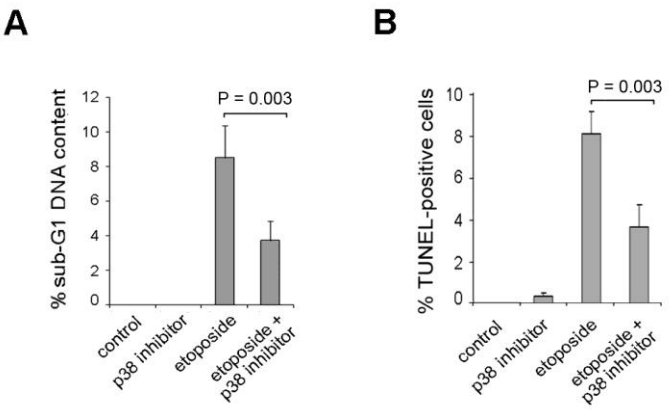

Figure 2.12 p38 inhibitor inhibits genotoxic stress-induced death

(**A**) Mel202 cells were treated with 4 µM etoposide, 30 µM of p38 inhibitor, or both for 48 h. Propidium iodide was used in order to determine the sub-G_1 DNA content by flow cytometry. (**B**) Mel202 cells were treated with 12.5 µM etoposide, 30 µM of p38 inhibitor, or both for 48 h. TUNEL staining was performed to determine the percent of apoptotic cells.

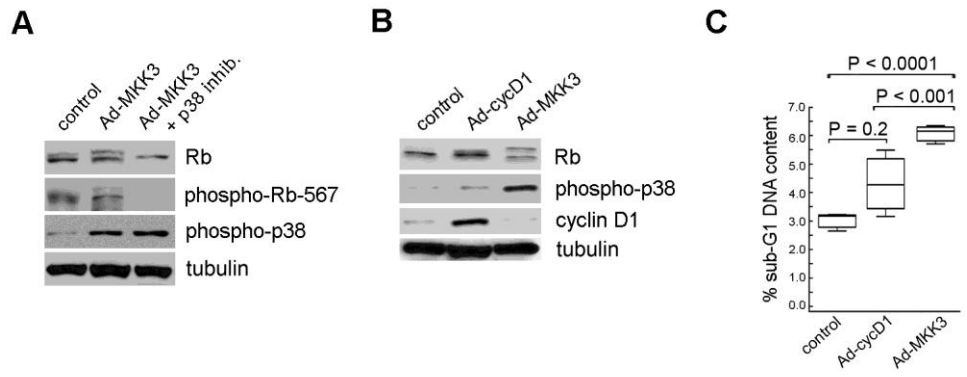

Figure 2.13 MKK3 promotes hyperphosphorylation of Rb and apoptosis in a similar manner to genotoxic stress

(**A**) Mel202 cells were treated with Ad-LacZ or Ad-MKK3 at an MOI of 100 with or without 30 µM of p38 inhibitor and immunoblotted for Rb, phospho-Rb-567 or phospho-p38. (**B**) Mel202 cells were treated with Ad-GFP, Ad-cycD1, or Ad-MKK3 at an MOI of 30 for 48 h and immunoblotted for total Rb, phospho-p38, or cyclin D1. (**C**) Mel202 cells were treated with Ad-cycD1 or Ad-MKK3 at an MOI of 30 for 48 h. Flow cytometry was performed using propidium iodide staining to determine the percent of cells in sub-G_1.

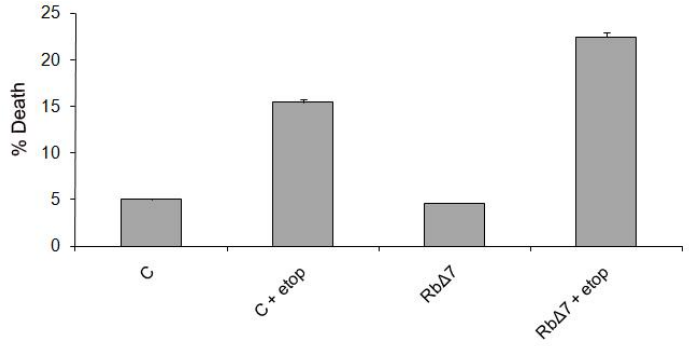

Figure 2.14 C-terminal sites are not necessary for genotoxic stress-induced induced death

Flow cytometry was performed in Mel202 cells using a mutant in which seven C-terminal sites, Ser780, Ser788, Ser795, Ser807, Ser811, Thr821, and Thr825 were mutated to alanines (RbΔ7) leaving only three remaining phosphoacceptor sites**;** Ser567, Ser608, and Ser612. Cells were transfected with CD20 and SKTT control or RbΔ7, the next day 12.5 μM of etoposide was added to the media. Cells were collected 48 h after etoposide treatment.

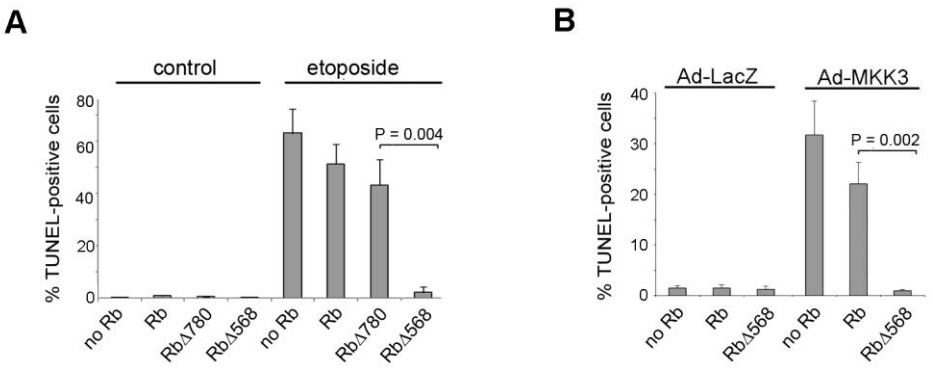

Figure 2.15 Phosphorylation of Rb on Ser567 is necessary for stress-induced death

(**A**) Mel501 cells were transfected with SKTT control, Rb, RbΔ780, or RbΔ568 for 72 h and treated with 12.5 μM etoposide for 48 h. TUNEL staining was performed. (**B**) Mel501 cells were transfected with SKTT control, Rb, or RbΔ568 for 72 h and treated with Ad-MKK3 or Ad-LacZ MOI 15 for 48 h. TUNEL staining was performed. Data were normalized to RbΔ568 for each virus.

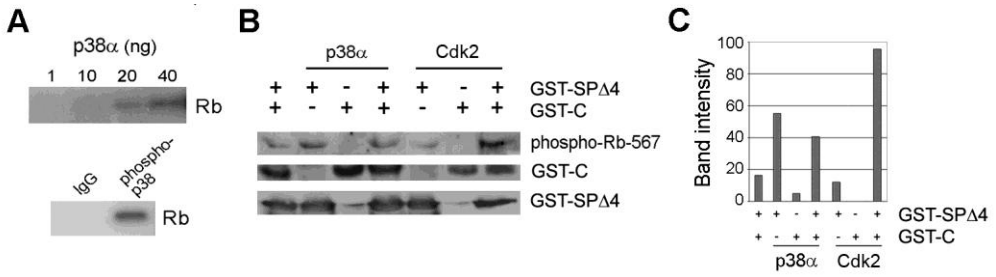

Figure 2.16 p38 phosphorylates Rb directly and by a different mechanism than cyclin E-Cdk2

(**A**) In vitro radioactive kinase assays revealed that p38 directly phosphorylated Rb. Top Panel: Purified p38α phosphorylated 0.5 µg of purified full length Rb. Bottom Panel: Endogenous phospho-p38 immunoprecipitated from Mel202 cells phosphorylated 0.5 µg of purified full length Rb. (**B**) Non-radioactive in vitro kinase assays were performed using 20 ng of purified p38 or cyclin E-Cdk2 kinase. GST-SPΔ4 (small pocket of Rb with all phosphoacceptor sites except Ser567 mutated to an alanine) and GST-C (C-terminus of Rb) were used as substrates. Lanes 1, 4, and 7 had 3.2 µg of GST-C, lanes 2 and 5 had no GST-C, lanes 3 and 6 had 1.6 µg of GST-C. 1 µg of GST- SPΔ4 was used. Samples were immunoblotted with an antibody against phospho-Rb-567 or an antibody against GST which recognizes both GST-SPΔ4 and GST-C. The GST-C and GST-SPΔ4 Rb fragments have been shown to interact in vitro (Harbour et al., 1999). GST-C is necessary for efficient phosphorylation of the small pocket by Cdk2, but is not required for phosphorylation by p38. (**C**) A graphical representation of the band intensity of the phospho-Rb-567 antibody in (**B**).

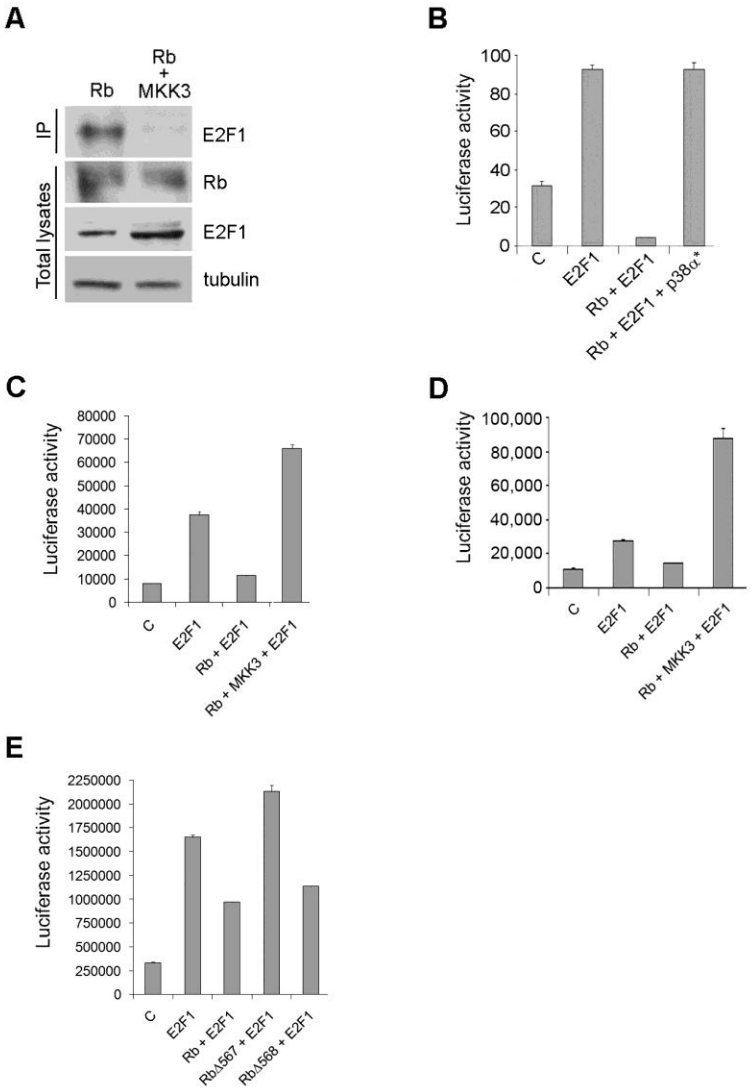

Figure 2.17 Phosphorylation of Rb by p38 derepresses E2F1-mediated transactivation

(**A**) Mel202 cells were transfected with Rb alone or with MKK3 for 48 h and immunoprecipitation for Rb was performed. The immunoprecipitation was immunoblotted for E2F1. Total lysates were immunoblotted for total Rb and E2F1. (**B**) An E2F reporter construct was used in luciferase assays. Empty vector (pCDNA3), Rb, E2F1, and constitutively active p38α expression constructs were transfected into Mel202 cells. The star represents constitutively active p38α. (**C**) An E2F reporter construct was used in luciferase assays. Empty vector (pCDNA3 and RSV2), Rb, E2F1, and MKK3 expression constructs were transfected into Mel202 cells. (**D**) An E2F reporter

construct was used in luciferase assays. Empty vector (pCDNA3 and RSV2), Rb, E2F1, and MKK3 expression constructs were transfected into C33A cervical cancer cells that lack functional Rb. (**E**) The E2F responsive Cyclin E reporter construct was used in luciferase assays. Empty vector (pCDNA3), Rb, RbΔ567, RbΔ568, and E2F1 expression constructs were transfected into Mel202 cells.

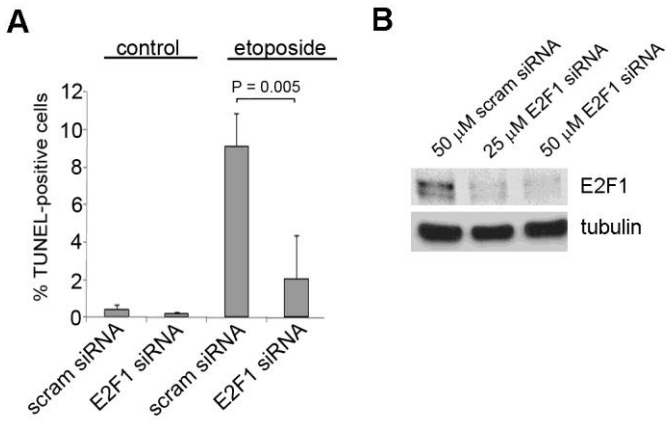

Figure 2.18 Phosphorylation of Rb by p38 leads to E2F1-mediated apoptosis

(**A**) Scrambled or E2F1 siRNA was used at a final concentration of 50nM for 72 h in Mel501 cells. The cells were treated with 12.5 μM of etoposide for 48 h and TUNEL staining was performed. (**B**) Knock down of E2F1 by siRNA was confirmed in Mel501 cells. 50 nM of scrambled siRNA, 25 nM of E2F1 siRNA, or 50 nM of E2F1 siRNA were transfected for 72 h.

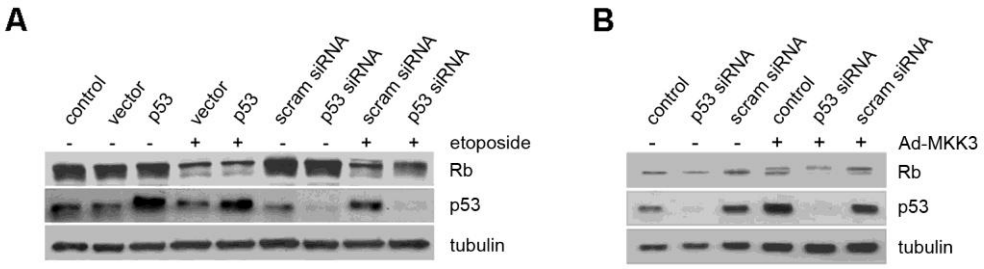

Figure 2.19 p38-mediated phosphorylation of Rb is p53-independent

(**A**) Mel202 cells were treated with 12.5 µM of etoposide for 48 h. Scrambled or p53 siRNA was used at a final concentration of 50 nM for 72 h. p53 expression vector or empty vector were transfected for 72 h. Samples were immunoblotted with antibodies against Rb and p53. (**B**) Mel202s were transfected with siRNA against p53 or scrambled siRNA for 72 h and treated with Ad-MKK3 MOI of 15 for 48 h. Samples were immunoblotted with antibodies against Rb and p53.

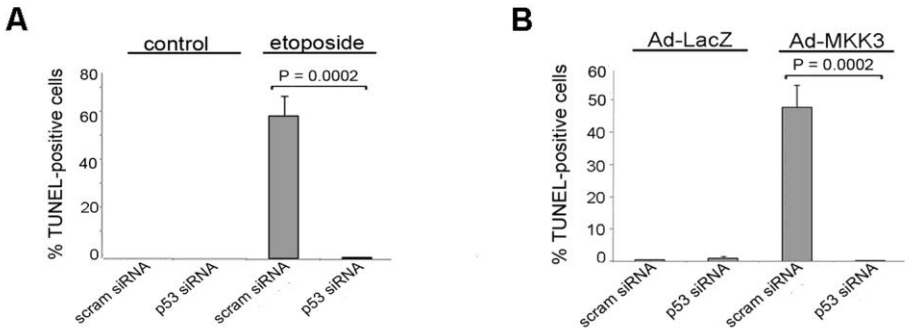

Figure 2.20 p38- induced apoptosis is p53-dependent

(**A**) Mel202 cells were transfected with p53 siRNA or scrambled siRNA for 72 h. Cells were treated with 12.5 µM of etoposide for 48 h and TUNEL staining was performed. (**B**) Mel202s were transfected with p53 siRNA or scrambled siRNA for 72 h and treated with Ad-LacZ or Ad-MKK3 at an MOI of 15. TUNEL staining was performed.

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Chapter 3

p38 phosphorylation of Ser567 on Rb in response to genotoxic stress leads to Rb-Hdm2 interaction and apoptosis

3.1 Abstract

While Rb has been classically studied in the cell cycle setting, this important tumor suppressor also regulates apoptosis. Cells were stressed with a DNA damaging reagent which resulted in degradation of Rb. Phosphorylation of Rb by the stress kinase p38, but not by the cell cycle kinase cyclin D-Cdk4, led to an increased interaction between Rb and Hdm2. Specifically, phosphorylation of Ser567 during stress enhanced the Rb-Hdm2 interaction. We provide new insights into the relationship between the oncoprotein Hdm2 and the tumor suppressors p53 and Rb and implicate Ser567 phosphorylation as a key event in the cellular response to stress. Consistent with our in vitro work, phosphorylation of Ser567, but not C-terminal sites, correlated with poor prognosis in uveal melanoma patients. Our data suggest that Ser567 may serve as a biomarker of stress in cancer patients.

3.2 Introduction

Rb was named the first tumor suppressor for the ability to inhibit cell division (Friend et al., 1986; Goodrich et al., 1991; Knudsen et al., 1998; Weinberg, 1991). In order for cells to divide, Rb must be temporarily inactivated by cyclin-Cdks, which phosphorylate Rb at up to 16 Ser/Thr-Pro phospho-acceptor sites, thereby allowing cells to traverse from G_1 into S phase (Chen et al., 1989; Hinds et al., 1992; Lin et al., 1991; Lundberg and Weinberg, 1998; Serrano et al., 1993). During cell cycle progression, Rb is phosphorylated by cyclin D/Cdk4 in early G_1, cyclin E/Cdk2 in late G_1, and finally by cyclin A/Cdk2 in S phase (DeCaprio et al., 1992; Kato et al., 1993; Sherr, 1996). Regulation of the cell cycle by Rb is intricately linked to the binding of E2F transcription factors (Stevaux and Dyson, 2002). Rb can bind the activators, E2F1-3 and the repressor, E2F4 to regulate transcription (Stevaux and Dyson, 2002). Rb inhibits the E2F transactivation function by directly binding and masking the transactivation domain and by recruiting chromatin remodeling factors to alter local chromatin structure to an inhibitory state (Almasan et al., 1995; Chellappan et al., 1991; Dyson, 1998; Hsieh et al., 1997; Irwin et al., 2000; Morgenbesser et al., 1994; Nevins, 1998; Shan et al., 1994; Tsai et al., 1998).

As well as acting as a cell cycle regulator, Rb also functions as an anti-apoptotic factor. Loss of Rb causes apoptosis in melanocytes as well as other cell types and Rb knockout mice have increased apoptosis (Bremner et al., 2004; Chau and Wang, 2003; Wu et al., 1996). To study the coordinate roles of Rb in cell cycle and apoptosis, we have selected the melanocyte lineage, where a number of observations point to a critical role for Rb in differentiation, survival and tumor suppression. The Cdk4/6 inhibitor $p16^{Ink4a}$ is frequently mutated in familial melanoma, and individuals with RB gene mutations are at increased risk for melanoma (Begg et al., 2005; Halaban, 1999; Luca et al., 1995; Reed et al., 1995; Reymond and Brent, 1995). Virtually all mouse models of melanoma require inhibition of Rb, such as by overexpression of large T antigen or by inactivation of $p16^{Ink4a}$, which allows unrestricted phosphorylation of Rb by Cdk4/6 (Castellano and Parmiani, 1999; Yu et al., 2003). Additionally, activating mutations in the RAS-

RAF-MAPK pathway, which inhibit Rb through activation of cyclin D, are ubiquitous in melanocytic tumors (Gupta et al., 2005; Pollock et al., 2003).

Hyperphosphorylation of Rb caused by a stress insult (**Chapter 2**) was accompanied by degradation of Rb. This degradation of Rb lends mechanistic insight into why cell die rather than arrest after genotoxic stress. We investigated the pathway that mediates Rb degradation and cell death in response to stress. While enzyme mediated-degradation was also considered, we found the degradation of Rb to be due to the proteosome. We focused on Hdm2, an E3 ligase that has been shown to play a role in the degradation of Rb. Hdm2 binds the C-terminus of Rb and ubiquitinates Rb, targeting it for degradation by the proteosome (Sdek et al., 2004; Xiao et al., 1995).

The mechanism of the Rb-Hdm2 interaction was investigated. We hypothesized that phosphorylation events drove this interaction as phosphorylation is key in regulating Rb. The residue Ser567 on Rb was of particular interest as it is the only phosphorylation site on Rb that, when phosphorylated or mutated, leads to localization of Rb in the cytoplasm rather than the nucleus (Ma et al., 2003). We found that phosphorylation of Rb on Ser567 leads to Hdm2-mediated degradation of Rb and apoptosis.

We determined that phosphorylation of Ser567 is important in human cancer. Uveal melanoma is the most common eye cancer (Onken et al., 2004). While presentation in the uveal tract of the eye accounts for only 4% of all melanoma, like cutaneous melanoma, it is highly resistant to radiation and chemotherapy and is therefore very difficult to treat. Half of uveal melanoma cases metastasize hematogenously to the liver where median survival is less than 6 months. There will be an estimated 200 deaths due to this disease in 2010 in the United States alone (ACS, 2010) (Gragoudas et al., 1991). Samples of primary uveal melanoma patients were obtained and increased phosphorylation of Rb on Ser567 correlated with poor prognosis in uveal melanoma by IHC. Our lab developed a peptide made up of Ser567 and the surrounding residues which mimics

the effect of Ser567 phosphorylation as a proof of principle for Rb targeted therapies. The peptide resulted in massive apoptosis of cancer cells. However, when Ser567 and Pro568 were mutated to alanines in an otherwise identical peptide the cells did not die, confirming that the pro-apoptotic activity is specific to Ser567. Understanding the mechanism behind Rb-mediated apoptosis in response to genotoxic stress could lead to life saving treatments.

3.3 Materials and methods

Please refer to Chapter 2 for materials and methods that have already been discussed.

Cell culture

A375 cutaneous melanoma, 10T/2 mouse fibroblasts, 92.1 uveal melanoma, and MCF-7 and MB-MDA-231 breast cancer cells were grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Sigma Aldrich), L-glutamine/antibiotics/anti-mycotics (Mediatech) under conditions of 5% carbon dioxide and 21% oxygen.

Inhibitors

When indicated, cells were treated with Hdm2 E3 Ligase Inhibitor 373225 (EMD), Z-VAD-FMK pan caspase inhibitor (Sigma Aldrich), or MG132 proteosome inhibitor (Sigma Aldrich).

Constructs and siRNA

An Rb-A expression plasmid was used (Harbour et al., 1999). Validated siRNA against Hdm2 or scrambled control (Ambion) were used.

Antibodies

Antibodies against Gal4 and Hdm2 were from Santa Cruz Biotechnology, Inc. Phospho-PKC-δ was from Cell Signaling.

Immunohistochemical Staining

Immunohistochemistry (IHC) was performed as previously described (Brantley et al., 2002). Rb-phospho-Ser567 polyclonal rabbit antibody was applied at a dilution of 1:50 (developed for our lab by Bethyl Laboratories as previously described (Ma et al., 2003). Rb-phospho-807-711 (Cell Signaling) was applied at a dilution of 1:100. Nibrin (Cell Signaling) was applied at a dilution of 1:75 as previously described (Ehlers and Harbour, 2005).

Ser567 Peptides

Transducible peptides were synthesized by Biomolecules Midwest (Waterloo, IL) as previously described and made up in serum-free media (Harbour et al., 2002). The Tat-S567 peptide comprises the 12 amino acid Tat transduction domain followed by a 20 amino acid fragment corresponding to amino acids 558 through 577 of the human Rb protein. The Tat-S567A was identical to the Rb567 peptide except for the substitution of an alanine for the serine corresponding to amino acid 567 of the parent protein. The Tat-S567A/P568A was identical to the Rb567 peptide except for the substitution of two alanines for the serine and proline residues corresponding to amino acids 567 and 568 of the parent protein. The Tat peptide was comprised only the Tat transduction domain.

3.4 Results

Genotoxic stress leads to degradation of Rb

In our experiments, genotoxic stress resulted not only in hyperphosphorylation of Rb, (**Chapter 2**) but also in a precipitous decline in total Rb protein levels, suggesting that p38-mediated phosphorylation causes degradation of Rb (**Figure 3.1**). Indeed, after 72 h of etoposide treatment, over half of the Rb in Mel202 uveal melanoma cells had been degraded (**Figure 3.1 A**). This result was confirmed in primary uveal melanocytes (**Figure 3.1 B**), in a second uveal melanoma cell line, 92.1 cells (**Figure 3.1C**), and in A375 cutaneous melanoma cells (**Figure 3.1 D**). This finding was not specific to the melanocyte lineage. Etoposide treatment resulted in Rb

degradation in U2OS osteosarcoma cells, 10T/2 mouse fibroblasts, and MCF-7 and MB-MDA-231 breast cancer cells (**Figure 3.2 A-D**).

Activation of p38 leads to Rb degradation

Etoposide activates many pathways therefore, Ad-MKK3 was used to directly active endogenous p38 and Rb protein levels were assessed. In corroboration with the etoposide data, Ad-MKK3 treatment also led to degradation of Rb (**Figure 3.3**).

Degradation of Rb during genotoxic stress is not due to enzymatic cleavage

We wondered if caspases were responsible for the Rb degradation seen in response to genotoxic stress. Rb is cleaved by caspase 3 and 7 on the DEAD886 recognition motif in the C-terminus releasing a p48 fragment made up of the N-terminus of Rb that is cytoplasmic and a p68 fragment made up of the large pocket of Rb that is nuclear and can still bind DNA but no longer interacts with E2F1 (An and Dou, 1996, Fattman, 1997). After the first cleavage event, Rb can undergo an additional internal cleavage at DSID349 by caspase 3 and 7 (Fattman et al., 2001). Caspase cleavage has been shown to be triggered by stress stimuli such as etoposide (Karpinich et al., 2002; Nakajima et al., 1994; Sawada et al., 2000). Although Rb has been shown to be cleaved by caspases under certain conditions (Fattman et al., 2001), the pan-caspase inhibitor Z-VAD-FMK did not completely block Rb degradation confirming that additional pathways are involved (**Figure 3.4 A**). Inhibition of phospho-PKC-δ, a downstream target of caspases, by ZVAD confirmed the inhibitor was working (**Figure 3.4 B**). While caspases may play a role, they are not the only pathway involved in degradation of Rb during genotoxic stress.

Rb undergoes Hdm2-mediated degradation in response to genotoxic stress

Rb interacts with the E3 ligase Hdm2, which targets both Rb and p53 for proteosomal degradation and can thereby act as an oncogene when overexpressed (Xiao et al., 1995, Sdek, 2004). The critical role Hdm2 plays in maintaining p53 at low cellular levels during normal conditions has been established (Momand et al., 1992, Oliner, 1993), but the physiologic

significance of the Hdm2-Rb interaction remains less certain. We hypothesized that Hdm2 may mediate the degradation of Rb that occurs in response to p38-mediated phosphorylation. Indeed, degradation of Rb was efficiently inhibited by a small molecule inhibitor of Hdm2 E3 ligase activity (**Figure 3.5 A**). In co-immunoprecipitation experiments, the physical interaction between Rb and Hdm2 was enhanced by etoposide, and by activating endogenous p38 by expression of MKK3, but not by activating endogenous Cdks by expression of cyclin D1 (**Figure 3.5 B-C**). Specifically, Ad-MKK3 treatment increased the interaction between Hdm2 and the hyperphosphorylated form of Rb (**Figure 3.5 C**). Knock down of Hdm2 using siRNA led to an increase in hyperphosphorylation of Rb (**Figure 3.6 A**) and cells treated with a proteosome inhibitor, MG132, resulted in increased levels of phosphorylated Rb (**Figure 3.6 B**) revealing that baseline degradation of Rb occurs in melanoma cells and that hyperphosphorylated forms of Rb were being degraded not dephosphorylated. Taken together, these data suggest that Hdm2 preferentially interacts with and degrades a phosphorylated form of Rb generated by p38- rather than Cdk-mediated phosphorylation, indicating a key mechanistic insight into how the cell cycle and apoptosis are regulated through Rb.

Phosphorylation of Rb on Ser567 by p38 induces its degradation by Hdm2

We speculated that Ser567 may be important in mediating the Rb-Hdm2 interaction. For all other phosphorylation sites on Rb except Ser567, mutation simply blocks phosphorylation. However, we previously showed that Ser567 plays a unique role in maintaining the conformation and activity of the pocket (Harbour et al., 1999). Thus, mutation of Ser567, as in our RbΔ567 construct, disrupts the pocket structure and function, thereby mimicking the effect of phosphorylating this site. In other words, both phosphorylation of Rb on Ser567 and the RbΔ567 mutant destabilize and inactivate the Rb protein. The RbΔ567 mutant interacted strongly with Hdm2 in co-immunoprecipitation experiments, whereas wildtype Rb interacted only weakly with Hdm2 (**Figure 3.7**). Further, RbΔ567 could induce a trimeric complex between Rb, Hdm2 and p53, but wildtype Rb could not (**Figure 3.7**). The A domain of Rb (Rb-A) was used as a negative control because the C-terminus of Rb, which is necessary for Hdm2 binding, is not present in this

mutant (Xiao et al., 1995). These results suggest that p38-mediated phosphorylation of Rb on Ser567 regulates Rb protein levels by modulating its interaction with and degradation by Hdm2.

Phosphorylation of Rb on Ser567 by p38 triggers Hdm2-mediated apoptosis

We wondered whether loss of Hdm2 would inhibit stress-induced apoptosis. We treated cells with a chemical inhibitor of Hdm2 and etoposide and found that the Hdm2 inhibitor inhibited cell death (**Figure 3.8 A**). Next, we directly targeted p38 and stressed cells with Ad-MKK3. Once again, the Hdm2 inhibitor inhibited apoptosis (**Figure 3.8 B**). To confirm these findings we used siRNA against Hdm2 and treated cells with etoposide or Ad-MKK3. Again, apoptosis was inhibited (**Figure 3.8 C-D**). Taken together, these results suggest that p38-mediated phosphorylation of Rb on Ser567 leads to Hdm2-dependent apoptosis.

Phosphorylation of Ser567, but not C-terminal sites, correlates with poor prognosis in uveal melanoma patients

Our lab has demonstrated that Rb phosphorylated on Ser567 is located mostly in the cytoplasm, where Rb is inactive and degraded by the proteosome. In contrast, C-terminal phosphorylated Rb is located mostly in the nucleus where it is active (Ma et al., 2003). Primary human uveal melanoma samples were evaluated by immunostaining for phosphorylation of Ser567 and the C-terminal phosphorylation sites using phospho-specific antibodies. Consistent with our cell culture data, Rb phosphorylated on Ser567 was localized to the cytoplasm, whereas Rb phosphorylated on the C-terminal sites Ser807 and Ser811 was located in the nucleus in tumor samples (**Figure 3.9 A**). Ser567 phosphorylation was strongly associated with metastatic death, with all patients whose tumor exhibited Ser567 phosphorylation dying of metastasis within five years. In contrast, 70% of patients without Ser567 phosphorylation were still alive after ten years (log rank test, $P = 0.0002$) (**Figure 3.9 B**). On the other hand, phosphorylation of the C-terminal sites Ser807 and Ser811 were not associated with metastasis or survival (**Figure 3.9 B**). One potential explanation for these findings is that Ser567 phosphorylation is an indicator of DNA damage, genomic instability and aneuploidy, which we previously showed was a strong predictor of metastatic death

(Ehlers and Harbour, 2005). Consistent with this hypothesis, Ser567 phosphorylation was strongly associated with expression of nibrin (**Figure 3.9 C**), which is a component of the MRE11/RAD50 double-strand break repair complex and is up-regulated in tissues with high levels of DNA double strand breaks (Wilda et al., 2000). Taken together, these findings suggest that Ser567 phosphorylation is a potential biomarker for aggressive tumors with high degrees of aneuploidy.

Ser567 peptide as proof of principle for Rb-directed therapy

Based on our finding that Ser567 induces apoptosis, at least in part by disrupting the pocket structure, we hypothesized that a compound that mimics this disruption of the pocket may allow targeted cell death by modulation of Rb. Such a strategy may be effective in treating cancers that are wildtype for Rb. We reasoned that a peptide containing the region surrounding Ser567 itself may compete with and thereby interfere with the interaction of Ser567 with other residues required for maintaining the tertiary structure of the pocket. Thus, we generated a 20 amino acid peptide fragment of Rb centered around Ser567, and control peptides in which Ser567 was converted to alanine (S567A) or Ser567 and Pro568 were both converted to alanine (S567A/P568A). To deliver these peptides efficiently into the nucleus of cultured cells, the Tat transduction domain was fused to the N-terminus of each peptide. Our lab has previously reported success with this approach (Harbour et al., 2002). Tat-S567 peptide, but not Tat-S567A or Tat-S567A/P568A peptides, efficiently induced apoptosis in Mel202 uveal melanoma cells (**Figure 3.10 A**). Also, the Tat-S567 peptide, but not Tat-S567A or Tat-S567A/P568A peptides, efficiently killed U2OS osteosarcoma cells (**Figure 3.10 B**). Additionally, the Tat-S567 peptide activated an E2F-dependent promoter-reporter more than Tat alone (**Figure 3.10 C**). Further work is needed to explore the precise mechanism by which the Ser567 peptide functions, but these findings provide promise for modulating Rb as an approach to targeted therapy.

3.5 Discussion

Genotoxic stress or direct activation of endogenous p38 using Ad-MKK3 results in degradation of Rb. Degradation occurs during stress-induced apoptosis but not during the cell cycle, thus indicating an important mechanistic insight into how these two processes are differentially regulated through Rb. This degradation in response to etoposide occurred in melanoma cells and primary melanocytes, as well as A375 cutaneous melanoma, 10T/2 mouse fibroblasts, 92.1 uveal melanoma, and MCF-7 and MB-MDA-231 breast cancer cells indicating this response is not limited to the melanocyte lineage. Although Rb has been shown to be cleaved by caspases under certain conditions (Fattman et al., 1997; Fattman et al., 2001), we found that the pan-caspase inhibitor Z-VAD-FMK could not block etoposide-induced Rb degradation. This is consistent with data demonstrating that fibroblasts from mice expressing the Rb D886A mutant, which blocks caspase cleavage of Rb, still undergo Rb degradation and apoptosis (Tan et al., 1997). Studies have shown that Rb can interact with the E3 ligase Hdm2, which targets proteins such as Rb and p53 for proteosomal degradation, such that it can act as an oncogene when overexpressed (Sdek et al., 2005; Sdek et al., 2004; Xiao et al., 1995).

There is some debate in the field as to whether Hdm2 binds the hypophosphorylated or hyperphosphorylated Rb and whether or not Hdm2 ubiquitinates Rb, but there is a consensus that Rb can undergo Hdm2- and proteosomal-dependent degradation. Sdek et al claim that Hdm2 preferentially interacts with hypophosphorylated Rb. However, their immunoprecipitations involve a GST-Rb fusion protein expressed in U2OS cells. We argue that our more physiological system of using endogenous Rb, while resulting in contradictory data, is more relevant (Sdek et al., 2004). This group agrees that the Hdm2-mediated degradation of Rb is dependent on the proteosome, but argues it is ubiquitin-independent (Sdek et al., 2005). However, their data demonstrating that the ligase-inactive Hdm2 RING finger mutant, in which cysteine464 is mutated to an alanine, abolishes degradation of Rb argues against their conclusion that this mechanism is independent of ubiquitination. The RING finger motif, consisting of cysteine and histidines which interact with two zinc ions, is important for ubiquitination activity (Fang et al., 2000; Itahana et al., 2007). Further, other groups have found that Hdm2 does, indeed, ubiquitinate Rb in an

overexpression setting and an endogenous setting (Uchida et al., 2005; von Willebrand et al., 2003).

While several groups have looked at the Rb-Hdm2 interaction, the phosphorylation sites and mechanism were previously unknown. We shed light on the mechanism behind the Rb-Hdm2 interaction by revealing that phosphorylation of Rb on Ser567 enhances the Rb-Hdm2 interaction. von Willebrand et al mutated 11 of the 16 possible phosphorylation sites and found that Rb was still ubiquitinated. Interestingly, the only sites they did not mutate were Ser567 and 4 sites in the N-terminus, supporting our findings that Ser567 plays a role in the ubiquitination and degradation of Rb (von Willebrand et al., 2003).

Not only does phosphorylation of Ser567 lead to an interaction between Rb and Hdm2, but it also leads to an Rb-p53 interaction. We confirm that a trimeric complex can be formed between Hdm2, Rb, and p53 in our system (Hsieh et al., 1999; Hsieh et al., 2002; Yap et al., 1999). Our data provide a mechanistic explanation for how Hdm2, an oncogene, links the two most prominent tumor suppressors. This interaction between Rb and Hdm2 and p53 represents a rare circumstance in which phosphorylation of Rb enhances, rather than abolishes, its interaction with binding partners.

Our cell culture findings are supported by work on human tissue demonstrating that Ser567 is a marker for high levels of stress in uveal melanoma patients. Our lab has performed gene expression profiling on uveal melanomas and found that the tumors can be divided into two classes which determine the likelihood of survival. Class 1 tumors have a spindle morphology and almost never metastasize (low grade). Class 2 tumors have an epithelioid morphology, and a high probably of metastasis and death (high grade) (Onken et al., 2004). We have found that phospho-Rb-Ser567 positive staining correlates with class 2 patients, while class 1 patients were negative for Ser567 phosphorylation. This information could be valuable in Rb-targeted cancer therapy. As proof of principle, our lab created short peptides of the amino acids surrounding

Ser567. Preliminary experiments revealed that the Ser567 peptide kills cancer cells. Taken together we have demonstrated that Ser567 phosphorylation promotes Hdm2-mediated degradation of Rb ultimately resulting in cell death, is a predictor of metastatic death in uveal melanoma, and is a potential target for therapeutic applications.

3.6 Figures

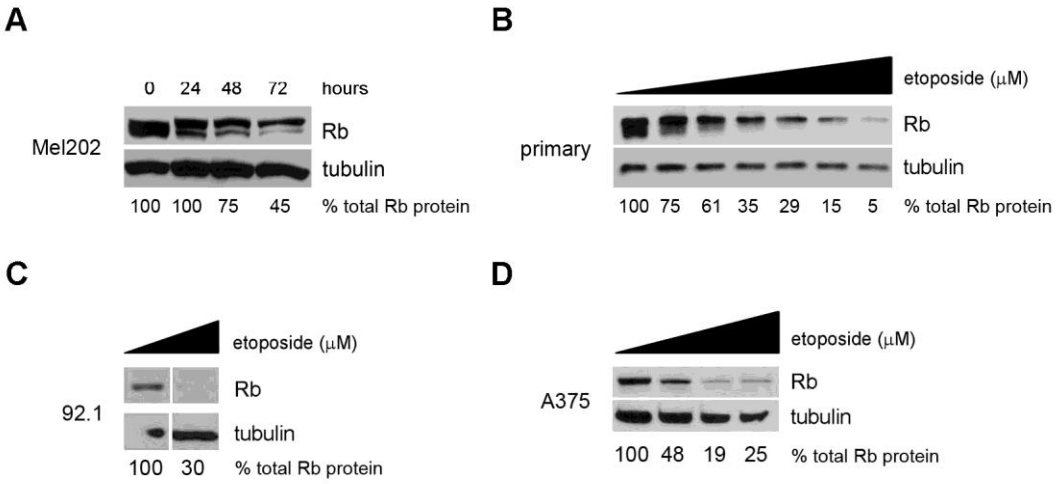

Figure 3.1 Genotoxic stress leads to degradation of Rb in melanoma cells and melanocytes

(**A**) Mel202 cells were treated with 12.5 μM etoposide for 0, 24, 48, 72 h and immunoblotted for Rb. (**B**) Primary human uveal melanocytes were treated with 0, 1.5, 3, 6, 12.5, 25, 50 μM etoposide for 48 h and immunoblotted for Rb. (**C**) 92.1 uveal melanoma cells were treated with 6 or 12.5 μM etoposide for 48 h and immunoblotted for Rb. (**D**) A375 cutaneous melanoma cells were treated with 0, 1.5, 6, 12.5 μM of etoposide for 48 h and immunoblotted for Rb.

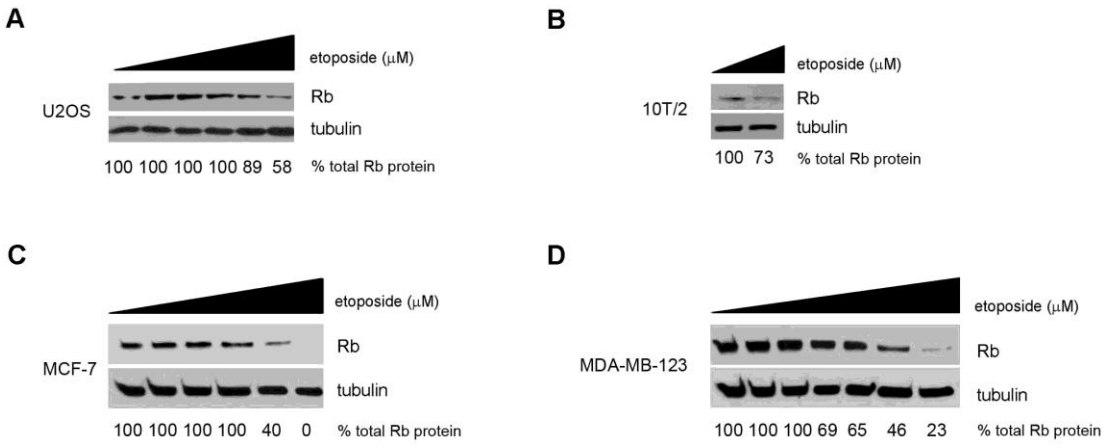

Figure 3.2 Genotoxic stress leads to degradation of Rb in other cell types

(**A**) U2OS osteosarcoma cells were treated with 0, 1.5, 3, 6, 12.5, 25 μM of etoposide for 48 h and immunoblotted for Rb. (**B**) 10T/2 mouse fibroblasts were treated with 1.5 or 3 μM of etoposide for 48 h and immunoblotted for Rb. (**C**) MCF-7 cells were treated with 0, 0.7, 1.5, 6, 12.5, 25 μM of etoposide for 48 h and immunoblotted for Rb. (**D**) MDA-MB-123 cells were treated with 0, 0.7, 1.5, 6, 12.5, 25, 50 μM of etoposide for 48 h and immunoblotted for Rb.

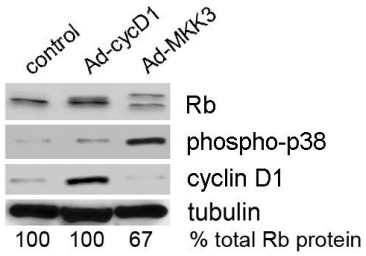

Figure 3.3 Activation of p38 leads to degradation of Rb

Mel202 cells were treated with Ad-GFP, Ad-cycD1, or Ad-MKK3 at an MOI of 30 for 48 h and immunoblotted for total Rb, phospho-p38, or cyclin D1.

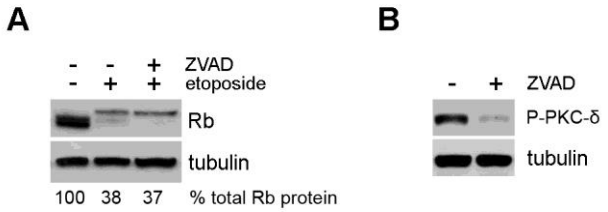

Figure 3.4 Caspase inhibition fails to block etoposide induced phosphorylation of Rb

(**A**) ZVAD-FMK and etoposide were used at 50 µM and 12.5 µM respectively for 72 h. Lysates were immunoblotted for Rb. (**B**) Control demonstrating ZVAD-FMK is active. 50 µM of ZVAD-FMK inhibited phospho-PKC-δ, a downstream caspase target.

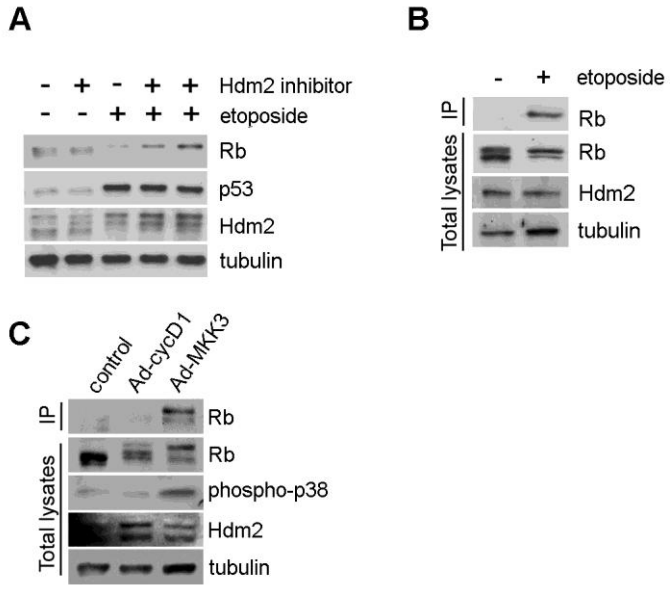

Figure 3.5 Phosphorylation of Rb by p38 enhances the Rb-Hdm2 interaction

(**A**) Mel202 cells were treated for 48 h with or without 12.5 µM of etoposide. 50 µM of Hdm2 inhibitor was used in lane 2, 5 µM in lane 4, and 10 µM in lane 6. No Hdm2 inhibitor was used in Lanes 1 and 3. (**B**) Mel202 cells were treated with 12.5 µM etoposide for 48 h. Immunoprecipitation for endogenous Hdm2 was performed and samples were immunoblotted for endogenous Rb. Total lysates were immunoblotted for Rb and Hdm2. (**C**) Mel202 cells were treated with Ad-cycD1 or Ad-MKK3 at an MOI of 15 for 48 h, immunoprecipitated for endogenous Hdm2, and immunoblotted for endogenous total Rb. Total lysates were immunoblotted for Rb, phospho-p38, and Hdm2.

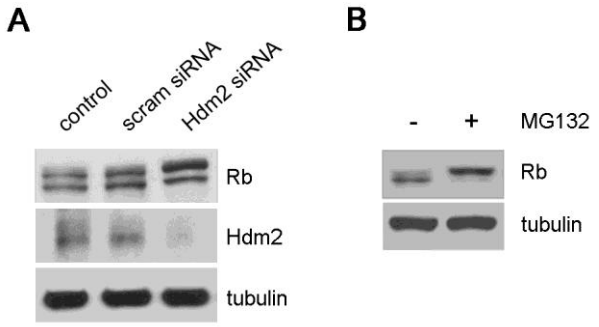

Figure 3.6 Rb is ubiquitinated and degraded in the proteosome

(**A**) Mel202 cells were treated with 50 nM of scrambled or Hdm2 siRNA for 72 h and immunoblotted for Rb or Hdm2. (**B**) Mel202 cells were treated with 50 µM of MG132 for 3 h and immunoblotted for Rb.

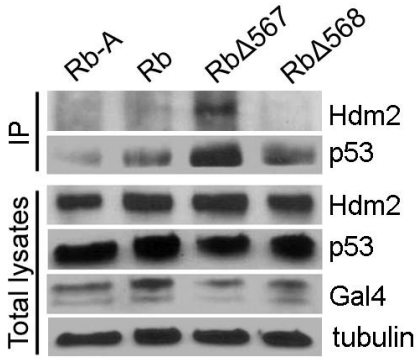

Figure 3.7 Phosphorylation of Rb on Ser567 by p38 enhances the Rb-Hdm2 interaction

Mel202 cells were transfected with Gal4-tagged Rb constructs: Rb-A (the A domain of Rb serves as a negative control), Rb, RbΔ567, or RbΔ568. Cells were immunoprecipitated for Gal4 to pull down Rb, and immunoblotted for endogenous Hdm2 and endogenous p53. Total lysates were immunoblotted for Hdm2, p53, and Gal4.

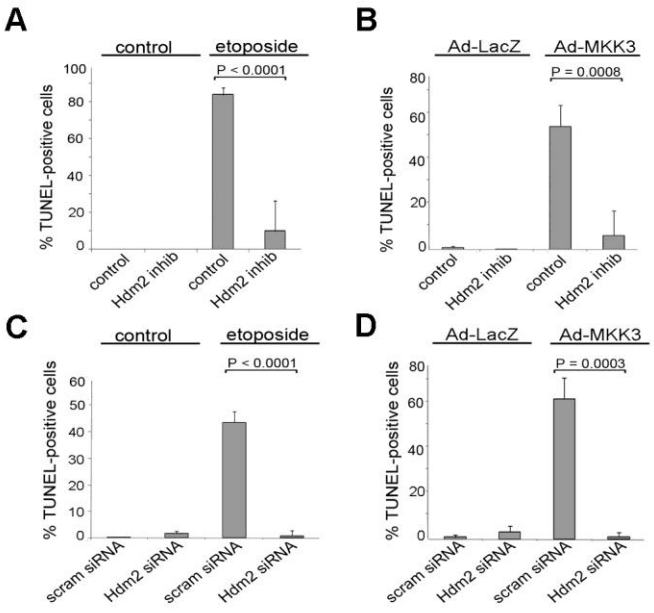

Figure 3.8 Stress-induced apoptosis is mediated by Hdm2

(**A**) Mel202 cells were treated with 10 µM Hdm2 inhibitor and 12.5 µM etoposide for 48 h and TUNEL staining was performed. (**B**) Mel202 cells were treated with 10 µM Hdm2 inhibitor for 72 h. Cells were treated Ad-LacZ or Ad-MKK3 at a MOI of 100 for 48 h and TUNEL staining was performed. (**C**) Mel202 cells were transfected with Hdm2 siRNA for 72 h and treated with 12.5 µM etoposide for 48 h and TUNEL staining was performed. (**D**) Mel202 cells were transfected with Hdm2 siRNA for 72 h and treated with Ad-LacZ or Ad-MKK3 at an MOI of 50 for 48 h and TUNEL staining was performed.

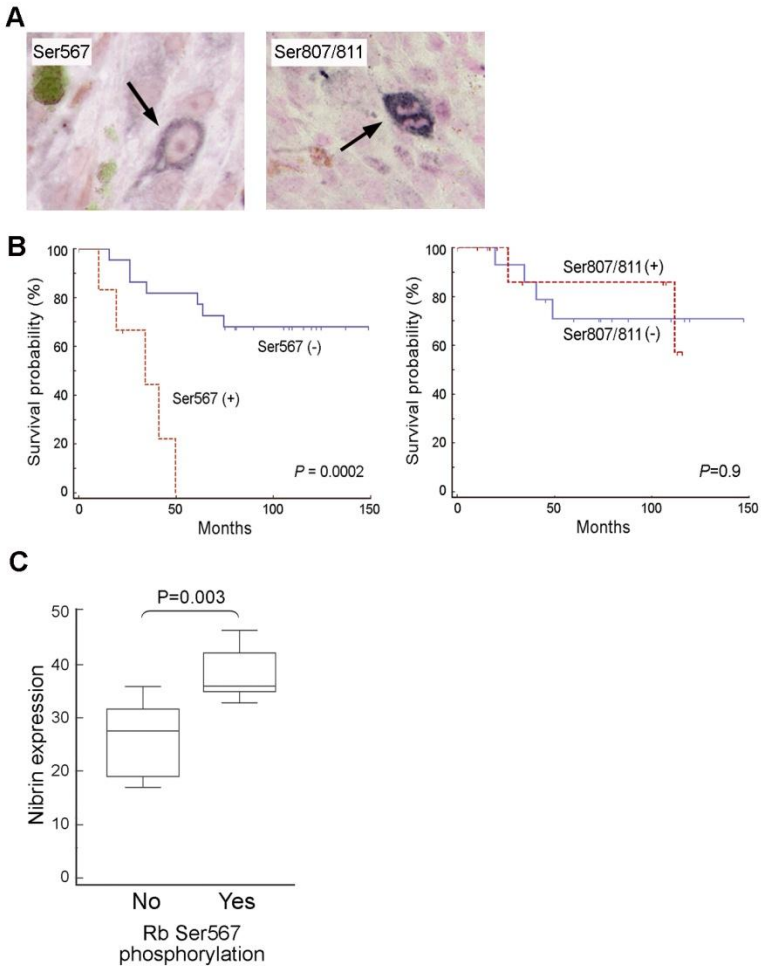

Figure 3.9 Phosphorylation of Ser567, but not C-terminal sites, correlates with poor prognosis in uveal melanoma patients

(**A**) IHC was performed on formalin-fixed, paraffin-embedded tissue sections on primary human uveal melanomas and stained for Rb-phospho-Ser567 or Rb-phospho-807-811. Arrow points to a cell staining positive for Rb-phospho-Ser567 in the cytoplasm or a cell staining positive for Rb-phospho-807-811 in the nucleus and mitotic bodies. (**B**) Kaplan Meier analysis reveals that patients with positive Rb-phospho-Ser567 staining have a worse prognosis than patients with negative staining while Rb-phospho-807-811 staining does not correlate with patient outcome. (**C**) Ser567 phosphorylation is associated with nibrin expression.

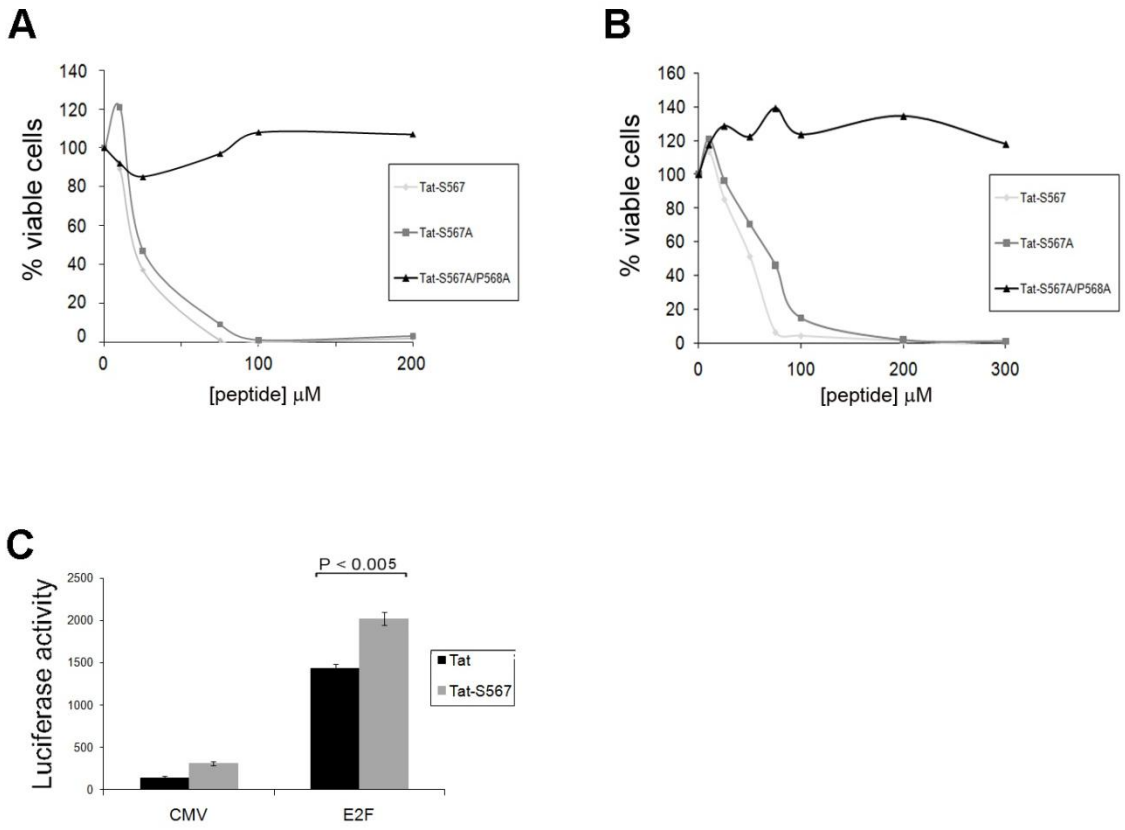

Figure 3.10 Ser567 peptide as proof of principle for Rb-directed therapy

(**A**) Tat-S567, Tat-S567A or Tat-S567A/P568A peptides were added to Mel202 cells and cell viability assays were performed 24 h later. (**B**) Tat-S567, Tat-S567A or Tat-S567A/P568A peptides were added to U2OS osteosarcoma cells and cell viability assays were performed 24 h later. (**C**) Tat alone or Tat-S567 peptides were used in a luciferase assay with the E2F-dependent cyclin E promoter. Cells were transfected with CMV empty vector or E2F1 expression construct. (M. Onken and L. Worley unpublished data)

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Chapter 4

Discussion and Future Directions

4.1 Discussion

Rb functions as a tumor suppressor by inhibiting cell division (Goodrich et al., 1991; Knudsen et al., 1998). In order for cells to divide, Rb is temporarily phosphorylated by Cdks, at up to 16 Ser/Thr-Pro phospho-acceptor sites, thereby allowing cells to traverse from G_1 into S phase (Chen et al., 1989; Hinds et al., 1992; Lin et al., 1991; Lundberg and Weinberg, 1998; Serrano et al., 1993). Most tumors co-opt this mechanism and maintain Rb in a partially hyperphosphorylated state, often by constitutive activation of cyclin-Cdks such as cyclin D1 or inactivation of Cdk inhibitors such as $p16^{Ink4a}$, allowing for continual cell cycle progression (Sherr and McCormick, 2002).

These findings are reflected in the prevailing view of Rb function as an 'on-off' regulator of the G_1/S transition in which Rb is completely inactivated every cell cycle by Cdks, releasing E2Fs to activate cell cycle genes. Though widely accepted in the Rb field, this traditional model is inadequate to account for many basic observations. The simple fact that cells do not undergo apoptosis with every attempt at cell division implies that Rb is not completely inactivated with every cell cycle as this would result E2F-mediated transcription of pro-apoptotic genes (Ginsberg, 2002; Zhao et al., 2005). The strikingly different phenotypes associated with complete Rb inactivation (by genetic deletion or excessive hyperphosphorylation) and partial Rb inhibition (by loss of $p16^{Ink4a}$ or overexpression of cyclin D) indicate that these proteins do not constitute a simple linear pathway, as previously envisaged (**Figure 4.1**) (Sherr, 2000).

We hypothesized that Rb is only partially phosphorylated and inhibited during normal cell division, and this incomplete inactivation is sufficient to block its cell cycle inhibitory function and to allow cell cycle progression without triggering apoptosis. The residual anti-apoptotic activity persists unless Rb is more completely phosphorylated (or if RB is deleted), suggesting that Rb may serve as a buffer against apoptosis during normal cell division and a checkpoint for triggering apoptosis under abnormal stress conditions such as excessive DNA damage in which apoptosis may be favored over proliferation or differentiation.

In order to test this hypothesis and explore why these mechanisms for inactivating Rb that have been assumed to be functionally equivalent result in such strikingly different phenotypic outcomes, we developed an experimental model system in which Rb degradation and apoptosis were induced by genotoxic stress in Mel202 uveal melanoma cells, which have been extensively characterized and shown to be wildtype for Rb and p53 (Sun et al., 2005). Uveal melanoma is the most common type of eye tumor with roughly half of the cases resulting in death due to liver metastasis (Harbour, 2006). Rb is necessary for melanocyte survival making it an attractive model to study Rb (Macleod et al., 1996; Yu et al., 2003). Several key experiments were confirmed using primary melanocytes or other cell lines, demonstrating our findings were not specific to Mel202 cells. We used etoposide, a DNA damaging reagent that causes double-stranded breaks to induce genotoxic stress. Our findings were confirmed using adenovirus expressing MKK3, a direct activator of p38, to induce stress.

We propose a new model, based on our hypothesis, in which Rb exists not only in fully active and fully inactive forms, but rather, in multiple phosphorylation states that have differing abilities to bind E2Fs and other proteins, and to inhibit cell division and apoptosis (Ianari et al., 2009; Lundberg and Weinberg, 1998; Ma et al., 2003). Evidence for this new model has been demonstrated. Partially phosphorylated forms of Rb bind E2Fs (Ezhevsky et al., 2001; Ezhevsky et al., 1997), and Rb continues to co-localize with E2Fs at certain promoters in vivo beyond the G_1/S transition despite being sufficiently phosphorylated to allow for cell cycle progression (Wells et al., 2000; Wells et al., 2003). Phosphorylation of Rb by Cdks during cell division does not lead to Rb degradation or apoptosis. This normal cycling depends on Rb remaining in a reversible, partially inactivated state such that it can still repress pro-apoptotic E2Fs (Wells et al., 2003; Young et al., 2003). The different phosphorylated forms of Rb appear to allow the cell to differentially control cell cycle genes and apoptotic genes, the former being derepressed by partial phosphorylation of Rb and the latter being activated by more complete phosphorylation of Rb (Young and Longmore, 2004a; Young et al., 2003; Zhang et al., 2000). The anti-proliferative and anti-apoptotic functions of Rb can be biochemically uncoupled and are both mediated largely

through interactions with E2Fs (Chau et al., 2006). Taken together, these observations indicate that the decision between proliferation and apoptosis is made, at least in part, by the manner in which Rb is phosphorylated.

These findings raise a key question: if Cdks do not completely phosphorylate nor fully inactivate Rb during the normal cell cycle, then under what physiologic conditions and by what mechanism does complete inactivation of Rb and concomitant activation of apoptotic genes occur? In recent years, other kinases capable of phosphorylating Rb have been identified, such as p38, which is activated during cellular stress and promotes apoptosis (Hou et al., 2002; Nath et al., 2003; Wang et al., 1999). p38 has been shown to phosphorylate Rb in response to stress and death receptor signaling in multiple cell types, such as endothelial cells, cerebellar neurons, Jurkat lymphocytic cells, colon cancer cells, and melanoma cells (Hou et al., 2002; Kishore et al., 2003; Lee et al., 2003; Nath et al., 2003; Wang et al., 1999; Yeste-Velasco et al., 2009). Rather than promoting cell division, phosphorylation of Rb by p38 appears to promote apoptosis under physiologic conditions of cellular stress (Bowen et al., 2002). While the ability of p38 to phosphorylate Rb has been established, it remains unclear why p38 favors apoptosis whereas Cdks promote cell division. One possibility is that p38 preferentially phosphorylates a different site or sites on Rb that confer different biochemical and, thus, physiologic effects.

Several lines of evidence point to the Ser567 phosphoacceptor site as playing an important role in Rb regulation. The Ser567-Pro568 motif is highly conserved across species (Ma et al., 2003), suggesting that it is the ability of Ser567 to be phosphorylated that is important in its function. Ser567 is the only phosphorylation site on Rb that is a target of naturally occurring mutations in human retinoblastoma (Templeton et al., 1991; Yilmaz et al., 1998), revealing that it plays a different role than the other 15 phosphorylation sites. Phosphorylation of this site Ser567 has not been reported to be phosphorylated during normal cell cycle progression (Lees et al., 1991). Ser567 is the only phosphorylation site on Rb that, when phosphorylated or mutated, disrupts the A-B box interaction, destabilizes the pocket structure, and abrogates interaction with E2Fs

(Harbour et al., 1999; Ma et al., 2003). Crystallographic studies have shown that Ser567 mediates critical contacts between the A and B boxes, and that phosphorylation of this site would be predicted to destabilize the pocket structure and eliminate the E2F binding site (Lee et al., 2002; Lee et al., 1998). Because Ser567 is buried in the pocket domain, rather than being exposed such as C-terminal residues, it is the least efficiently phosphorylated site by Cdks (Harbour et al., 1999). Phosphorylation of Ser567 by Cdks requires prior phosphorylation of the C-terminus, which contains Cdk docking sites that bring cyclin-Cdk complexes into proximity of Ser567 through an intramolecular interaction between the C-terminus and the pocket (Harbour et al., 1999). We demonstrate that p38 efficiently phosphorylates Ser567 under conditions favoring apoptosis, and this phosphorylation does not depend on the intramolecular interactions and conformational changes associated with Cdk-mediated phosphorylation. This would allow p38 to bypass the Cdks and directly regulate Rb in a cell cycle-independent manner during apoptotic signaling. The ability of p38 to phosphorylate Ser567 may be due to its small size, allowing it to access Ser567, which is located in a recess at the interface of the A and B boxes of the pocket, where it would likely be inaccessible to the much larger cyclin-Cdk complexes under normal circumstances (Harbour et al., 1999; Lee et al., 2002). Importantly, inhibition of Ser567 phosphorylation, but not of Ser780 a C-terminal site phosphorylated during the cell cycle, inhibited etoposide-induced apoptosis. Taken together, these findings suggest that p38 phosphorylates Rb in a cell cycle-independent manner and that it is the ability of p38 to phosphorylate Ser567 efficiently that distinguishes the pro-apoptotic effect of p38 phosphorylation from the proliferative effect of Cdk phosphorylation. Consistent with this hypothesis, Nath and colleagues showed that an Rb mutant with all available phosphorylation sites mutated to prevent their phosphorylation except Ser567 was unable to rescue p38-mediated inactivation of Rb (Nath et al., 2003). Similarly, von Willebrand and colleagues showed that a phosphorylation defective Rb mutant was not resistant to ubiquitination despite elimination of most of the phosphorylation sites; Ser567 was one of the few sites that were not mutated (von Willebrand et al., 2003). The ability of p38, but not Cdks, to phosphorylate Rb on Ser567 efficiently may provide a key to understanding the different effect that p38 has on Rb compared to Cdks.

In order to further characterize the mechanism of Rb-mediated apoptosis in response to genotoxic stress we investigated the roles of both E2F1 and p53—two transcription factors intricately linked to Rb. Stress led to release of E2F1 confirming our hypothesis that while Rb is only partially phosphorylated and still bound to E2F1 during the cell cycle, a more complete phosphorylation by Ser567 results loss of the Rb-E2F interaction, allowing for transcription of pro-apoptotic genes. Reduction in p53 or E2F1 levels inhibited stress-mediated apoptosis corroborating with data demonstrating that etoposide activates p53 (Lowe et al., 1993), activates E2F1 (Ginsberg, 2002), and causes E2F1 to favor pro-apoptotic target genes such as p73 rather than cell cycle target genes (Pediconi et al., 2003).

We demonstrate that phosphorylation of Rb on Ser567 enhances its interaction with Hdm2, an oncogene that is overexpressed in many human tumors including uveal and cutaneous melanoma (Brantley and Harbour, 2000; Polsky et al., 2002; Polsky et al., 2001). Herein, we provide new insights into the relationship between Hdm2, an E3 ligase and oncoprotein, and the tumor suppressors p53 and Rb. Our finding that genotoxic stress promotes interaction between Rb and Hdm2 parallels recent findings that a p53 activating compound promotes Mdm2-mediated degradation of $p21^{Cip1}$, both ultimately leading to cell death (Enge et al., 2009). It has long been known that Hdm2 interacts with and triggers the degradation of both p53 and Rb (Momand et al., 1992; Sdek et al., 2005; Uchida et al., 2005; von Willebrand et al., 2003; Xiao et al., 1995). In particular, Hdm2 binds and ubiquitinates Rb, targeting it for degradation (Sdek et al., 2004; von Willebrand et al., 2003; Xiao et al., 1995). We show that phosphorylation of Ser567 and consequent disruption of the Rb pocket may be a critical event that triggers interaction of Rb with Hdm2. This represents a rare circumstance in which phosphorylation of Rb enhances, rather than abolishes, its interaction with a binding partner. Further, it is tempting to speculate that the dual role of Hdm2 in regulating protein levels of two major tumor suppressors – Rb and p53 – is physiologically relevant, but this has not been confirmed experimentally. In this model, (**Figure 4.2**) during quiescence, Rb is hypophosphorylated and active, preventing cell cycle progression.

In parallel, Hdm2 maintains p53 at low levels by proteosomal degradation. In normal cycling cells, Rb is phosphorylated and partially inactivated by Cdks, and Hdm2 continues to maintain p53 at low levels. During low levels of stress cycle arrest would be favored through disruption of the Hdm2-p53 interaction and subsequent accumulation of activated p53. But under these conditions, p38 would not phosphorylate Rb, and Hdm2 would thus not interact with Rb. The result would be active Rb that could cooperate with p53 to trigger cell cycle arrest. At higher stress levels, apoptosis would be favored as a mechanism for eliminating a potentially deleterious cell. Disruption of the Hdm2-p53 interaction would activate p53, and release Hdm2; however, in this setting, activated p38 would phosphorylate Rb on Ser567, allowing Hdm2 to interact with Rb triggering its degradation. Furthermore, it has been shown that Rb-Hdm2 binding stabilizes p53 and promotes p53-dependent apoptosis (Hsieh et al., 1999; Yap et al., 1999). MK2, a direct transcriptional target of p38, phosphorylates Hdm2 and enhances its activity (Weber et al., 2005), thus p38 may promote the degradation of Rb through multiple complementary mechanisms. Loss of Rb and the consequent release of pro-apoptotic E2Fs, coupled with activation of p53, then tips the balance of cellular events in favor of apoptosis over cell cycle arrest (Sun et al., 2010). Without such a mechanism, activation of p53 might indiscriminately trigger both cell cycle arrest and apoptotic programs, leading to a chaotic and inefficient response to stress. Consistent with this model, p53 and Rb compete for binding to Hdm2 (Uchida et al., 2005), p53 requires Rb for efficient induction of cell cycle arrest (Harrington et al., 1998), and activation of p53 triggers apoptosis in the absence of Rb (Macleod et al., 1996).

There are ongoing efforts to target p38 in a clinical setting in a wide range of diseases and therefore insights gleaned from this work could provide new therapeutic insights. Stimulating or mimicking p38 signaling to Rb promotes cancer cell death and sensitizes cancer cells to therapy. Compounds that preferentially activate MKK3 and/or p38α in Rb positive cancer cells versus normal cells such as anisomycin (an antibiotic produced by Streptomyces griseolus) (Mauro et al., 2002) or Resveratrol (found in red wine and grapes) (Bai et al. 2010), may sensitize cells to chemotherapy and radiation therapy (Olson and Hallahan, 2004). There is evidence that the p38

pathway plays a critical role not only in tumorigenesis, but also in metastasis (Horak et al., 2008). MKK4, 6, and 7, direct activators of p38, have been shown to inhibit metastasis by promoting tumor cell dormancy. p38 activators may provide therapy against metastasis as well as tumorigenesis.

On the other hand, compounds that inhibit p38 are also currently being researched and are in clinical trials. Rb hyperphosphorylation and degradation mediates neuronal cell death in multiple neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease and stroke (Giovanni et al, 2000; Hayashi et al, 2000; Hoglinger et al, 2007; Liu & Greene, 2001; O'Hare et al, 2000; Park et al, 2000; Ramalho et al, 2004; Thakur et al, 2008). Further, p38 can regulate Rb and modulate cell death in neurons, and pharmacologic blockade of p38 has a neuroprotection effect (Hou et al, 2002; Yeste-Velasco et al, 2009). Similar mechanisms may govern the development of atherosclerosis and cardiomyocyte apoptosis that occur in association with myocardial infarction (Hauck et al, 2002; Proctor et al, 2008). p38 is upregulated in inflammatory diseases such as rheumatoid arthritis (RA) and BIB796 (Boeringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT, USA), which prevents ATP binding, and is in stage III trials for treating RA (Cuadrado and Nebreda, 2010; Pargellis et al., 2002). In some cases p38 promotes cell migration and implantation in cancer and therefore inhibiting p38, rather than activating p38 as previously discussed, would be valuable. For example, ARRY-797 (Biopharma, Rockville, MD, USA) is in phase II trials to treat cytokine driven cancers in combination with cisplatin and SCIO-469 (Scios Inc, Johnson and Johnson, New Brunswick, NJ, USA) is in phase II to treat multiple myeloma (Coulthard et al., 2009).

Although there are over a dozen p38 inhibitors currently in clinical trials many are causing adverse effects such as liver toxicity. New ways of inhibiting p38 are being developed in order to thwart this problem. One method of avoiding toxicity is to use a drug delivery system that allows for low levels of the inhibitors to be maintained in specific organs as has been recently shown for cardiac dysfunction (Cuadrado and Nebreda 2010). Another method that was recently published is the use of a fusion peptide with a p38 docking sequence and Tat transmembrane sequence

which has been shown to be anti-inflammatory in mice (Fu et al., 2008a). A third approach is to instead target downstream effectors of p38 such as the pro-inflammatory MAPKAPK2 (Coulthard et al., 2009).

In this study, we provide evidence for a pro-apoptotic signaling pathway induced by genotoxic stress, in which p38 phosphorylates Rb in a manner that triggers E2F1-, p53-, and Hdm2-mediated cell death. The Rb residue Ser567 is implicated as a key target of this p38-mediated phosphorylation, providing a molecular explanation for why Rb phosphorylation by p38 differs mechanistically and physiologically from that catalyzed by Cdks. This p38-mediated pathway could provide a rapid, cell cycle-independent means for overriding the cell cycle and triggering apoptosis in response to genotoxic stress. These findings may provide new insights for therapeutic intervention, both to inhibit cell death in degenerative diseases and to promote cell death in cancer where manipulation of this mechanism could kill cells directly or sensitize them to radiation or chemotherapy.

4.2 Future directions

Determine if other E2F family members play a role in our experimental model

Our studies determined that phosphorylation of Rb on Ser567 led to derepression of E2F1. E2F1 was investigated because it is the main E2F member involved in apoptosis and is required for p53-depedent apoptosis. Loss of E2F1 causes a massive reduction in apoptosis and overexpression of E2F1 increases apoptosis (Pan et al., 1998; Stevaux and Dyson, 2002). Interestingly, Rb has a separate binding site contained in the C-terminus, rather than spanning the pocket domain and C-terminus, that only binds the E2F1 family member. E2F1 levels are increased during etoposide treatment and the binding to Rb on this separate site is lost (Dick and Dyson, 2003). Future work will be necessary to determine if other E2F family members play a role.

Determine if other stress kinases phosphorylate Rb on Ser567

We chose to focus on p38 because there are many lines of evidence revealing that p38 phosphorylates Rb during stress. While it was not known that p38 phosphorylates Rb in our system, p38 had been shown to phosphorylate Rb in several other cell types (Hou et al., 2002; Kishore et al., 2003; Lee et al., 2003; Nath et al., 2003; Wang et al., 1999; Yeste-Velasco et al., 2009). However, this mechanism is not mutually exclusive with other strategies that the cell may employ for differentially regulating cell proliferation and apoptosis, such as through PI3K/Akt signaling to block the E2F1 apoptotic program during proliferation (Dick and Dyson, 2003; Hallstrom et al., 2008; Hallstrom and Nevins, 2003) or through ATM which has been shown to affect Rb phosphorylation levels (Pizarro et al. 2010). Other MAPK members such as ERK1/2 or JNK could also be tested (Bowen et al., 2002; Chauhan et al., 1999; Wang et al., 1999). Additional stress kinases could work in concert with p38 to promote Rb degradation and apoptosis. It would be interesting to examine if these kinases phosphorylate Rb directly on Ser567 in a cell cycle-independent manner as p38 does.

An unbiased approach to identify kinases that phosphorylate Rb during stress could be employed by using a serine/threonine kinase array (Invitrogen Protoarray, Carlsbad, CA) (Jones et al., 2006). In vitro kinase assays would be performed to validate array results. Investigating other kinases that phosphorylate Ser567 will allow for additional therapeutic targets.

Unbiased approach to look at differential phosphorylation

We specifically looked at Ser567 for a number of reasons; it is not phosphorylated during the normal cell cycle, it is a target of missense mutations in tumors, and it is located at a unique location at the interface of the A and B boxes in the pocket domain (Lee et al., 2002; Lee et al., 1998; Templeton et al., 1991; Yilmaz et al., 1998). However, there are 15 other phosphoacceptor sites on Rb. An unbiased approach to look at all 16 phosphorylation sites in both a cell cycle setting and a stress setting would be an interesting future experiment. We performed preliminary mass spectrometry experiments in collaboration with Reid Townsend and obtained excellent coverage of the Rb protein, but unfortunately we were not able to obtain phosphorylated Rb

fragments. Further troubleshooting will need to be done in order to obtain Rb phospho-peptides by mass spectrometry.

An unbiased approach to determine how differential phosphorylation of Rb by Cdks versus p38 regulates its interaction with E2Fs and its occupation of cell cycle versus apoptotic gene promoters

ChIP-chip or chIP-sequencing (chIP-seq) could be performed as an unbiased approach to look at what promoters Rb is bound to during cell cycle conditions versus apoptotic conditions. Previous experiments have investigated Rb promoter occupation by chIP, but none to date have addressed this question. The Farnham lab performed chIP experiments on Rb during different stages of the cell cycle (Wells et al., 2000). Other groups have looked at Rb on specific promoters after stress, for example, in doxorubicin treated MCF-7 breast cancer cells and T98G glioblastoma cells (Ianari et al., 2009; Jackson and Pereira-Smith, 2006) or by TNF-α stimulation in *Ramos* Burkitt's lymphoma *cells (Dasgupta et al., 2004),* but all of these studies looked at only a handful of promoters, rather than using a promoter array.

Several groups have used chIP-chip or chIP-seq in order to look at Rb promoter occupation in an unbiased manner. ChIP-chip experiments have been performed on Rb in G_0 or S phase arrested cells (Wells et al., 2003) and on E1A treated fibroblasts (Young and Longmore, 2004b). The newly developed chIP-seq has been used to investigate the role of the pocket protein family in quiescence and senescence in fibroblasts (Chicas et al. 2010). While these studies added valuable knowledge to the Rb field none of them address how differential phosphorylation may regulate Rb promoter occupation. We performed chIP experiments for Rb in cells that were treated with etoposide or untreated and looking at E2F targets such as TP53 and APAF1. We found that TP53 and APAF1 were bound to Rb in untreated cells but not in treated cells. The samples were sent to NimbleGen (HG18 RefSeq Promoter C4226-00-01), but unfortunately the experimental samples were not enriched compared to the input and no data was obtained. Further troubleshooting will need to be performed in order to complete this experiment.

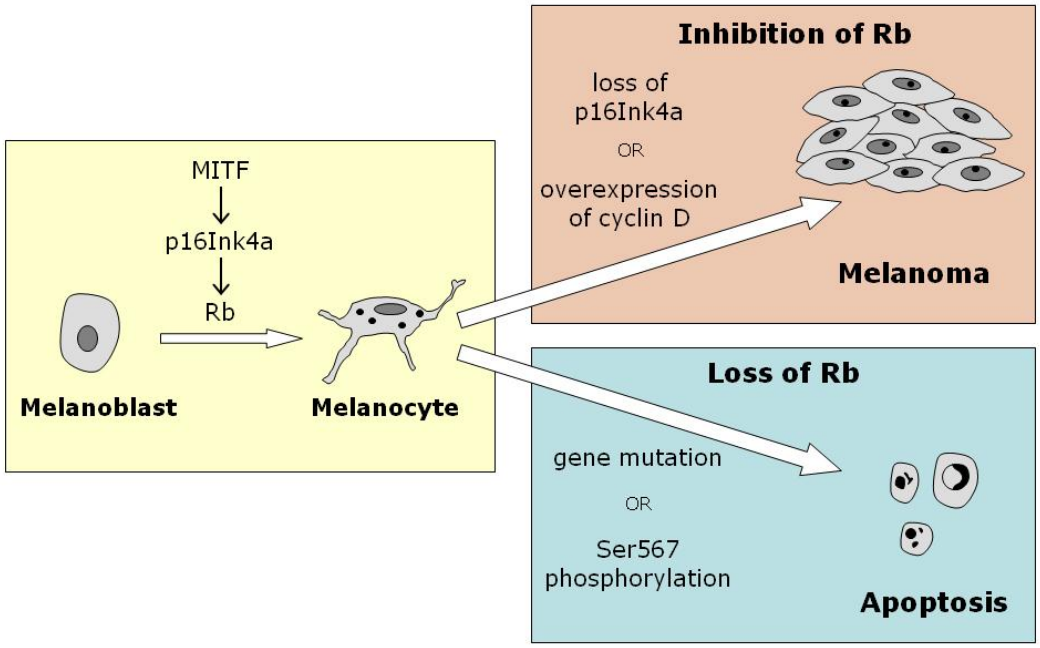
Look at the role of Ser567 phosphorylation in other cancers

We investigated the role of Rb hyperphosphorylation in patient prognosis. A phospho-Rb-567 specific antibody was used in IHC on uveal melanoma samples from Dr. Harbour's patients and Ser567 phosphorylation correlated with patient outcome. Phosphorylation at this site may be an indicator of severely stressed and abnormal cells in other cancers as well.

Investigate the Role of the p38-Rb pathway in vivo

p38α floxed mice (Engel et al., 2005) could be crossed with Tyrosinase-Cre mice (Tonks et al., 2005; Tonks et al., 2003) in order to obtain conditional knockout (CKO) mice with p38α specifically knocked out in melanocytes. Tyrosinase is an enzyme involved in the early stages of melanogenesis (Pawelek et al., 1980). A p38α conditional mouse would be used because the p38α knockout is embryonic lethal at day E10.5 due to vascularization defects (Adams et al., 2000; Allen et al., 2000; Mudgett et al., 2000; Tamura et al., 2000). First, the phenotype of these mice would be assessed as these mice have never been crossed before. Rb phosphorylation status, amount of death, and amount of proliferation could be examined. Additionally, the initiating agent 7,12-dimethyl-benz[a]anthracene (DMBA) followed by the promoting agent the phorbol myristate acetate (PMA) could be administered to the skin of the mice to induce melanoma formation (Abel et al., 2009; Broome Powell et al., 1999). Differences in Rb phosphorylation status, apoptosis, proliferation, and tumor burden would be investigated. We would predict that loss of p38α would result in a loss of ability to remove melanocytes exposed to carcinogenic DNA damage. Loss of p38α may accelerate tumor progression through more rapid accumulation of DNA damage and genomic instability. Consistent with this hypothesis, p38α loss has been shown to induce cardiomyocyte proliferation in vivo (Engel et al., 2005).

4.3 Figures



4.1 Rb is inactivated by several mechanisms

Rb plays a role in melanocyte differentiation, proliferation, and apoptosis. In melanocytes, Mitf stimulates Rb hypophosphorylation through activation of $p16^{Ink4a}$, which couples cell cycle exit with differentiation. Rb is also necessary to maintain a differentiated state, since partial phosphorylation of Rb by loss of $p16^{Ink4a}$ or overexpression of cyclin D causes melanocytes to re-enter the cell cycle. Complete inactivation of Rb through genetic mutations or through excessive phosphorylation (e.g., Ser567 phosphorylation) leads to high levels of free E2F and favors apoptosis over proliferation. Adapted from (Delston and Harbour, 2006).

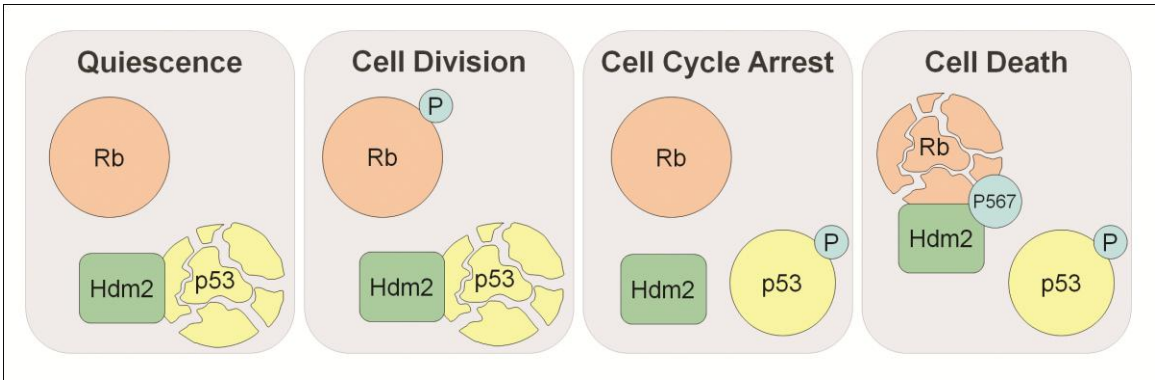


Figure 4.2 Hypothesis for how p38-mediated phosphorylation of Rb on Ser567 provides a death signal, rather than the Cdk-mediated proliferative signal, and how shuttling of Hdm2 between p53 and Rb may tightly regulate the cellular response to stress

In quiescent cells, Rb is hypophosphorylated and active, preventing cell cycle progression. In parallel, Hdm2 maintains p53 at low levels by proteosomal degradation. In normal cycling cells, Rb is phosphorylated and partially inactivated by Cdks, and Hdm2 continues to maintain p53 at low levels. When cells are exposed to low levels of genotoxic and perhaps other forms of stress, p53 is phosphorylated and released from Hdm2, allowing cellular levels of p53 to increase. A major effect of this increase in p53 is transactivation of $p21^{Cip1}$ which, in turn, promotes hypophosphorylation and activation of Rb. Rb is available because it has not been degraded by activation of p38. The net result is stress-induced cell cycle arrest. When cells are exposed to higher levels of stress, p53 accumulates and, in addition, p38 is activated to phosphorylate Rb on Ser567, which promotes interaction with Hdm2, degradation of Rb, and E2F1-mediated apoptosis. With Rb degraded and unavailable to cooperate with p53 to induce cell cycle arrest, the apoptotic genes under basal inhibition by Rb are derepressed, leading to an apoptotic response. Taken from (Delston et al., 2010)

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RACHEL BAKER DELSTON

6931 Amherst Ave, St. Louis, MO 63130
rdelston@gmail.com **314-303-9625**

EDUCATION

Washington University in St. Louis, MO, Ph.D. Candidate, Molecular and Cellular Biology, expected to graduate Fall of 2010

Oberlin College, Oberlin, OH, B.A. May, 2002, Majors: Biochemistry and Biology

EXPERIENCE

Washington University in St. Louis, MO, Dr. J. William Harbour
PhD Candidate, August 2003-present
Project: p38 phosphorylates Rb on Ser567 by a novel, cell cycle-independent mechanism that triggers Rb-Hdm2 interaction and apoptosis

University of California, San Francisco, Dr. Rik Derynck
Research Assistant, July 2002-July 2003
Project 1: Determine the relationship between Interferon Regulatory Factors and Smads
Project 2: In vivo expression of Histone Deacetylase 4 and 5

National Cancer Institute, National Institutes of Health, Dr. Anita Roberts
Summer Cancer Research Training Award, Summer 2001
Research Intern, January 2001
Project: The role of Smad3 in cell inflammation using irradiated primary keratinocytes with and without Smad3

Food and Drug Administration, Washington, D.C., Dr. Ben Tall
Summer Research Intern, Summer 2000, Summer 1999
Research Intern, January 1999
Project: Identified and characterized two hemagglutinin factors, a hemolysin and a protease, expressed by Vibrio tubiashii, a clam pathogen

Oberlin College
Reference Assistant at Oberlin College Science Library, September 2001-May 2002
Tutor for Cellular and Molecular Biology Course, September 2002-May 2002
Research Assistant, October 2000-May 2001
Project: Separation of nitrogen-containing compounds from bomb residues using Reverse Phase High Performance Liquid Chromatography

FUNDING AWARDS

Washington University Vision Training Grant, December 2006-December 2009
- Paid for 3 years of my stipend, travel money, and research money
- Involved a class and journal club

Washington University Cancer Pathway Fellowship, September 2004- September 2006
- Paid for one year of my stipend

- Involved classes, journal clubs, and shadowing physicians

MEETINGS ATTENDED

Pre-ARVO (The Association for Research in Vision and Ophthalmology) Ocular Oncology Course, May 2009, Fort Lauderdale, FA

- Invited Speaker

NIH Graduate Student Research Festival, September 2008, Bethesda, MD

- Poster presenter

Gordon Conference on Cell Growth & Proliferation, June 2007, Bidderford, ME

- Poster presenter

AACR Special Conference, TGF-beta in Cancer and other Diseases, February 2006, La Jolla, CA

American Society for Microbiology 100th General Meeting, May 2000, Los Angeles, CA

- Selected for Student Travel Grant Award to attend the meeting
- Poster presenter

OTHER

- Reviewed papers for Molecular and Cellular Biology and Oncogene in 2007, Molecular Cancer Research in 2008
- Reviewed grants for Wellcome Trust Grant in 2005 and 2007
- Mentored undergraduate students and rotation students in the Harbour lab, 2005-present
- Peer mentor for MCB program at Washington University, 2004-2009
- Teaching assistant for Bio 3411 Principles of the Nervous System, Washington University, 2004

PUBLICATIONS

Delston, R.B., Matatall, K., Sun, Y., Onken, M.D., Harbour, J.W. p38 phosphorylates Rb on Ser567 by a novel, cell cycle-independent mechanism that triggers Rb-Hdm2 interaction and apoptosis. *Oncogene*. Accepted.

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