The Role of the Retinoblastoma Protein in Mitochondrial Apoptosis

By

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Abstract

The retinoblastoma protein (pRB) tumor suppressor is deregulated in the vast majority of human tumors. pRB is a well-established transcriptional co-regulator that influences many fundamental cellular processes. It has been most well characterized in its ability to block cell proliferation by inhibiting the E2F family of transcription factors. Importantly, pRB also plays a pivotal role in apoptosis. This function has been extensively characterized in the context of genotoxic stress. Specifically, these studies have revealed that pRB can act in both an anti-apoptotic manner by inducing cell cycle arrest, and a pro-apoptotic manner by transcriptionally co-activating pro-apoptotic genes. Here, we show that pRB can also promote TNFα-induced apoptosis. Moreover, this investigation led us to uncover a novel, non-transcriptional and non-nuclear role of pRB in the induction of apoptosis. Specifically, we found that pRB can enhance TNFα-induced apoptosis even in the presence of an inhibitor of translation, and that a fraction of endogenous pRB is localized at the mitochondria both in the absence and presence of treatment with apoptotic stimuli. Further characterization revealed that pRB can directly bind to and activate BAX, resulting in mitochondrial outer membrane permeabilization and apoptosis. Importantly, targeting ectopically expressed pRB specifically to the mitochondria generated a separation-of-function mutant deficient for pRB's classic, nuclear roles. Remarkably, we found that this mito-tagged pRB mutant can promote apoptosis in response to many apoptotic stimuli, arguing that mitochondrial pRB is a general mediator of apoptosis. Moreover, expression of this mito-pRB mutant in vivo was sufficient to suppress tumorigenesis. Taken together, our data uncover a role for pRB in the direct activation of mitochondrial apoptosis. To our knowledge, this is the first characterization of a non-nuclear and transcription-independent function for pRB. Moreover, most human tumors are wild-type for pRB, but contain alterations that result in constitutive phosphorylation of pRB. While this functionally inactivates pRB's cell cycle function, we show that pRB's mitochondrial role is unaffected. This raises the possibility that this novel pro-apoptotic pRB mechanism can be exploited for chemotherapeutic treatment.

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Chapter One

Introduction
The retinoblastoma protein (pRB) tumor suppressor is affected in most cancers. pRB functions as a transcriptional co-regulator in many cellular processes including proliferation and apoptosis. In investigating its role in TNFα-induced apoptosis, I have uncovered a novel function of pRB in inducing apoptosis directly at the mitochondria. In this chapter I will introduce the pRB tumor suppressor and the apoptotic pathway from a historical perspective, with a particular emphasis on discussing the myriad cellular roles of pRB as well as the regulation of mitochondrial apoptosis induction. I will conclude with a summary of our current understanding of pRB's role in apoptosis.

**Part I: The Retinoblastoma Tumor Suppressor Protein**

**A: Discovery of the Retinoblastoma Protein**

The retinoblastoma protein derives its name from retinoblastoma, a malignant tumor of the eye and one of several heritable forms of childhood cancer. About half of the cases occur in young children from affected families, where retinoblastoma can occur bilaterally. The remaining cases typically occur unilaterally and sporadically in the general population. Analysis of the hereditary pattern of retinoblastoma led Alfred Knudson to postulate the two-hit hypothesis (Knudson, 1971), which argues that retinoblastoma is a result of two mutational events, now known to inactivate both copies of a single gene. In the sporadic form of the disease, both copies of the gene must be inactivated in the same cell. In contrast, in the inherited or childhood form of the disease, one inactive copy of the gene is inherited. Thus, only the second copy needs to be lost somatically, an event referred to as loss of heterozygosity, for retinoblastoma to develop. This results in a dramatically higher chance of developing the disease at multiple sites and in both eyes (bilaterally). Analysis of a subset of retinoblastoma samples
mapped susceptibility to retinoblastoma to chromosome 13q14 (Benedict et al., 1983; Dryja et al., 1984), allowing for the subsequent cloning of the retinoblastoma protein gene, RB-1, as the first tumor suppressor (Friend et al., 1987; Fung et al., 1987; Lee et al., 1987b). Further molecular analysis of RB-1 revealed that it encodes a primarily nuclear phosphoprotein that is approximately 110 kilodaltons in size (Lee et al., 1987a). It was later shown that pRB function is deregulated in most, if not all human tumors (Sherr and McCormick, 2002). However, inactivating mutations in RB-1 itself are only found in about one-third of human tumors and are most prevalent in retinoblastoma, osteosarcoma, and small cell lung carcinoma (Chauveine et al., 2001; Kaye and Harbour, 2004; Weinberg, 1992). In contrast, the majority of human tumors carry mutations in the upstream regulatory pathway, which results in inactivation of some, but not all of the functions described below (Sherr and McCormick, 2002).

B: Functions of the Retinoblastoma Protein

Much of the early structural and functional analyses of pRB were informed by the study of how small DNA tumor viruses cause transformation of cells in vitro and tumor formation in vivo. Identification of the viral oncoproteins E1A, large T-antigen, and E7 in adenovirus, simian virus 40, and human papillomavirus, respectively, led to the discovery of pRB as one of their main cellular targets and suggested that pRB normally functions to suppress cellular transformation and tumor formation (DeCaprio et al., 1988; Dyson et al., 1989; Whyte et al., 1988). It was also found that pRB underwent phosphorylation in a cell cycle dependent manner, suggesting that this tumor suppressing activity relates to regulation of proliferation (Buchkovich et al., 1989; Chen et al., 1989; DeCaprio et al., 1989; Mihara et al., 1989). Finally, it was shown that some viral oncoproteins preferentially bind hypophosphorylated pRB (Ludlow et al., 1990) and mutational analysis of these viral oncoproteins identified a highly conserved LxCxE motif
that is necessary for pRB binding (Dyson et al., 1990; Dyson et al., 1989; Munger et al., 1989; Stabel et al., 1985; Vousden and Jat, 1989). This led to the hypothesis that the tumor suppressor pRB can be inactivated in three ways: mutation of RB-1, binding to a viral oncoprotein, or phosphorylation (either as part of the normal cell cycle or constitutively as a result of mutations in the regulatory pathways).

i. **Structure and Posttranslational Modifications of the Retinoblastoma Protein**

Detailed biochemical analysis of the 928 amino acid pRB protein revealed that it consists of three structural domains – the N-terminal domain, the Small Pocket, and the C-terminal domain (Figure 1). Naturally-occurring mutations in RB-1 and the LxCxE binding cleft recognized by viral oncoproteins are located in the Small Pocket (Hu et al., 1990; Huang et al., 1990; Kaelin et al., 1990), suggesting that this region is particularly important for tumor suppression. The Small Pocket in turn contains the A and B folds, which interact to form a dumbbell shaped globular domain (Lee et al., 1998). The two folds are highly conserved amongst all members of the pocket protein family (pRB, p107 and p130) and are separated by a poorly conserved region called the spacer (Dynlacht et al., 1994; Mulligan and Jacks, 1998; Zhu et al., 1995). The Small Pocket and C-terminal domain compose the Large Pocket, which has been shown to be both necessary and sufficient for tumor suppression (Hiebert, 1993; Qin et al., 1992; Yang et al., 2002). The bipartite nuclear localization signal of pRB maps to the C-terminal domain. The highly conserved LxCxE binding cleft that was identified by virtue of being recognized by viral oncoproteins is now known to mediate interactions with many cellular proteins that also contain the LxCxE motif and that play a role in many cellular processes including proliferation, apoptosis, and differentiation.
Figure 1. Structure of the Retinoblastoma Protein. pRB is comprised of three general domains, the N-terminal domain, the Small Pocket, and the C-terminal domain. Together the Small Pocket and C-terminal domain form the Large Pocket, which is both necessary and sufficient for tumor suppression. The LxCxE binding cleft is localized in the Small Pocket and the Nuclear Localization Signal is localized in the C-terminal domain. pRB contains several CDK-phosphorylation sites, and their approximate localization is denoted by a “P”.
As mentioned above, pRB is phosphorylated in a cell-cycle dependent manner, which inactivates its cell cycle function (described in detail in the next subsection). pRB is hypophosphorylated in non-proliferating cells and in early G1, and becomes increasingly more phosphorylated as cyclin-CDKs become activated and the cell enters the cell cycle (Sherr, 1994). During mitosis, pRB is dephosphorylated, reactivating its cell cycle function (Ludlow et al., 1993). Phosphorylation does not affect the stability of the protein, but instead affects pRB activity by disrupting intermolecular interactions and promoting intramolecular interactions (Chinnam and Goodrich, 2011). To date, 16 putative CDK phosphorylation sites have been identified and mutation of 11 of these sites together results in a constitutively active pRB protein with regard to cell cycle regulation (Knudsen and Wang, 1997). Cyclin-CDK kinases are in turn regulated by CDK inhibitors, including p16 (Lukas et al., 1995; Serrano et al., 1993). Importantly, several tumorigenic events, including inactivating mutations affecting CDK inhibitors or gene amplifications and gain-of-function mutations affecting cyclin-CDKs, result in constitutive hyperphosphorylation of pRB and thus abnormal proliferation (Figure 2).

Interestingly, hyperphosphorylated pRB appears to be less nuclear than hypophosphorylated pRB (Mittnacht and Weinberg, 1991). It is unclear whether this reflects a loss of nuclear tethering of hyperphosphorylated pRB as a result of differential protein and chromatin interactions or a more active export mechanism (Fulcher et al., 2010; Jiao et al., 2006; Jiao et al., 2008; Roth et al., 2009). Notably, this cytoplasmic pRB species includes pRB that is localized to mitochondria (Ferecatu et al., 2009). This thesis provides evidence that this mitochondrial pRB species functions in induction of apoptosis.

pRB also contains phosphorylation sites recognized by kinases other than CDKs. Furthermore, pRB is also subject to posttranslational modifications other than phosphorylation.
Figure 2. Tumorigenic events resulting in inactivation of pRB’s cell cycle function. pRB plays an important role in regulating entry into the cell cycle. Phosphorylation of pRB by cyclin-CDKs inactivates that function resulting in progression into $S$ phase. Tumorigenic events that result in constitutive activation of cyclin-CDKs (loss-of-function mutations in the CDK inhibitor p16 or gain-of-function mutations affecting cyclin-CDKs) therefore induce formation of constitutively phosphorylated pRB and abnormal cell proliferation.
pRB is both phosphorylated and acetylated in response to DNA damage, although the functional consequence of these modifications is poorly understood (Chan et al., 2001; Inoue et al., 2007; Markham et al., 2006). Additionally, pRB can be sumoylated (Ledl et al., 2005), but the function of this modification is also unclear. Finally, it has been shown that pRB contains a caspase cleavage site near the C-terminus, which is cleaved upon TNFa treatment resulting in removal of the C-terminal 42 amino acids of pRB (Janicke et al., 1996; Tan et al., 1997). A knock-in mouse model expressing a non-cleavable form pRB is impaired for apoptosis (Chau et al., 2002). Thus, the cleavage site likely plays a role in apoptosis though the mechanistic consequence of cleavage is poorly understood.

ii. Regulation of Cell Cycle Entry

As described above, pRB is phosphorylated in a cell-cycle dependent manner and preferentially bound by viral oncoproteins when hypophosphorylated. This suggested that pRB’s tumor suppressive activity may relate to the regulation of cell cycle entry. It had also been shown that adenoviral infection resulted in increased activity of the cellular transcription factor E2F, which was known to regulate expression of genes important for proliferation (Kovesdi et al., 1986; La Thangue and Rigby, 1987). Further studies linked these observations, establishing that in normal cells E2F exists in a protein complex with pRB (Bandara and La Thangue, 1991; Chellappan et al., 1991). This allowed for the cloning of the gene encoding the E2F1 transcription factor (Helin et al., 1992; Kaelin et al., 1992; Shan et al., 1992). Subsequently, it was shown that E2F1 interacts with hypophosphorylated pRB and this interaction results in repression of E2F1 activity both by masking its transactivation domain (Dynlacht et al., 1994; Flemington et al., 1993; Helin et al., 1993; Hiebert et al., 1992; Zamanian and La Thangue, 1993) and by recruiting chromatin modifiers such as histone deacetylases that result in formation...
of more condensed chromatin (Brehm et al., 1998; Luo et al., 1998a; Magnaghi-Jaulin et al., 1998). It was also shown that E2F1 requires heterodimerization with a second protein, a DP family member, to activate transcription (Bandara and La Thangue, 1991; La Thangue and Rigby, 1987). To date, eight E2F family members and three DP family members have been identified. E2F transcription factors fall into three functional groups based on their ability to stimulate transcription and their temporal association with promoters—activator E2Fs (1, 2, 3), repressor E2Fs (4, 5), and a third group whose members (6, 7, 8) function predominantly as repressors but act independently of pocket proteins (Trimarchi and Lees, 2002).

E2F transcription factors play an important role in regulating entry into the cell cycle. Overexpression of E2F1 is sufficient to drive quiescent cells to proliferate and to prevent cells from exiting the cell cycle in response to serum starvation (Johnson et al., 1993; Qin et al., 1994; Singh et al., 1994; Xu et al., 1995). E2F binding sites have been identified in several genes important for proliferation, including CDC2, DHFR (dihydrofolate reductase), TK (thymidine kinase), DNA polymerase α, and cyclin A (Nevins, 1992). In non-proliferating cells and early G1, hypophosphorylated pRB binds and represses E2Fs (Figure 3). Upon mitogenic signaling, cyclin-CDKs become activated, resulting in phosphorylation of pRB, release of E2Fs, and transcriptional activation of genes important for cell cycle entry (Weinberg, 1995).

Solving of the crystal structure of the pRB binding domain of E2F bound to the Small Pocket of pRB (Lee et al., 2002; Xiao et al., 2003) allowed for the generation of a pRB mutant deficient for general E2F binding. Surprisingly, expression of this pRB mutant was still anti-proliferative, despite loss of ability to repress E2F target genes (Chau et al., 2006; Dick and Dyson, 2003). This suggested that pRB can also regulate proliferation in an E2F-independent manner. Subsequently, it was shown that pRB can interact with Cdhl, resulting in degradation
Figure 3. The Retinoblastoma Protein regulates entry into the cell cycle. During G0 and early G1, pRB binds to and represses E2F transcription factors. Upon mitogenic signaling, cyclin-CDKs become activated, resulting in hyperphosphorylation of pRB, release of E2Fs, and transcriptional activation of genes essential for progression into S phase.
of Skp2 by the Anaphase Promoting Complex and accumulation of the CDK inhibitor p27 (Alexander and Hinds, 2001; Binne et al., 2007; Ji et al., 2004). Thus, pRB regulates cell cycle entry through both E2F-dependent and -independent mechanisms and the contexts in which and extents to which these two functions contribute to the regulation of proliferation and tumor suppression remain to be elucidated. In addition to its role in regulating cell cycle entry, E2F1 has also been shown to play an important role in many other cellular processes including apoptosis. In fact, characterization of the pRB mutant deficient for general E2F binding allowed for the identification of a second interaction site specific for E2F1 that may be important for E2F1-induced apoptosis (Carnevale et al., 2012; Chau et al., 2006; Dick and Dyson, 2003; Julian et al., 2008). Furthermore, pRB has also been shown to play important roles in many other cellular processes, both in an E2F-dependent and independent manner. In fact, more than 150 pRB-interacting proteins have been identified to date (Chinnam and Goodrich, 2011). A number of these non-canonical pRB roles are discussed below.

iii. Non-canonical functions of the Retinoblastoma Protein

In addition to regulating proliferation, pRB has been shown to function in a rapidly expanding list of other cellular processes including terminal differentiation, senescence, genome stability, mitochondrial biogenesis, metabolism, and apoptosis. The relative contribution of these functions to tumor suppression remains an active area of research.

In general, pRB impinges on these diverse processes both via its ability to function as a transcriptional co-factor and by serving as an adaptor protein to influence chromatin structure both locally and genome-wide (Chinnam and Goodrich, 2011). pRB’s ability to affect gene expression via multiple mechanisms is best illustrated by its ability to repress E2F activity. As mentioned above, pRB physically interacts with and blocks the transactivation domain of E2F.
In addition, pRB also affects gene expression as an adaptor protein by recruiting chromatin modifiers to E2F binding sites. pRB has been shown to interact directly with numerous chromatin modifiers and some of these interactions have been mapped to the LxCxE binding cleft (Dahiya et al., 2000). Specifically, pRB represses E2F target genes by recruiting histone modifiers such as histone deacetylases (HDAC1, HDAC2), histone methyltransferases (DNMT1), histone demethylases (RBP2), and histone remodeling complexes (Brg1, Brm) (Benevolenskaya et al., 2005; Brehm et al., 1998; Dunaief et al., 1994; Luo et al., 1998a; Magnaghi-Jaulin et al., 1998; Robertson et al., 2000; Strober et al., 1996; Trouche et al., 1997). Thus, pRB influences expression of genes, including E2F target genes important for cell cycle progression, via multiple mechanisms.

pRB can also promote both stress- and oncogene-induced cellular senescence (Chicas et al., 2010; Narita et al., 2003; Robertson et al., 2000), a stable form of cell cycle arrest and an important barrier to cellular transformation (Braig et al., 2005; Collado et al., 2005). Specifically, re-expression of pRB in cancer cells can result in induction of senescence and, conversely, loss of pRB in senescent mouse embryonic fibroblasts (MEFs) causes cell cycle re-entry (Sage et al., 2003; Xu et al., 1997). pRB functions in senescence by regulating expression of E2F target genes involved in DNA replication as well as regulating formation of senescence associated heterochromatic foci (Chicas et al., 2010; Narita et al., 2003). Thus, pRB regulates induction of senescence via multiple mechanisms.

Additional studies indicate that loss of pRB function results in chromosome instability and aneuploidy (Bester et al., 2011; Hernando et al., 2004; Manning and Dyson, 2011). pRB functions in maintaining genome stability in part by regulating expression of mitotic genes, including MAD2, an E2F target gene and spindle assembly checkpoint protein (Schvartzman et
Furthermore, pRB regulates expression of E2F target genes important for DNA replication and loss of pRB has been shown to result in insufficient nucleotide biosynthesis and consequently replication fork stalling (Barbie et al., 2004; Bester et al., 2011; Knudsen et al., 2000). Finally, pRB loss also results in a general defect in chromatin condensation particularly at telomeric and centromeric regions, as a result of pRB's interaction with both chromatin modifiers and condensin II (Coschi et al., 2010; Garcia-Cao et al., 2002; Gonzalo et al., 2005; Isaac et al., 2006; Longworth et al., 2008; Manning et al., 2010).

pRB also plays an important role in regulating terminal differentiation. Rb-mutant embryos die in utero and display severe defects, including abnormal proliferation and differentiation of neuronal tissues and the lens (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). pRB’s importance in differentiation is in part a consequence of its role in mediating cell cycle exit. However, pRB can also directly interact with and activate or repress lineage-specific transcription factors. pRB plays an active role in muscle differentiation by potentiating MyoD activity (Zhang et al., 1999a; Zhang et al., 1999b). pRB also regulates cell fate commitment into fat and bone via direct interaction with the lineage-specific transcription factors C/EBP, PPARγ, and Runx2, respectively (Berman et al., 2008; Calo et al., 2010; Thomas et al., 2001).

More recently, pRB has been shown to regulate mitochondrial biogenesis and metabolism. Specifically, loss of pRB is associated with mitochondrial defects in part via its interaction with RBP2 and regulation of expression of genes encoding mitochondrial proteins (Lopez-Bigas et al., 2008; Sankaran et al., 2008; Takahashi et al., 2012). Similarly, loss of pRB is also associated with changes in expression of genes involved in oxidative phosphorylation and glutathione metabolism (Blanchet et al., 2011; Nicolay et al., 2013). Thus, there is a newly
appreciated role of pRB in regulating mitochondrial functions, albeit all by affecting nuclear gene expression.

Finally, pRB is also an important regulator of apoptosis and can act in both a pro-apoptotic and anti-apoptotic manner. This dichotomous nature of pRB in regulating apoptosis is discussed in detail below. Briefly, pRB was originally characterized as an anti-apoptotic factor and loss of pRB in MEFs results in an increased sensitivity to genotoxic stress (Knudsen and Knudsen, 2008), perhaps as a consequence of failure to arrest the cell cycle and increased chromosomal instability (Bosco et al., 2004; Burkhart and Sage, 2008; Knudsen et al., 2000; Manning and Dyson, 2011). In contrast, and more consistent with its tumor suppressive function, pRB can also promote apoptosis in highly proliferative cells by activating transcription of pro-apoptotic E2F1 target genes in response to apoptotic stimuli such as DNA damage (Araki et al., 2008; Carnevale et al., 2012; Ianari et al., 2009; Knudsen et al., 1999; Korah et al., 2012). This thesis provides evidence that extends the pro-apoptotic role of pRB to a previously unappreciated, transcription-independent function of pRB directly at the mitochondria.
Part II: Apoptosis and the Retinoblastoma Protein

A: Cell Death and Apoptosis

Apoptosis or programmed cell death is a conserved cellular process in all metazoans. It is essential for organogenesis and tissue formation in development and for cellular homeostasis in adults. Excessive cell death may result in degenerative diseases, immunodeficiency, and infertility, while insufficient cell death contributes to cancer and autoimmune diseases (Danial and Korsmeyer, 2004).

Apoptosis is characterized by distinct morphological changes including cell membrane blebbing, cell shrinkage and rounding, chromatin condensation, and DNA fragmentation, ultimately resulting in the formation of apoptotic bodies which are engulfed by macrophages or neighboring cells (Kerr et al., 1972). The biochemical processes that lead to these morphological changes are discussed in detail below. However, in addition to apoptosis, cells can also undergo cell death via other processes including necrosis and autophagy. In contrast to apoptosis, necrosis involves loss of membrane integrity and disruption of the cell, resulting in an inflammatory response (Leist and Jaattela, 2001). Finally, cells can also undergo cell death via a process called autophagy, a conserved lysosomal pathway involved in protein and organelle turnover.

i. Apoptosis

The original genetic understanding of apoptosis came from studies performed in C. elegans, when it became apparent that the same 131 cells of 1090 cells die during development (Brenner, 1974; Sulston, 1976). This lead to the identification of genes involved in regulating this process (Ellis and Horvitz, 1986). Specifically, CED-3 and CED-4 were shown to be
required for apoptosis (Figure 4). CED-4 acts upstream of CED-3 and can be inhibited by the anti-apoptotic protein CED-9 (Hengartner and Horvitz, 1994a). Finally, the pro-apoptotic protein EGL-1 can inhibit CED-9 by displacing CED-4 (Conradt and Horvitz, 1998).

Cloning and characterization of ced-3 revealed that it was related to the mammalian interleukin 1β converting enzyme (ICE, caspase 1) and that expression of either in mammalian cells resulted in induction of apoptosis (Yuan et al., 1993). Subsequently, other mammalian homologs for nematode apoptotic proteins were identified. CED-4 activity could be reconstituted in vitro by 3 mammalian protein, Apaf-1, cytochrome c, and caspase 9 (Li et al., 1997; Zou et al., 1997). The anti-apoptotic gene ced-9 turned out to be the worm homolog of the mammalian oncogene Bcl2 (Hengartner and Horvitz, 1994b; Vaux et al., 1992), first identified at the chromosomal breakpoint of t(14;18) in human follicular B cell lymphoma (Bakhshi et al., 1985; Cleary and Sklar, 1985; Tsujimoto et al., 1985). Finally, the mammalian homolog of EGL-1 is a BH3-only protein.

There are two general apoptotic pathways in mammals, the intrinsic or mitochondrial pathway and the extrinsic or death receptor pathway. These two pathways are described in detail below. However, two protein families (the caspase family and the Bcl2 family) are central to our understanding of these two pathways and will therefore be introduced first.

Caspases are main effectors of apoptosis. As noted above, the mammalian CED-3 homolog ICE or Caspase 1 is the founding member of this family of proteases. There are 14 mammalian caspases which fall into three functional groups: inflammatory caspases, initiator apoptotic caspases, and effector apoptotic caspases. The name caspase derives from the fact that the catalytic triad contains a cysteine and that cleavage occurs after an aspartic acid residue (Thornberry and Lazebnik, 1998). All caspases are synthesized as inactive zymogens composed
Figure 4. Apoptotic pathway components in *C. elegans* and mammals. The apoptotic pathway is highly conserved. Pathway components were first identified in *C. elegans* via genetic screens. Simplified schematic of mammalian apoptotic pathway shows nematode homologs.
of a pro-domain, a large subunit, and a small subunit. Caspase cleavage (auto-catalytic or as part of a cascade) results in the formation of an active tetramer that is composed of two heterodimers, which in turn consist of a large and small subunit with the active site at the interface (Danial and Korsmeyer, 2004). Initiator caspases are auto-catalytic, while effector caspases are activated by initiator caspases and in turn process more than 100 substrates that result in the characteristic morphological changes associated with apoptosis, including apoptotic proteins, structural proteins, DNA repair proteins, and cell cycle related proteins (Hotti et al., 2000; Kothakota et al., 1997; Ku et al., 1997; Levkau et al., 1998; Li et al., 1998; Luo et al., 1998b; Rao et al., 1996; Schmeiser et al., 1998; Zhou et al., 1998).

The founding member of the Bcl2 family is the mammalian CED-9 homolog Bcl2, which also founded a new class of oncogenes, regulators of cell death. The realization that Bcl2 localizes to mitochondria, lead to the recognition of the central importance of this organelle to apoptosis. The identification of BAX as an interactor of Bcl2 and its subsequent characterization established that the Bcl2 family contains both pro- and anti-apoptotic proteins (Oltvai et al., 1993). Furthermore, the mammalian homolog of EGL-1, a BH3-only protein, is also a Bcl2 family member. The Bcl2 family is particularly important for the intrinsic apoptotic pathway and will be discussed in more detail below.

It should be noted that activation of apoptosis via either the intrinsic or extrinsic pathways does not require new protein synthesis. However, changes in gene expression in response to various stimuli can alter the concentration of apoptotic regulators, including caspases and Bcl2 family members, and in this way modulate the sensitivity of cells to apoptotic stimuli.
a) **The Mitochondrial Pathway**

The intrinsic or mitochondrial pathway is activated in response to diverse signals of stress and damage. Mitochondria are of central importance to this pathway by sequestering pro-apoptotic proteins in the intermembrane space. Bcl2 family members regulate activation of the mitochondrial pathway by regulating permeability of the mitochondrial outer membrane and thus release of these pro-apoptotic proteins from the intermembrane space into the cytoplasm where they allow for activation of initiator caspase 9 (Figure 5A).

Bcl2 family members are divided into three subclasses based on the presence of 1-4 homology domains (BH1-4), which roughly correspond to α-helices (Figure 5B). The multidomain pro-apoptotic family members (BAX, BAK) contain BH1-3. In the inactive state, BAX is monomeric and cytoplasmic, while BAK is monomeric and mitochondrial. Activation of BAX and BAK is a multistep process that involves conformational changes, translocation to and insertion in the mitochondrial outer membrane (in the case of BAX), and oligomerization (Youle and Strasser, 2008). The resulting pore permeabilizes the mitochondrial outer membrane (Mitochondrial Outer Membrane Permeabilization, MOMP) and releases apoptotic proteins into the cytoplasm. Thus, BAX and/or BAK are required for intrinsic apoptosis (Lindsten et al., 2000; Wei et al., 2001). Among the pro-apoptotic proteins released upon MOMP is cytochrome c, identified as one of the three mammalian factors required for reconstituting CED-4 activity in vitro. In the cytoplasm cytochrome c binds to Apaf-1 and caspase 9, forming the apoptosome. This results in auto-catalytic activation of the initiator caspase 9 and subsequent activation of effector caspases and apoptosis. BAX/BAK can be inhibited by another subclass of Bcl2 family proteins called anti-apoptotic Bcl2 family members (e.g. Bcl2). These contain BH1-4, forming a hydrophobic groove that can accommodate BH3 domains of pro-apoptotic family members.
**Figure 5. Intrinsic/Mitochondrial Pathway.** (A) Activation of BH3-only proteins by a variety of apoptotic stimuli results in activation of multidomain pro-apoptotic proteins BAX and BAK, MOMP, cytochrome c release and activation of the caspase cascade. (B) Schematic representation of Bcl2 family members and homology domains BH1-4. TM denotes transmembrane domains.
Finally, pro-apoptotic BH-3 only proteins (e.g. BID, BAD) only contain the BH3 domain and are the upstream sensors of the intrinsic pathway. Different apoptotic stimuli activate different BH3-only proteins via different mechanisms, including transcriptional upregulation, changes in subcellular localization, and posttranslational modifications (Nakano and Vousden, 2001; Oda et al., 2000; Puthalakath et al., 1999; Raff, 1992; Yu et al., 2001; Zha et al., 1996). There are two types of BH3-only proteins, activators (e.g. BID, BIM) and sensitizers (e.g. BAD, NOXA) (Letai et al., 2002; Llambi et al., 2011). Activator BH3-only proteins can directly bind and activate BAX and BAK. In contrast, sensitizer BH3-only proteins bind anti-apoptotic Bcl-2 family members, relieving inhibition of activator BH3-only proteins and/or multidomain pro-apoptotic family members BAX and BAK. It should be noted that specific Bcl2 family members display specificity in their interactions with other family members (Certo et al., 2006; Chen et al., 2005; Kuwana et al., 2005; Opferman et al., 2003).

Other (non-Bcl2 family) proteins can also activate BAX and BAK, either by acting like a BH3-only activator or sensitizer. The absence of an identified BH3 domain precludes these proteins from being Bcl2 family proteins. One example is p53, an important tumor suppressor and mediator of many cellular processes including proliferation and apoptosis. p53 has been shown to promote apoptosis via two mechanisms. In response to DNA damage, p53 can activate transcription of pro-apoptotic genes including BH3-only proteins PUMA and NOXA (Vousden and Prives, 2009). In addition, p53 also translocates directly to mitochondria where it can act as a direct activator of BAX or as a sensitizer by interacting with anti-apoptotic Bcl2 family members Bcl2 and BclXL (Caelles et al., 1994; Chipuk et al., 2004; Chipuk et al., 2003; Dumont et al., 2003; Haupt et al., 1995; Mihara et al., 2003). Neither a mitochondrial targeting sequence
nor a BH3 domain has been identified on p53. Similarly, the orphan nuclear steroid receptor Nur77 can promote apoptosis both by activating transcription of pro-apoptotic genes (Rajpal et al., 2003) and by translocating to mitochondria and interacting with Bcl2 (Li et al., 2000; Lin et al., 2004). Since Nur77 does not have a mitochondrial targeting sequence, it has been suggested that its interaction with Bcl2 localizes it to the mitochondria. No BH3 domain has been identified yet. Another nuclear protein, histone H1.2, has also been implicated in inducing apoptosis directly at the mitochondria. Histone H1.2 has been shown to trigger MOMP in a BAK-dependent manner (Gine et al., 2008; Konishi et al., 2003; Okamura et al., 2008). The exact mechanism of this action remains to be elucidated, including the interaction domain.

Finally, nucleophosmin, a nucleolar phosphoprotein, has been shown to locate to the cytoplasm in response to apoptotic stimuli and participate in the activation of BAX (Kerr et al., 2007).

Thus, activation of the multidomain pro-apoptotic Bcl2 family members BAX and BAK can be regulated by a wide variety of proteins. These include activator BH3-only Bcl2 family members as well as non-Bcl2 family members. The relative importance, in particular in vivo, of these non-Bcl2 activators of apoptosis compared to BH3-only proteins remains to be determined.

b) The Extrinsic Apoptotic Pathway

Generation of monoclonal antibodies against cell surface receptors allowed for the identification of some antibodies that induced apoptosis (Trauth et al., 1989). This lead to the identification and cloning of cell surface death receptors, including Fas, TNFR, and DR4 and 5 (Itoh et al., 1991; Suda et al., 1993), which mediate the extrinsic or death receptor apoptotic pathway. In general, death ligand binding results in oligomerization of the receptor, recruitment of the death-inducing signaling complex (DISC) which contains initiator caspase 8, and autocatalytic activation of caspase 8 (Ashkenazi and Dixit, 1998). Initiator caspase 8 can promote
apoptosis via two mechanisms. Caspase 8 can cleave and activate effector caspases directly or it can cleave the BH3-only protein BID to form tBID, which translocates to the mitochondrial outer membrane and activates the intrinsic/mitochondrial pathway (Li et al., 1998; Luo et al., 1998b). Thus, the two apoptotic pathways are interconnected and affect each other.

The death ligand TNFα is secreted by cells of the immune system in response to bacterial infection. It binds the death receptor TNFR. There are two TNFRs, TNFR1 which is ubiquitously expressed in most tissues and TNFR2 which is expressed by cells of the immune system (Wajant et al., 2003). Binding of TNFα to TNFR1 induces a primary pro-inflammatory response and a secondary pro-apoptotic response (Figure 6). Briefly, activation of TNFR1 results in recruitment of IKK to the signaling complex and phosphorylation and subsequent degradation of I-κB, an inhibitor of NF-κB. This allows NF-κB to translocate to the nucleus and activate transcription of pro-inflammatory proteins. NF-κB also activates transcription of anti-apoptotic proteins, inhibiting apoptosis. Prolonged exposure to TNFα, results in the recruitment of DISC and activation of caspase 8 (Aggarwal, 2003).

Apoptosis is an important cellular process and deregulation of apoptosis can result in diseases like cancer. Over the past few decades, many essential components involved in apoptosis have been identified and a general picture of how these components work together to regulate apoptosis has emerged. Nevertheless, new regulators of apoptosis are still being discovered. This thesis provides evidence for the tumor suppressor pRB as a novel regulator of mitochondrial apoptosis by acting as a direct activator of BAX.
Figure 6. Schematic of extrinsic/death receptor pathway induced by TNFα. TNFα treatment elicits a primary NF-κB-mediated pro-inflammatory response which is anti-apoptotic. Prolonged exposure to TNFα activates the extrinsic apoptotic pathway, which can also activate the intrinsic/mitochondrial pathway via the BH3-only protein tBID.
The Retinoblastoma Protein functions in Apoptosis

As described above, apoptosis is one of the many cellular processes ascribed to pRB. In fact, pRB has been characterized as both an anti-apoptotic and pro-apoptotic factor. The evidence supporting these two opposing mechanisms of action is discussed in detail below.

i. The Retinoblastoma Protein as an Anti-apoptotic Factor

Earliest evidence for the anti-apoptotic role of pRB emerged from the characterization of the Rb-null embryo, which displays ectopic proliferation and increased levels of apoptosis in several tissues including the nervous system, lens, and skeletal muscle (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Subsequent studies using tetraploid aggregation and conditional knockout mouse models revealed that apoptosis in many tissues is rescued by providing the Rb-null embryo with a wildtype placenta and thus is mostly non-cell autonomous (de Bruin et al., 2003; Wenzel et al., 2007; Wu et al., 2003). Further inspection of the Rb-null placenta revealed that loss of pRB in trophoblasts results in excessive proliferation and consequently disruption of the normal placental architecture and vascularization. This results in decreased placental transport function and hypoxia in embryonic tissues, causing the increased levels of apoptosis observed in many Rb-null tissues. However, while providing the Rb-null embryo with a wildtype placenta rescued most of the apoptotic phenotype in the central nervous system, it did not rescue increased levels of apoptosis observed in the lens and skeletal muscles. Further inspection showed that there was a coexistence of cell death and ectopic mitosis, suggesting that apoptosis was linked to a defect of cells to growth arrest and respond to differentiation signals.

As described above, pRB’s role in promoting cell cycle arrest and terminal differentiation has been well established. These observations lead to the postulation of the conflict model, which
argues that pRB’s anti-apoptotic role is an indirect consequence of its ability to promote cell cycle arrest and undergo differentiation. Thus, there are two explanations for the increased levels of apoptosis observed in germline Rb-null embryos. First, the hypoxic stress caused by the Rb-null placenta (a result of pRB’s role in regulating proliferation of trophoblasts) contributes to increased levels of apoptosis observed in some tissues including the neuronal tissue. Second, there is a conflict of signals in some tissues, such as the lens and skeletal muscle, which is caused by an inability to undergo cell cycle arrest and terminal differentiation in response to differentiation signals in the absence of pRB. This triggers apoptosis as a default outcome of ectopic mitosis. Analysis of conditional knockout Rb mouse models supports this hypothesis.

For example, loss of pRB in the central nervous system results in increased levels of proliferation but no apoptotic defect, arguing that the cell cycle defect is cell-autonomous while the apoptotic phenotype observed in the Rb-null embryo was not (MacPherson et al., 2003). However, loss of pRB in the lens results in both ectopic mitosis and increased levels of apoptosis in areas of the lens that normally contain differentiated lens fiber cells (MacPherson et al., 2003). Similarly, loss of pRB in proliferating myoblasts causes elevated levels of apoptosis in vivo and induction of differentiation in cultured Rb-deficient primary myoblasts results in high levels of apoptosis (Huh et al., 2004). In contrast, loss of pRB after differentiation (in differentiatiated myotubes in vivo) results in a normal muscle phenotype (Huh et al., 2004). Thus, pRB’s anti-apoptotic role appears to be an indirect consequence of its cell cycle and differentiation functions.

Consistent with these in vivo data, analysis of the role of pRB in apoptosis in cultured cells also shows that pRB can act in an anti-apoptotic manner. Early studies showed that pRB protects cultured primary murine fibroblasts from apoptosis induced by genotoxic stress (Almasan et al., 1995; Bosco et al., 2004; Knudsen et al., 2000). Further analysis revealed that in
response to treatment with genotoxic agents, MEFs and mouse adult fibroblasts that are deficient for pRB fail to activate DNA damage checkpoints and inappropriately proceed into S-phase. Since this triggers apoptosis as a default outcome, pRB deficiency increases sensitivity to genotoxic stress. Thus, pRB can act in an anti-apoptotic manner both in vivo and in vitro, though this is at least in part an indirect consequence of pRB’s role in mediating cell cycle arrest.

It has also been proposed that pRB can directly inhibit apoptosis by repressing E2F1, a known activator of apoptosis. E2F1 can promote apoptosis in a manner in a p53-dependent and -independent manner. Specifically, E2F1 can activate transcription of p14ARF, resulting in inhibition of Mdm2-mediated degradation of p53 and consequently induction of apoptosis (Bates et al., 1998). E2F1 can also promote apoptosis in a p53-independent manner by directly activating transcription of apoptotic proteins including Apaf-1, caspase 7, and p73 (Ginsberg, 2002; Moroni et al., 2001; Muller et al., 2001). Given pRB’s role in repressing E2F’s proliferative function during quiescence and early G1, it was proposed that pRB can also act to directly suppress E2F1’s pro-apoptotic function in response to apoptotic stimuli. This model predicts that pRB is inactivated in response to an apoptotic stimulus. Consistent with this hypothesis, it has been described that pRB is cleaved at the C-terminus and degraded after prolonged treatment with an apoptotic stimulus (Fattman et al., 2001; Janicke et al., 1996; Tan et al., 1997). However, while the appearance of cleaved pRB correlates with induction of apoptosis, degradation may occur later (Janicke et al., 1996). This argues that cleavage rather than degradation inactivates pRB function. Finally and most convincingly, a pRB mutant that is not cleavable displays decreased sensitivity to TNFα treatment both in cultured cells and in a knock-in mouse model (Chau et al., 2002; Tan et al., 1997). These data are consistent with the hypothesis that pRB needs to be cleaved for E2F1 to promote TNFα-induced apoptosis.
However, cleaved pRB can still bind E2F1 and repress its transcriptional activity (Janicke et al., 1996). It remains to be seen how exactly pRB cleavage by caspases affects pRB’s many cellular functions to impinge on the apoptotic response.

Taken together, it is apparent that the analysis of the role of pRB in apoptosis is complicated by its pleiotropic cellular activities. While pRB’s anti-apoptotic function is well established, this is likely an indirect effect of pRB’s ability to regulate proliferation and differentiation. However, pRB can also act in a pro-apoptotic manner in some cellular settings. These data are discussed below.

ii. The Retinoblastoma Protein as a Pro-apoptotic Factor

There have been a number of reports revealing a pro-apoptotic role of pRB. Overexpression of pRB in head and neck squamous cell carcinoma, glioblastoma, prostate, and cervical cancer cell lines results in increased levels of apoptosis and sensitizes to radiation treatment (Araki et al., 2008; Bowen et al., 2002; Bowen et al., 1998; Ianari et al., 2009; Knudsen et al., 1999; Li et al., 2002). Thus, while pRB inhibits apoptosis in MEFs (Almasan et al., 1995; Bosco et al., 2004; Knudsen et al., 2000), it promotes apoptosis in several cancer cell lines. Similarly, expression of retinoblastoma protein in vivo can both promote and inhibit apoptosis and this may be dependent on the cellular context. For example, expression of the drosophila homolog of pRB in wing and eye imaginal discs has different effects depending on the proliferation status of the cells; there is an increase in the levels of apoptosis in proliferative tissues, while there is no effect on apoptosis in post-mitotic, differentiated cells (Milet et al., 2010). Moreover, loss of pRB in the intestinal epithelium impairs DNA-damage induced apoptosis (Ianari et al., 2009). Taken together, this led to the hypothesis that the ability of pRB
to promote or repress apoptosis may be dependent on the proliferation status of the cell. The mechanism of how pRB promotes apoptosis is discussed below.

E2F1 is a well-characterized transcriptional activator of apoptosis. Analysis of the role of pRB in response to genotoxic and oncogenic stress in both primary and transformed cell lines revealed that these stimuli stabilized the formation of a complex containing pRB and E2F1, which localized to promoters of pro-apoptotic, E2F1 target genes that were transcriptionally active (Ianari et al., 2009). Another study showed that in response to DNA damage, E2F1 exists as both a free and a pRB-bound species depending on specific post-translational modifications (Carnevale et al., 2012). Remarkably, both species of E2F1 contribute toward maximal induction of apoptosis including transcriptional activation of pro-apoptotic, E2F1 target genes (Carnevale et al., 2012). These data reconcile the facts that ectopic expression of E2F1 alone is apoptotic and that a pRB:E2F1 complex can also promote apoptosis in the context of genotoxic stress. Finally, the pro-apoptotic function of pRB has been extended to apoptotic stimuli other than DNA damage. Specifically, it was also shown that apoptosis induced by TGFβ requires the formation of a pro-apoptotic complex that contains pRB, E2F1, and the transcriptional co-activator P/CAF (Korah et al., 2012). Taken together these data show that pRB can inhibit apoptosis in an indirect manner and promote apoptosis by directly co-activating transcription of pro-apoptotic genes (Figure 7).

It is unclear how pRB differentially interacts with E2F1 to either suppress proliferation or activate apoptosis. However, as mentioned above, pRB and E2F1 can form two distinct complexes (Carnevale et al., 2012; Dick and Dyson, 2003; Julian et al., 2008). Since pRB represses E2F1's proliferative activity by blocking its transactivation domain, it is unlikely that this pRB-E2F1 complex can activate transcription of pro-apoptotic genes. It remains to be seen
whether the second, E2F1-specific interaction domain mediates pRB’s pro-apoptotic function and how the formation of such a complex is regulated. Alternatively, it is possible that both free and pRB-bound E2F1 localizes to promoters of pro-apoptotic genes and pRB contributes to transcriptional activation by functioning as an adaptor and recruiting transcriptional co-activators. Moreover, it is unknown whether the pro-apoptotic function of pRB is restricted to participation in a transcriptionally active, pro-apoptotic E2F1 complex or if pRB can also promote apoptosis via other, non-E2F-mediated mechanisms. Finally, pRB’s role in apoptosis has primarily been evaluated in response to genotoxic stress. This thesis provides evidence that pRB can also promote apoptosis in response to TNFα. Furthermore, pRB can promote TNFα-induced apoptosis in a transcription-independent manner by directly activating BAX and triggering mitochondrial apoptosis.
Figure 7. The role of pRB in apoptosis. pRB can act in both an anti-apoptotic manner and in a pro-apoptotic manner. In response to DNA damage pRB can protect against apoptosis by inducing cell cycle arrest and promote apoptosis by co-activating transcription of pro-apoptotic genes.
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Chapter Two

The retinoblastoma protein induces apoptosis directly at the mitochondria

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K.I.H. conducted all of the cell and in vivo studies, including xenograft assays with S.N. and p16 knockdown studies with M.A.M. E.S.L. contributed Fig. 5B-D. E.C. developed the OS cell xenograft assay. A.I. and L.D.W. were involved in the study design and data analysis. K.I.H. and J.A.L. designed the study, analyzed the data and wrote the paper.

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Abstract

The retinoblastoma protein gene, RB-1, is mutated in one third of human tumors. Its protein product, pRB, functions as a transcriptional co-regulator in many fundamental cellular processes. Here, we report a non-nuclear role for pRB in apoptosis induction via pRB’s direct participation in mitochondrial apoptosis. We uncovered this activity by finding that pRB potentiates TNFα-induced apoptosis, even when translation was blocked. This pro-apoptotic function was highly BAX-dependent, suggesting a role in mitochondrial apoptosis, and accordingly a fraction of endogenous pRB associated with mitochondria. Remarkably, we found that recombinant pRB was sufficient to trigger the BAX-dependent permeabilization of mitochondria or liposomes in vitro. Moreover, pRB interacted with BAX in vivo, and it could directly bind and conformationally activate BAX in vitro. Finally, by targeting pRB specifically to mitochondria we generated a mutant that lacked pRB’s classic nuclear roles. This mito-tagged pRB retained the ability to promote apoptosis in response to TNFα and also additional apoptotic stimuli. Most importantly, induced expression of mito-tagged pRB in Rb-/-;p53-/- tumors was sufficient to block further tumor development. Together, these data establish a non-transcriptional role for pRB in direct activation of BAX and mitochondrial apoptosis in response to diverse stimuli, which is profoundly tumor suppressive.
Introduction

Regulation of pRB is perturbed in most, if not all, cancers (Sherr and McCormick, 2002). pRB functions in many cellular processes and is a key regulator of the cell cycle by interacting with and inhibiting E2F transcription factors (van den Heuvel and Dyson, 2008). Upon mitogenic signaling, cdk/cyclin complexes phosphorylate pRB, resulting in release of E2Fs and cell cycle progression (van den Heuvel and Dyson, 2008). Notably, the two thirds of human tumors that are RB-1 wildtype typically carry mutations in upstream regulators of pRB (p16^Ink4a, cyclinD or cdk4) that promote cdk/cyclin activation and thus pRB phosphorylation (Sherr and McCormick, 2002). Since these mutations all inactivate pRB’s anti-proliferative function, little attention has been paid to the status of RB-1 in considering tumor treatment. However, we note that pRB also functions as a transcriptional co-regulator of differentiation, senescence, and apoptosis genes (Calo et al., 2010; Gordon and Du, 2011; Ianari et al., 2009; Viatour and Sage, 2011). In addition, a portion of the pRB protein exists in the cytoplasm (Fulcher et al., 2010; Jiao et al., 2006; Jiao et al., 2008; Roth et al., 2009) and even at mitochondria (Ferecatu et al., 2009), but no known roles have been assigned to these species.

This current study concerns the role of pRB in the regulation of apoptosis. It is already well established that pRB can either promote or suppress apoptosis through both direct and indirect transcriptional mechanisms (Gordon and Du, 2011; Ianari et al., 2009). Earliest evidence for the anti-apoptotic role of pRB emerged from the characterization of the Rb-null mouse, which exhibits increased levels of apoptosis in the nervous system, lens, and skeletal muscle (Jacks et al., 1992). Subsequent studies showed that this phenotype is mostly non-cell autonomous, resulting from the deregulation of cell cycle genes and the consequent over-proliferation of placental tissues that disrupts its normal architecture and vascularization, and
causes hypoxia in embryonic tissues (de Bruin et al., 2003; Wenzel et al., 2007; Wu et al., 2003). Loss of pRB in mouse embryonic fibroblasts (MEFs) results in increased sensitivity to genotoxic stress (Knudsen and Knudsen, 2008). However, this is thought to be an indirect consequence of failure to prevent cell cycle entry in the absence of pRB, as well as increased chromosomal instability (Bosco et al., 2004; Burkhart and Sage, 2008; Knudsen et al., 2000; Manning and Dyson, 2012). In contrast, and more consistent with a tumor suppressor role, pRB can also act in a pro-apoptotic manner in highly proliferative cells (Araki et al., 2008; Carnevale et al., 2012; Ianari et al., 2009; Knudsen et al., 1999; Milet et al., 2010). In this context, pRB and also hyperphosphorylated pRB contributes directly to apoptosis by functioning in a transcriptionally active pRB:E2F1 complex that promotes expression of pro-apoptotic genes, such as caspase 7 and p73, in response to DNA damage (Ianari et al., 2009). Taken together, these studies suggest that the ability of pRB to promote or repress apoptosis, at least in response to genotoxic stress, may be dictated by the cellular context.

The pro-apoptotic role of pRB has been primarily investigated in the context of DNA damage. The findings in this current study followed from our analysis of pRB’s apoptotic function in response to another apoptotic stimulus, TNFa. TNFa can promote apoptosis via both the extrinsic and mitochondrial/intrinsic pathways (Jin and El-Deiry, 2005). The extrinsic pathway involves direct activation of the caspase cascade. In contrast, the intrinsic pathway depends on activation of the Bcl-2 protein family members BAX and BAK, which trigger mitochondrial outer membrane permeabilization (MOMP) and release of pro-apoptotic factors, such as cytochrome c, that lead to effector caspase activation (Brunelle and Letai, 2009; Chipuk et al., 2010; Jin and El-Deiry, 2005; Martinou and Youle, 2011; Wyllie, 2010). Notably, in addition to its pro-apoptotic response, TNFa also induces a NFkB-mediated, pro-inflammatory
response, which inhibits apoptosis (Karin and Lin, 2002). Thus, to study its apoptotic function, TNFα is commonly used in conjunction with a factor that abrogates the pro-inflammatory response such as the proteasome inhibitor MG-132 or the translational inhibitor cycloheximide (Karin and Lin, 2002; Traenckner et al., 1994).

In this current study, we show that pRB enhances apoptosis in response to TNFα, and can exert this effect in the presence of translation inhibition. This finding led us to discover a novel, non-nuclear function for the pRB protein. Specifically, pRB is associated with mitochondria and it can induce MOMP by directly binding and activating BAX. By localizing pRB specifically to the mitochondria, we showed that this mitochondrial apoptosis function is capable of yielding potent tumor suppression in the absence of pRB’s canonical nuclear functions. Importantly, mitochondrial pRB can respond to a wide variety of pro-apoptotic signals, suggesting that this represents a general and potent tumor suppressive mechanism. Additionally, we find that cdk-phosphorylated pRB is present at the mitochondria and pRB’s mitochondrial apoptosis function remains intact in the presence of tumorigenic events, such as p16 inactivation, that promote pRB phosphorylation. Thus, we believe that it may be possible to exploit pRB’s mitochondrial apoptosis role as a therapeutic treatment in the majority of human tumors that retain wildtype Rb-1.
Results

pRB is pro-apoptotic in response to TNFα treatment even in the presence of an inhibitor of translation.

Our initial interest in exploring pRB’s role in TNFα-induced apoptosis stemmed from prior reports that a phosphorylation-site mutant version of pRB played a pro-apoptotic role in the TNFα response (Masselli and Wang, 2006). We began our studies using a stable variant of an immortalized rat embryonic fibroblast cell line, RAT16, in which doxycycline withdrawal induces ectopic pRB expression. RAT16 cells with basal or induced pRB expression were treated with TNFα in concert with the proteasome inhibitor MG132 to block the TNFα-induced activation of NFκB and its pro-inflammatory response. We found that ectopic pRB expression significantly enhanced TNFα-induced apoptosis as measured by Annexin V staining (Fig. 1A). Additionally, we note that pRB also caused a subtle increase in apoptosis even in the absence of TNFα treatment (Fig. 1A). We further confirmed these data by analysis of cleaved effector caspases 3 and 7 protein levels (Fig. 1B). We wanted to confirm that this was not simply a consequence of pRB overexpression, and thus also assessed the role of the endogenous pRB using knockdown cell lines. Notably, even partial knockdown of endogenous pRB in RAT16 cells was sufficient to significantly impair TNFα-induced apoptosis (Fig. 1C), without any detectable disruption of cell cycle phasing (Fig. 1D). Similar results were observed in a second cell line, human primary IMR90 fibroblasts, in which pRB knockdown also suppressed TNFα-induced apoptosis (Fig. 1E). Thus, modulation of pRB levels by either overexpression or knockdown is sufficient to enhance or depress the apoptotic response to TNFα.
Figure 1. pRB is pro-apoptotic in response to TNFα and MG132. (A) RAT16 cells with and without induced expression of pRB for 24 hours were treated with TNFα and MG132 for 48 hours and analyzed for apoptosis by AnnexinV staining. Induced expression of pRB resulted in increased levels of apoptosis without TNFα treatment and greatly enhanced TNFα/MG132-induced apoptosis. (B) Western blot showing induction of pRB expression in RAT16 cells and procaspase 3, cleaved caspase 3, procaspase 7, and cleaved caspase 7 protein levels. Induced expression of pRB for 24 hours resulted in increased levels of cleaved caspase 3 and 7 protein levels in response to 48 hours of TNFα/MG132 treatment. (C) Stable pRB knockdown in RAT16 cells decreased levels of apoptosis in response to treatment with TNFα/MG132 for 48 hours and (D) did not affect cell cycle phasing. Western blot verifying knockdown of pRB in inset. (E) Stable pRB knockdown in IMR90 cells decreased TNFα/MG132-induced. Western blot verifying knockdown of pRB in inset. (F) Induced expression of pRB does not significantly change expression levels of apoptotic, inflammatory, or autophagic genes with or without TNFα/MG132 treatment as assessed by RT-qPCR and normalized to ubiquitin. (A-F) Bars, average of at least three independent experiments (±SD).
We anticipated that pRB's contribution to the pro-apoptotic TNFα response would reflect its ability to transcriptionally activate pro-apoptotic genes, as observed in our prior DNA damage studies (Ianari et al., 2009). However, we did not detect any significant changes in the levels of apoptotic, inflammatory, or autophagic mRNAs, including known pRB:E2F1 targets, in response to pRB induction in our RAT16-TNFα experiments (Fig. 1F). This led us to consider that perhaps pRB might be acting independently of transcription. To explore this possibility, we took advantage of the fact that cycloheximide (CHX) can be used instead of MG132 to block activation of NFκB (Karin and Lin, 2002; Traenckner et al., 1994). Since CHX acts to block translation, this would preclude any effects that required gene expression changes. Strikingly, induction of pRB in RAT16 cells was able to potentiate apoptosis in response to TNFα and CHX (Fig. 2A), with control experiments verifying translation inhibition by CHX (Fig. 2B). Moreover, pRB knockdown in IMR90s impaired TNFα/CHX-induced apoptosis (Fig. 2C). Thus, taken together, our data show that pRB synergizes with TNFα to promote apoptosis in both primary and immortalized human and rodent cell lines in the absence of translation. This strongly suggests that pRB can act through a previously unappreciated mechanism.

**pRB activates mitochondrial apoptosis in a BAX-dependent manner.**

TNFα can induce apoptosis through both the extrinsic and mitochondrial pathways. To further pinpoint pRB’s role, we exploited the fact that the mitochondrial pathway is highly dependent on BAX and/or BAK (Chipuk et al., 2010; Martinou and Youle, 2011). Specifically, we generated stable pools of immortalized wildtype, Bak−/−, Bax−/−, or Bax−/−; Bak−/− MEFs that would allow for doxycycline-inducible pRB expression (Fig. 3A) and assayed for apoptosis in the absence and presence of TNFα/CHX treatment. To ensure that we were assaying the
Figure 2. pRB promotes TNFα-induced apoptosis in a transcription-independent manner. (A) Induced expression of pRB for 24 hours in RAT16 cells increased apoptosis resulting from 24 hours of TNFα and CHX treatment. (B) Upper panel: Schematic of experiment. Lanes 1 and 2, pRB expression was induced for 24 hours and subsequently cells were left untreated or treated with TNFα/CHX, respectively. Lane 3 and 4, pRB expression was induced and concurrently cells were left untreated or treated with TNFα/CHX, respectively. Lane 5 and 6, pRB expression was never induced and cells were left untreated or treated with TNFα/CHX, respectively. Samples were collected after 24 hours of TNFα/CHX treatment and analyzed by western blotting (middle panel) and by FACS (lower panel). The addition of CHX concurrently to induction of pRB expression in Lane 4 resulted in no visible pRB protein, similar levels of apoptosis as no pRB expression (Lane 6) and less apoptosis than pRB expression for 24 hours prior to treatment (Lane 2). This suggests that CHX inhibited expression of pRB. (C) Stable knockdown of pRB in IMR90 cells decreased apoptosis induced by 24 hour treatment with TNFα and CHX. (A, C) Graph bars represent average of at least three independent experiments (±SD).
Figure 3. pRB promotes mitochondrial apoptosis in a BAX-dependent manner.
(A) pRB expression was induced for 24 hours in stable pools of wild-type, Bak\(^{-/-}\), Bax\(^{-/-}\), and
Bax\(^{-/-}\);Bak\(^{-/-}\) immortalized MEFs by doxycycline addition and confirmed by western blotting
using antibodies against pRB, BAK, BAX, and tubulin. (B) Wild-type, Bak\(^{-/-}\), Bax\(^{-/-}\), and
Bax\(^{-/-}\);Bak\(^{-/-}\) immortalized MEFs with or without 24 hours of pRB expression were left
untreated or treated with TNF\(\alpha\) and CHX for 10 hours and analyzed for apoptosis by
AnnexinV staining. Induction of pRB in wildtype and Bak\(^{-/-}\), but not Bax\(^{-/-}\) or Bax\(^{-/-}\);Bak\(^{-/-}\),
MEFs sensitized to TNF\(\alpha\)/CHX-induced apoptosis. Each MEF variant was independently
generated twice. Graph bars represent average of three representative, independent
experiments (±SD).
BAX/BAK-dependence of any pRB effect, rather than a general difference in the apoptotic potential of the various Bak genotypes, we conducted these experiments using treatment conditions that yielded minimal response to TNFα/CHX in the uninduced (basal pRB) cells (Fig. 3B). Remarkably, pRB induction enhanced TNFα-induced apoptosis in both wildtype (p=0.01) and Bak (p<0.005) MEFs, but had no effect in either Bak or Bak;Bak−/− MEFs (Fig. 3B). This analysis yielded two important conclusions. First, pRB exerts its effect on TNFα-induced apoptosis by acting specifically on the mitochondrial pathway. Second, pRB requires BAX, but not BAK, for this activity.

pRB is thought of primarily as a nuclear protein, but it also exists in the cytoplasm (Fulcher et al., 2010; Jiao et al., 2006; Jiao et al., 2008; Roth et al., 2009). Indeed, pRB has even been reported at mitochondria (Ferecatu et al., 2009), albeit without any known function. Thus, we speculated that pRB might act directly at mitochondria. To validate and further explore the mitochondrial localization of pRB, we first fractionated IMR90 cells. We note that our goal was not to necessarily recover all mitochondria, but rather to obtain a mitochondrial fraction free of nuclear and cytoplasmic contamination, as confirmed by western blotting for various nuclear, cytoplasmic and mitochondrial markers (Fig. 4). Intriguingly, we detected a portion of endogenous pRB in the isolated mitochondria, and found that this exists even in the absence of treatment with TNFα or genotoxic agents (Fig. 4A, B). Furthermore, using phospho-specific pRB antibodies, we showed that this mitochondrial pRB includes the cdk-phosphorylated species (Fig. 4A). Based on the relative loading and western blot signals, we estimate that about 5% of the total cellular pRB is present in the mitochondrial fraction. Since we optimized the mitochondrial fractionation for purity, not completeness, this is likely a conservative estimate of the level of mitochondrial pRB.
Figure 4. A fraction of pRB localizes to mitochondria. (A) IMR90 cell mitochondria were fractionated and equal amounts (in μg) of mitochondrial fraction (mito) versus total lysate were analyzed by western blotting using an antibody against pRB and a cocktail of antibodies against phosphorylated pRB. A fraction of pRB, including phospho-pRB localizes to mitochondria. The purity of the mitochondrial fraction was verified using control nuclear and cytoplasmic markers. (B) Mitochondria of IMR90 cells treated with camptothecin (CPT; 5 hours) or TNFa (24 hours) were isolated and analyzed by western blotting. The levels of mitochondrial pRB are unaffected by treatment with these drugs. (C) Mitochondria were isolated from mouse livers and analyzed by western blotting. A fraction of pRB is present at mouse liver mitochondria. (D) Mouse liver mitochondria were subfractionated into mitoplast and non-mitoplast (SN). Mitochondrial pRB localizes outside the mitoplast. (A-D) Data are representative of at least three independent experiments.
To further validate the mitochondrial localization of pRB in vivo, we next examined mitochondria from mouse livers. Again, western blotting showed that a fraction of pRB existed within the mitochondrial fraction (Fig. 4C). Finally, subfractionation of mouse liver mitochondria localized pRB outside of the mitoplast (inner mitochondrial membrane and enclosed matrix; Fig. 4D), consistent with the notion that it is associated with the outer mitochondrial membrane and therefore coincident with the site of BAX/BAK action.

**pRB directly activates Bax and induces cytochrome c release from isolated mitochondria.**

Having established that pRB is associated with mitochondria in vivo, we hypothesized that pRB might act to trigger mitochondrial outer membrane permeabilization (MOMP). To test this, we performed in vitro cytochrome c release assays. First, we isolated mitochondria from wildtype mouse livers and added recombinant monomeric BAX, because BAX is not associated with mitochondria in unstressed conditions (Walensky et al., 2006). As expected, addition of BAX alone did not result in cytochrome c release (Fig. 5A). Remarkably, co-addition of purified, baculovirus-expressed human pRB and monomeric BAX was sufficient to induce cytochrome c release (Fig. 5A). This was comparable to the effect of cleaved BID, a BH3-only member of the BCL-2 family that is a physiologic trigger of MOMP. Thus, recombinant pRB can induce MOMP in isolated mitochondria supplemented with monomeric BAX.

Next, we wanted to investigate the BAX/BAK-dependence of this activity. As isolated mitochondria contain monomeric BAK, we repeated this assay using mitochondria isolated from \(Alb-cre^{pos};Bax^{-};Bak^{-}\) mouse livers (Walensky et al., 2006). Consistent with our cellular studies, pRB (up to 500nM) only induced cytochrome c release when these mitochondria were supplemented with BAX (Fig. 5B). Thus, pRB induces MOMP in a dose- and BAX-dependent manner.
**Figure 5. pRB induces MOMP by directly activating BAX.** (A) Mouse liver mitochondria were isolated, supplemented with recombinant, monomeric BAX and incubated with and without recombinant cleaved BID (positive control) or baculovirus-expressed, recombinant human pRB. Cytochrome c release was assessed following incubation and centrifugation by western blotting of pellet versus supernatant. Addition of either pRB or cleaved BID was sufficient to release cytochrome c into the supernatant. (B) Mitochondria isolated from Bax−/−;Bak−/− mouse livers were incubated with indicated amounts of recombinant pRB only or recombinant pRB and monomeric BAX. Recombinant pRB promotes cytochrome c release in a dose-dependent and BAX-dependent manner. Data are representative of two independent experiments. (C) ANTS/DPX-loaded liposomes were incubated with 50, 100, and 250nM recombinant pRB in the presence or absence of recombinant monomeric BAX. ANTS/DPX release was assessed over time. pRB yielded dose-dependent liposome permeabilization in a BAX-dependent manner. (D) *In vitro* binding assay using recombinant pRB and recombinant, monomeric BAX. Activated BAX was immunoprecipitated using an active-conformation specific BAX antibody (6A7) and binding was assessed by western blotting using an antibody against total BAX (inactive + active) and pRB. When co-incubated with inactive BAX, pRB bound to (lower panels) and stimulated formation of (upper panels) conformationally active BAX. (E) Co-immunoprecipitation experiment using IMR90 cells treated with TNFα/CHX for 3 hours. Endogenous pRB was immunoprecipitated from cell extracts and endogenous BAX association was assessed by western blotting. pRB and BAX form an endogenous complex *in vivo* in TNFα/CHX-treated cells. (A, C-E) Data are representative of at least three independent experiments.
To further explore this BAX specificity, we performed a liposome release assay in which freshly prepared ANTS/DPX-loaded liposomes were incubated with increasing concentrations of purified pRB in the absence or presence of recombinant, monomeric BAX (Fig. 5C). As expected, addition of either BAX or pRB (up to 250nM) alone was insufficient to yield ANTS/DPX release. In contrast, upon co-addition, pRB was sufficient to trigger BAX-dependent liposome permeabilization in a dose- and time-dependent manner.

We wanted to further investigate pRB’s ability to directly activate BAX. It is well established that BAX undergoes a conformational change upon activation that can be detected using an antibody (6A7) specific for the active conformation of BAX (Hsu and Youle, 1997). Thus, we performed an in vitro binding assay in which recombinant pRB and/or monomeric BAX were incubated separately or together in the absence of mitochondria or liposomes. We then screened for BAX activation by immunoprecipitation with 6A7, and assayed pRB association by western blotting (Fig. 5D). Notably, we found that addition of pRB promoted formation of the active BAX conformation (Fig. 5D, upper panels). Moreover, pRB co-immunoprecipitated with this active BAX species (Fig. 5D, lower panels). Thus, pRB can both associate with BAX, and trigger its conformational activation. Since the liposome and co-immunoprecipitation assays were both conducted in the absence of any other proteins, we can conclude that pRB is sufficient to directly activate BAX and induce BAX-mediated membrane permeabilization.

We next sought to validate the pRB:BAX interaction in the in vivo context and with endogenous proteins. Endogenous interactions between BAX and pro-apoptotic proteins, including BH3-only proteins, are notoriously difficult to observe due to the dynamic, ‘hit and run’ nature of these interactions (Eskes et al., 2000; Perez and White, 2000; Walensky and
Gavathiotis, 2011). Thus, to enable detection of such transient interactions, we treated primary human IMR90 fibroblasts with TNFα/CHX and then crosslinked using the amine-reactive crosslinking agent DSP. Using this approach, we were able to detect BAX within immunoprecipitates of the endogenous pRB protein (Fig. 5E). Thus, taken together, our in vitro and in vivo experiments show that pRB can bind directly to BAX and induce it to adopt the active conformation that triggers MOMP and cytochrome c release.

The mitochondrial function of pRB induces apoptosis even in the absence of pRB’s nuclear functions.

Our data have identified a mitochondrial function for pRB. We next wanted to address how this function contributes to pRB’s tumor suppressive activity. First, we asked which region of pRB is required for transcription-independent apoptosis. The tumor suppressor pRB is traditionally divided up into three domains (Fig. 6A): the N-terminus (residues 1-372), the small pocket domain (residues 373-766) and the C-terminus (residues 767-928). The small pocket is required for pRB’s known biological functions and most of the mutations found in human tumors disrupt this domain. We expressed the three domains (RB_N, RB_SP, and RB_C) in an inducible manner using stable RAT16 cell lines (Fig. 6A, B). Notably, expression of the small pocket of pRB, but not the N-terminal or C-terminal domains promoted apoptosis in response to TNFα and CHX (Fig. 6C). Thus, pRB’s ability to induce transcription-independent apoptosis mapped to the small pocket domain. Since this domain is essential for pRB’s tumor suppressive activity, these data are consistent with functional significance. Unfortunately, the small pocket is also required for most other pRB functions.
Figure 6. The Small Pocket of pRB is sufficient to induce transcription-independent apoptosis. (A) Schematic of pRB domains: the N-terminus (RB_N; residues 1-372), the small pocket domain (RB_SP; residues 373-766) and the C-terminus (RB_C; residues 767-928). (B) Stable variants of RAT16 cells allowing for inducible expression of HA-tagged full-length pRB, RB_N, RB_SP, and RB_C by doxycycline withdrawal were generated and expression was verified after 24 hours by western blotting using an HA-antibody. (C) RAT16 cells with and without expression of pRB, RB_N, RB_SP, and RB_C for 24 hours were treated with TNFα and CHX for 24 hours and levels of apoptosis were assessed by AnnexinV staining. Induced expression of full-length pRB and RB_SP, but not RB_N or RB_C, sensitized to TNFα/CHX-induced apoptosis. (B-C) Each RAT16 variant was independently generated three times. Graph bars represent average of three representative, independent experiments (±SD).
As an alternative approach, we sought to study the impact of the mitochondrial pRB activity in the absence of pRB's canonical nuclear functions by targeting pRB specifically to the mitochondria. Mutation of the NLS and direct targeting to the outside of the mitochondria using the transmembrane domain of Bcl2 proved insufficient for exclusive mitochondrial localization (data not shown), and instead we combined NLS mutation with fusion to the mitochondrial leader peptide of ornithine transcarbamylase (mitoRBANLS; Fig. 7A). We note that this leader peptide targets to the matrix of mitochondria, but it has been used successfully to study protein function at the outer mitochondrial membrane, presumably by allowing resorting to other mitochondrial compartments (Marchenko et al., 2000). We confirmed that expression of mitoRBANLS did not cause general mitochondrial cytotoxicity, as judged by the absence of increased ROS production (data not shown). We also localized a control protein, REL, to mitochondria to further control for any non-specific effect of mitochondrial targeting. Stable, inducible RAT16 cell lines were used to express mitoRBANLS, wildtype pRB, or mitoREL upon doxycycline withdrawal. Importantly, we confirmed specific targeting of mitoRBANLS and the control mitoREL protein to mitochondria (Fig. 7B, C). Consistent with its restricted localization, mitoRBANLS was unable to perform nuclear pRB functions, as judged by its inability to mediate the transcriptional repression of cell cycle genes that are known pRB targets (cdc2, mcm3, mcm5, mcm6, PCNA, and cyclinA2) in stark contrast to the wildtype pRB protein (Fig 7D). Importantly, mitoRBANLS retained the ability to enhance TNFα/CHX-induced apoptosis (Fig. 7E). This was not a non-specific effect of mitochondrial targeting, as the mitoREL control did not modulate apoptosis (Fig. 7E). We note that wildtype pRB was expressed at much higher levels than the mitoRBANLS protein, even when considering only the mitochondrially-localized fraction (Fig. 7C). Nevertheless, mitoRBANLS was almost as efficient as wildtype pRB at promoting
Figure 7. Mitochondria-targeted pRB is deficient for pRB’s nuclear function, but induces apoptosis in response to various apoptotic stimuli. (A) Schematic of mitoRBANLS construct. pRB was targeted to mitochondria by fusion to the mitochondrial leader peptide of ornithine transcarbamylase and mutation of the NLS. (B) Stable variants of RAT16 cells allowing for inducible expression of mitoRBANLS, wildtype pRB, mitoREL, and wildtype REL were generated and cellular localization following 24 hours induction was assessed by immunofluorescence using antibodies against pRB and REL. Mitochondria were visualized using MitoTracker. mitoRBANLS and mitoREL localized to mitochondria. (C) Western blotting showing induced expression levels of mitoRBANLS and wildtype pRB at mitochondria and total lysate. mitoRBANLS expressed at lower levels than wildtype pRB, even when considering the mitochondrial fraction. (D) pRB, but not mitoRBANLS, repressed E2F target genes cdc2, mcm3, mcm5, mcm6, PCNA, cycA2 as measured by RT-qPCR and normalized to ubiquitin. Average of two independent experiments (±SD). (E) Induced expression of mitoRBANLS and wildtype pRB, but not mitoREL or REL, sensitized to apoptosis induced by 24 hour treatment with TNFα and CHX. (B-E) Each RAT16 variant was independently generated three times. Graph bars represent average of three representative, independent experiments (±SD). (F) mitoRBANLS expression was induced by doxycycline addition for 24 hours in stable variants of wild-type, Bak−/−, Bax−/−, and Bax−/−.
TNFα/CHX-induced apoptosis. As an additional control against non-specific mitochondrial cytotoxicity, we wanted to verify that mitoRBΔNLS works through the same directed mechanism as wildtype pRB. Thus, we assessed the BAX/BAK dependence of this protein by generating stable variants of immortalized wildtype, Bak−/−, Bax−/−, or Bax−/−;Bak−/− MEFs to allow doxycycline-inducible mitoRBΔNLS expression (Fig. 7F). Expression of mitoRBΔNLS in wildtype and Bak−/−, but not Bak−/− or Bax−/−;Bak−/− MEFs, enhanced TNFα-induced apoptosis (Fig. 7G). Thus, mitoRBΔNLS promotes apoptosis in a BAX-dependent manner, exactly like wildtype pRB. Targeting pRB directly to the mitochondria therefore successfully generates a separation of function mutant that is deficient for pRB’s classic nuclear functions but retains the ability induce apoptosis in a transcription-independent, BAX-dependent manner in response to TNFα.

In our earlier experiments, induction of wildtype pRB and RB_SP consistently yielded a small, but significant increase in apoptosis even in the absence of TNFα treatment. This led us to hypothesize that mitochondrial pRB may function in a broader manner in apoptosis. We therefore evaluated the effect of mitoRBΔNLS expression on apoptosis induced by apoptotic factors other than TNFα. Immortalized wildtype or Bax−/−;Bak−/− MEFs with and without mitoRBΔNLS expression were treated with two drugs, etoposide and staurosporine (STS), that work via distinct mechanisms. Notably, mitoRBΔNLS expression enhanced both etoposide and STS-induced apoptosis (Fig. 7H). Thus, mitochondrial pRB can be activated in response to a

[Bak−/−] immortalized MEFs and confirmed by western blotting using antibodies against pRB, BAK, BAX, and tubulin. (G) Induction of mitoRBΔNLS in wildtype and Bak−/−, but not Bax−/− or Bax−/−;Bak−/−, MEFs sensitized to 10 hour treatment with TNFα and CHX. (H) Wildtype and Bax−/−;Bak−/− MEFs with and without induced expression of mitoRBΔNLS were treated with staurosporine (STS; 1μM) for 6 hours or etoposide (25μM) for 12 hours. Induction of mitoRBΔNLS in wildtype, but not Bax−/−;Bak−/− MEFs enhanced apoptosis in response to STS and etoposide. (F-H) Each MEF variant was independently generated twice. Graph bars represent average of three representative, independent experiments (±SD).
variety of apoptotic stimuli, including both intrinsic and extrinsic stimuli. This argues that mitochondrial pRB function may contribute to pRB-induced apoptosis in various settings.

**Mito-targeted pRB suppresses tumor growth *in vivo.*

We next wanted to evaluate whether the mitochondrial function of pRB can contribute to tumor suppression *in vivo.* For this analysis, we used a murine Rb⁻/⁻;p53⁻/⁻ osteosarcoma cell line (DKO-OS) that is capable of forming xenografts in mice. We generated stable variants of DKO-OS cells that would allow doxycycline inducible expression of mitoRBANLS or control proteins (Fig. 8A, B). Importantly, mitoRBANLS localized specifically to mitochondria in these tumor cells and it, but not mitoREL, promoted apoptosis in either the presence or absence of TNFa treatment (Fig. 8C and data not shown). We injected DKO-OS-mitoRBANLS or DKO-OS-wtRb cells into immuno-compromised mice (12 injections/cell line) and switched half of the animals to doxycycline once the tumor volume reached approximately 0.05cm³ to yield tumors without (herein referred to as basal) or with mitoRBANLS and wildtype pRB induction. We note that these tumor cells were not treated with apoptotic stimuli but presumably were subject to oncogenic stress. Control experiments (with parental DKO-OS cells) showed that the doxycycline treatment itself was not tumor suppressive (Fig. 8D). Strikingly, expression of wildtype pRB, versus mitoRBANLS, was similarly efficient in blocking further tumor expansion (Fig. 9A). Specifically, after 11 days, the tumor volume with basal expression was three fold greater than the tumor volume with either mitoRBANLS or pRB expression (Fig. 9A).

Importantly, despite this equivalent impact on tumor growth, examination of tumor sections confirmed that there were clear differences in the cellular response to mitoRBANLS versus pRB expression (Fig. 9B). Ki67, a proliferation marker and pRB-repression target, was
Figure 8. Mitochondria-targeted pRB induces apoptosis in transformed Rb^-;p53^- osteosarcoma cells. (A) Representative image showing localization of mitoRBANLS and pRB in transformed, Rb^-;p53^- osteosarcoma cells by immunofluorescence. mitoRBANLS localized to mitochondria and not the nucleus. (B) Western blot showing relative expression levels of mitoRBANLS and pRB after 24 hours of induction. mitoRBANLS expressed poorly compared to pRB. (C) Expression of mitoRBANLS or pRB, but not mitoREL, in transformed, Rb^-;p53^- osteosarcoma cells for 24 hours resulted in increased levels of apoptosis, even in the absence of TNFα treatment. Graph bars represent average of three independent experiments (±SD). (D) Rb^-;p53^- osteosarcoma cells (parental) were injected into the flank of nude mice. Once a tumor volume of approximately 0.05 cm^3 was reached, half the mice were fed dox food and tumor volume was monitored for 11 days (±SEM). Doxycycline had no effect on the growth of xenografted tumors. Tumor volume normalized to Day 1.
Figure 9. Mitochondria-targeted pRB induces apoptosis in vivo and is tumor-suppressive. (A) Rb\(^{-/-}\);p53\(^{-/-}\) osteosarcoma cell variants allowing for doxycycline inducible expression of mitoRBANLS or wildtype pRB were injected into the flank of nude mice (2 injection sites per mouse; 12 injections per cell line variant). Once a tumor volume of approximately 0.05 cm\(^3\) was reached, mitoRBANLS or wildtype pRB expression was induced in half the mice and tumor volume was monitored for 11 days (n=6/condition, ±SEM). Induced expression of mitoRBANLS or wildtype pRB suppressed growth of xenografted tumors. Tumor volume normalized to Day 1. (B) Induced expression of mitoRBANLS or wildtype pRB resulted in increased levels of apoptosis as measured by immunohistochemistry using a cleaved caspase 3 antibody (top). Tumors expressing mitoRBANLS also contained small areas with very high levels of apoptosis (inset). Induced expression of wildtype pRB, but not mitoRBANLS decreased proliferation as measured by Ki67 staining (bottom). Images representative of respective 6 tumor sections. (C) Murine p16 was knocked down in immortalized wildtype MEF variants that allow for doxycycline-inducible expression of pRB. Phosphorylation status of pRB (as judged by mobility) and knockdown of p16 using two distinct siRNAs was confirmed by western blotting. Induced expression of pRB enhances TNFα/CHX-induced apoptosis in the presence and absence of p16 and this activity is not inactivated by pRB phosphorylation. Bars, average of two independent experiments (±SD).
downregulated in tumors expressing wildtype pRB, but completely unaffected by mitoRBΔNLS (Fig. 9B, lower panels), in accordance with mitoRBΔNLS's inability to perform nuclear functions. Both pRB proteins promoted apoptosis, as judged by cleaved caspase 3 staining, but we observed qualitative differences in this response (Fig. 9B, upper panels). Tumors with wildtype pRB showed a uniform increase in apoptosis (2.5 fold increase relative to basal, p<0.01). Tumors with mitoRBΔNLS had regions comparable to the wildtype pRB response (2.3 fold increase relative to basal, p<0.001), but also included smaller regions (Fig. 9B, insert box) that were profoundly apoptotic. Thus, wildtype pRB promotes both cell cycle arrest and apoptosis, while mitoRBΔNLS is solely apoptotic. Importantly, despite its restricted biological activity, mitoRBΔNLS was as efficient, if not more efficient, than wildtype pRB at mediating tumor suppression in vivo.

These observations raise the possibility that the mitochondrial function of pRB could be employed as a tumor suppressive mechanism, even in p53-deficient cells, at least when re-expressed in Rb null cells. This is very intriguing because approximately two thirds of human tumors are wildtype for the Rb-1 gene, but instead carry alterations in upstream regulators of pRB (p16^{ink4A}, cyclin D or cdk4) that promote its cdk-phosphorylation. Thus, we wanted to determine whether pRB can mediate its mitochondrial apoptosis function in the presence of such tumorigenic changes. This question was further spurred by our finding that cdk-phosphorylated pRB is observed in the mitochondrial fractions (Fig. 4A). To address the influence of pRB phosphorylation on mitochondrial pRB function we used two distinct siRNAs to knockdown p16 in our immortalized MEF populations that allow for doxycycline-inducible expression of pRB. We confirmed that these siRNAs yielded near complete p16 knockdown and promoted hyper-phosphorylation of the doxycycline-induced pRB (Fig. 9C). Importantl, pRB enhanced
TNFα/CHX-induced apoptosis with similar efficiency in MEFs with p16-knockdown versus those transfected with a negative control duplex (Fig. 9C). These data strongly suggest that cdk-phosphorylated pRB is competent to induce mitochondrial apoptosis and that this function will persist in the majority of human tumors that contain wildtype, constitutively phosphorylated pRB.
Discussion

We have known for more than two decades that pRB is localized in both the nucleus and cytoplasm. However, prior studies have largely focused on pRB's nuclear functions, particularly its widespread transcriptional roles. To our knowledge, no biological function has been assigned to cytoplasmic pRB. In fact, it has been suggested that this species is essentially inactive, and its existence simply reflects the loss of nuclear tethering of cdk-phosphorylated pRB (Mittnacht and Weinberg, 1991; Templeton, 1992). By following the interplay between pRB and TNFα-induced apoptosis, we have uncovered clear evidence for a biological role for pRB at mitochondria in various cellular settings including normal, immortalized, and tumor cells. Initially, we found that pRB not only enhanced TNFα-induced apoptosis, but could do so even in the presence of cycloheximide. This argued that pRB could act in a novel manner to promote apoptosis. We then discovered that this pRB function was BAX-dependent, indicating crosstalk to the mitochondrial/intrinsic apoptotic pathway. Importantly, additional observations argue that pRB plays a direct, and broadly applicable, role in this pathway. First, a fraction of endogenous pRB, including some cdk-phosphorylated pRB, is localized on the outside of mitochondria. Second, recombinant pRB is able to induce MOMP and liposome permeabilization in vitro. Third, pRB can directly bind and activate BAX in vitro in the absence of any other proteins, and we confirm an interaction between the endogenous pRB and BAX proteins in vivo. Fourth, targeting pRB to mitochondria generates a separation of function mutant that is deficient for pRB's nuclear functions but able to induce apoptosis in response to various stimuli including TNFα, etoposide, staurosporine, and presumably (since it is active in tumor cells) oncogenic stress. Finally, and most importantly, this mitochondrially-tethered pRB is sufficient to suppress tumorigenesis. Given these findings, we believe that endogenous, mitochondrial pRB acts non-transcriptionally,
and in a broadly engaged manner, to promote apoptosis by activating BAX directly and inducing MOMP. To our knowledge, this is the first reported non-transcriptional and non-nuclear function for pRB.

Of course, we are not arguing that pRB is essential for mitochondrial apoptosis; this process is consistently impaired, but not ablated, by pRB-deficiency. Instead, we conclude that pRB is one of a growing list of proteins that are able to modulate the activity of core apoptotic regulators. We suspect that pRB acts at the mitochondria to fine-tune the apoptotic threshold, because its effects are dose-dependent (both in vitro and with overexpression/knockdown in vivo), it is localized to mitochondria both in the absence and presence of apoptotic stimuli, and it can potentiate many different pro-apoptotic signals. pRB’s pro-apoptotic role is highly reproducible, but the fold change in our cell studies could be judged as relatively modest (often 2-3 fold). However, we note that these experiments sample only one snapshot in time. Indeed, in the context of the in vivo xenograft experiments, mitoRBANLS yielded the same modest increase in the frequency of apoptotic cells within tumor sections (2-3 fold), but we can now see the cumulative effects of pRB action and it is profoundly tumor suppressive.

As described above, our data provide some insight into the underlying mechanism of mitochondrial pRB action; it requires BAX, but not BAK, both in vivo and in vitro, and pRB can directly bind to and activate formerly inactive monomeric BAX. Clearly, additional questions remain regarding this activity. The first concerns the precise nature of the pRB-BAX interaction. Given pRB’s preference for BAX over BAK, we conclude that the pRB binding site is either specific to BAX, and not BAK, or it represents a shared domain that is somehow masked in the BAK protein. We note that we have recently identified a novel, and unique, activation site on the BAX protein (Gavathiotis et al., 2010; Gavathiotis et al., 2008) and thus are interested to
learn whether this might mediate pRB binding. In the case of pRB, we have shown here that the small pocket domain is both necessary and sufficient for transcription-independent apoptosis and thus likely contains sequences essential for BAX binding. This is gratifying, because the small pocket is essential for pRB's tumor suppressive activity. However, this region is still relatively large and mediates interactions with many other known pRB-associated proteins. Thus, additional analysis will be required to further define the critical BAX-interacting sequence(s) and determine how this might compete with the binding of other pRB targets. The second key question concerns the mechanisms by which pro-apoptotic signals trigger mitochondrial pRB to activate BAX. One obvious candidate is post-translational modifications. Our data show that cdk-phosphorylated pRB is present at mitochondria and capable of promoting mitochondrial apoptosis, based on the retention of mitochondrial pRB activity in p16 knockdown MEFs. However, we have yet to understand whether phosphorylation by cdkks (or other kinases) is simply permissive for, or actively enables, pRB's mitochondrial apoptosis role. We also note that it has been previously reported that TNFα treatment induces cleavage of pRB at the C-terminus releasing a 5kDa fragment (Huang et al., 2007; Tan et al., 1997). We were intrigued by the possibility that this cleavage might be responsible for the activation of mitochondrial pRB function by TNFα, presumably by exposing/activating the required small pocket domain. However, our exploratory analyses of both pre-cleaved (i.e. delta 5kDa) and uncleavable forms of pRB in stable inducible cell lines were inconsistent with the notion that cleavage is necessary and sufficient for pRB activation (data not shown). Thus, it remains an open question how pro-apoptotic signals including TNFα trigger the mitochondrial pRB response.
Regardless of the remaining mechanistic questions, our findings considerably expand our appreciation of pRB’s role in apoptosis. It is already clear that pRB can either suppress apoptosis by enforcing cell cycle arrest, or promote DNA damage-induced apoptosis by transcriptionally co-activating pro-apoptotic genes (Ianari et al., 2009). Here, we show that in addition to these transcriptional mechanisms, pRB can also promote apoptosis directly at mitochondria. We believe that pRB’s overall pro-apoptotic function is likely a result of the combined effect of nuclear and mitochondrial pRB. The relative extent to which these two functions contribute to apoptosis is likely context specific. However, our mito-tagged pRB experiments clearly showed that the mitochondrial function of pRB can contribute to apoptosis in response to a broad range of stimuli, including the oncogenic context of tumor cells. Most importantly, our in vivo xenograft studies establish the tumor suppressive potential of mitochondrial pRB in the absence of classic, nuclear pRB functions. Given these observations, we conclude that mitochondrial apoptosis represents a novel, and bona fide, mechanism of tumor suppression for pRB. This adds to a growing list of ways in which pRB has the potential to be tumor suppressive, including its classic cell cycle function (Burkhart and Sage, 2008), transcriptional co-regulation of apoptosis, autophagy, and metabolic genes (Blanchet et al., 2011; Ciavarra and Zacksenhaus, 2011; Takahashi et al., 2012; Tracy et al., 2007; Viatour and Sage, 2011), and its ability to control fate commitment by modulating the transcriptional activity of core differentiation regulators (Calo et al., 2010; Viatour and Sage, 2011). The extent to which each of these mechanisms of pRB action contributes to overall tumor suppression remains to be fully elucidated. The ability to localize pRB specifically to the mitochondria allowed us to study pRB’s mitochondrial role in the absence of all other known pRB functions. Remarkably, at least in this context, this mito-specific pRB was as efficient, if not more efficient, than wildtype pRB.
at suppressing tumorigenesis. This unequivocally establishes the potential potency of pRB’s mitochondrial apoptosis function. However, it does not disavow the potential contribution of other pRB functions. It remains an open question whether the myriad roles of pRB collaborate consistently in tumor suppression or whether, in specific contexts, one or more functions have more physiological relevance than others. It is also interesting to note that the multiple functions of pRB are highly reminiscent of those of the p53 tumor suppressor. p53 was also initially linked to cell cycle arrest and then shown to play a central role in promoting apoptosis both through the transcriptional activation of pro-apoptotic genes and by directly inducing MOMP at the mitochondria in a transcription-independent manner (Chipuk et al., 2004; Leu et al., 2004; Mihara et al., 2003; Speidel, 2010; Vousden and Prives, 2009). Our data now suggest that the direct promotion of mitochondrial apoptosis is a general mechanism of tumor suppression.

Finally, we believe that our findings have significant implications for therapeutic treatment in the approximately two-thirds of human tumors that retain wildtype pRB but instead carry mutations that promote cdk/cyclin activation and pRB phosphorylation (Sherr and McCormick, 2002). Historically, little attention has been paid to RB-1 status in chemotherapeutic response because the absence of pRB or the presence of phospho-pRB similarly inactivates pRB-mediated G1 arrest. However, we have previously found that phospho-pRB can activate transcription of pro-apoptotic genes (Janari et al., 2009). In this current study, we show that the endogenous mitochondrial pRB includes the cdk-phosphorylated form, and it retains its pro-apoptotic role in highly proliferative tumor cells and after inactivation of the cdk inhibitor p16. Moreover, both the transcriptional and the mitochondrial, pro-apoptotic functions of pRB occur independent of p53. Thus, we believe that it should be possible to develop chemotherapeutic strategies for the majority of human tumors that retain wildtype RB-1,
which engage phospho-pRB and promote apoptosis through both transcriptional and mitochondrial mechanisms.
Materials and Methods

Cell Culture and Drug Treatment

RAT16 and IMR90 cells were grown in MEM with Earle’s Salts and 10% FBS, Pen-Strep, L-Glutamine, Sodium Pyruvate, and NEAA. All other cell lines were grown in DMEM with 10% FBS and Pen-Strep. For TNFα treatments, 50ng/ml of recombinant mouse TNFα (Sigma) was used with 0.1μM MG132 (Calbiochem) for 48 hours or 0.5μg/ml CHX (Sigma) for 24 hours unless noted otherwise. MEFs were treated with TNFα/CHX for 10 hours, 1μM staurosporine (Sigma) for 6 hours, or 25μM etoposide (Sigma) for 12 hours. TET System approved FBS (Clontech) and 1μg/ml doxycycline (Clontech) were used for inducible cell lines unless noted otherwise. Overexpression was induced for 24 hours prior to TNFα treatment. 20μg/ml and 15μg/ml doxycycline were used in wildtype, Bax⁻⁻, and Bak⁻⁻ MEFs to induce expression of pRB and mitoRBΔNLS, respectively. 5μg/ml doxycycline was used in Bax⁻⁻;Bak⁻⁻ MEFs to adjust for expression levels.

FACS Apoptosis Analysis

Cell suspensions were stained with AnnexinV-FITC or APC (Becton Dickinson) and Propidium Iodide (Sigma) or 7AAD (Becton Dickinson). Total apoptotic cells were assessed by gating for AnnexinV positive using a FACScan or FACSCalibur System (Becton Dickinson). Similar trends were observed for all experiments when only early apoptotic (AnnexinV⁺;PI/7AAD⁻) cells were considered (data not shown). For cell cycle profiling, cell suspensions were processed as previously described (Ianari et al., 2009) and analyzed by FACScan and FlowJo.
Plasmid and Stable Cell Line Generation

Human pRB knockdown was performed as described previously (Chicas et al., 2010) and the rodent Rb target sequence was TATAATGGGAATCAAACTCCTC, Luc control GAGCTCCCGTGAATTGGAATCC. 10μM siRNAs (IDT, murine p16) were transfected into MEFs using RNAiMax (Invitrogen) according to manufacturer recommendations. The lentiviral vector pCW22 (tet-on) was used for expression studies. Since RAT16 cells already contained tTA (tet-off), the rtTA was removed by AgeI and XmaI digestion. mitoRBANLS was generated by amplifying the mitochondrial leader peptide of ornithine transcarbamylase (cacaagggcatctgtggatcactgtgaag, ggaagggccctgctgtttaatctgagga, ggaaggccctgctgtttaatctgagga, ggaaggccctgctgtttaatctgagga) and human RB (catagggcccctgctgtttaatctgagga, gatttttaattaatcatttctcttctttgt). The NLS was mutated using gcaaaactttgcatctgttgaag, tatcaagggcatctgttgccagttg, followed by HindIII digestion. mitoREL was generated by amplifying the mitochondrial leader peptide of ornithine transcarbamylase (cacaagttaatgctgtgtaatctgagga, gtttcggaggccctgctgtttttag, gtttcggaggccctgctgtttttag, gtttcggaggccctgctgtttttag) and human REL (tgcccctggagctgtgtaatctgagga, gatttttaattaatcatttctcttcttttag). pCW22 3HA was generated using 3HA peptide (IDT) and ligated to RB_N (cacaagtgcatcctgctgtaatctgagga, gatttttaattaatcatttctcttcttttag), RB_SP (cacaagtgcatcctgctgtaatctgagga, gatttttaattaatcatttctcttcttttag), and RB_C (cacaagtgcatcctgctgtaatctgagga, gatttttaattaatcatttctcttcttttag). Cells infected with the inducible construct underwent blasticidin (Invitrogen) selection. All RAT16 variants were independently generated three times and all MEF variants were independently generated twice.
Mitochondrial Fractionations

IMR90 mitochondria were fractionated as follows: cells were resuspended in cold Buffer A [250mM Sucrose, 20mM HEPES pH 7.5, 10mM KCl, 1.5mM MgCl₂, 1mM EDTA, 1mM EGTA, 1mM DTT, protease inhibitors (Roche)] and homogenized using 20 strokes in a ¼” cylinder cell homogenizer (H&Y Enterprises, Redwood City, CA), 0.1558” ball. Nuclei and unlysed cells were removed by low-speed centrifugation and mitochondria and 10% of initial cell suspension (for Total Lysate) were lysed using RIPA buffer (0.5% Sodium Deoxycholate, 50mM Tris HCl pH 7.6, 1% NP40, 0.1% SDS, 140mM NaCl, 5mM EDTA, 100mM NaF, 2mM NaPγ, protease inhibitors) and quantified using BCA Protein Assay reagent (Pierce).

Mitochondria were isolated from livers of 2-6 month old mice maintained on a mixed C57Bl/6 x129sv background as follows: Livers were minced in Buffer A (0.3M Mannitol, 10mM HEPES/K pH 7.4, 0.1% BSA, 0.2mM EDTA/Na pH 8.0) and homogenized using 3 strokes in a Teflon Dounce Homogenizer. A small fraction was removed for total lysate. Nuclei and unlysed cells were removed from the remaining supernatant by low-speed centrifugation and mitochondria were washed in Buffer B (0.3M Mannitol, 10mM HEPES/K pH 7.4, 0.1% BSA). For whole mitochondria analysis, mitochondria and total lysate were lysed in RIPA buffer. For subfractionation, the mitochondrial pellet was resuspended at 2mg/ml in Hypotonic Buffer (10mM KCl, 2mM HEPES/K pH7.9), incubated on ice for 20 minutes and centrifuged at 14000rpm. The pellet was washed twice with Wash Buffer (150mM KCl, 2mM HEPES/K pH7.9) and all three supernatants combined yielded the non-mitoplast fraction. The mitoplast was resuspended in Hypotonic/Wash Buffer and equal fractions were analyzed by SDS-PAGE.
Mitochondria were isolated from livers of Alb-cre<sup>pos</sup>;Bax<sup>−/−</sup>;Bak<sup>−/−</sup> mice as reported previously (Walensky et al., 2006).

**In Vitro Cytochrome C Release Assay**

The assay was performed as previously described (Chipuk et al., 2005). Briefly, wildtype mitochondria were resuspended in Mitochondrial Isolation Buffer (200mM Mannitol, 68mM Sucrose, 10mM HEPES/K pH 7.4, 100mM KCl, 1mM EDTA, 1mM EGTA, 0.1%BSA) to a concentration of 1µg/µl and incubated with pRB (Sigma), GST-Bax (Sigma), or cleaved BID (R&D Systems) for 1 hour, 37°C. DKO mitochondria were resuspended in experimental buffer (125mM KCl, 10mM Tris-MOPS [pH 7.4], 5mM glutamate, 2.5mM malate, 1mM KPO4, 10µM EGTA-Tris [pH 7.4]) to a concentration of 1.5 mg/ml, and incubated with monomeric BAX (purified as previously described (Gavathiotis et al., 2008)) and pRB (ProteinOne) for 45 min, room temperature. Samples were centrifuged at 5500g and pellets versus supernatants were analyzed by SDS-PAGE and immunoblotting.

**Liposomal Release Assay**

The liposomal release assay was performed as described previously (LaBelle et al., 2012; Lovell et al., 2008). Briefly, large unilamellar vesicles (LUVs) were generated from a lipid mixture of 48% phosphatidylcholine, 28% phosphatidylethanolamine, 10% phosphatidylinositol, 10% dioleoyl phosphatidylserine and 4% tetraoleoyl cardiolipin as chloroform stocks (Avanti Polar Lipids). The lipid mixture was dried in glass test tubes under nitrogen gas and then under vacuum for 15 h. The fluorescent dye ANTS (6.3 mg) and the quencher DPX (19.1 mg) were added to 1 mg of dry lipid film, and the mixture resuspended in assay buffer (200mM KCl, 1mM
MgCl2, 10mM HEPES, pH 7.0). After five freeze-thaw cycles, the lipid mixtures were extruded through a 100 nm nucleopore polycarbonate membrane (Whatman) using a mini extruder (Avanti). Liposomes were isolated by gravity flow SEC using a crosslinked Sepharose CL-2B column (Sigma Aldrich). LUVs (5 μl) were treated with the indicated concentrations of BAX and pRB in 384-well format (Corning) in a total reaction volume of 30 μl. After time-course fluorescence measurement on a Tecan Infinite M1000 spectrophotometer (excitation 355 nm, emission 520 nm), Triton X-100 was added to a final concentration of 0.2% (v/v) to determine maximal release.

**Immunoprecipitation**

For the *in vitro* binding assay, recombinant, monomeric BAX (1 μM) and pRB (1 μM) were mixed in 10μl TBS (50 mM Tris, 150 mM NaCl) and pre-incubated for 30 minutes, room temperature. The samples were diluted with 1% BSA in TBS to 80μl, and incubated with pre-equilibrated Protein A/G-agarose beads (Santa Cruz) and 5μl 6A7 antibody (sc-23959, Santa Cruz) with rotation for 1 h, room temperature. Beads were collected and washed three times with 0.5 ml 1% (w/v) BSA/TBS buffer.

For the endogenous interaction study, IMR90 cells were treated with 50ng/ml TNFα and 0.5μg/ml CHX for 3 hours total and crosslinked with 1mg/ml DSP (Pierce) for 1 hour. Proteins were extracted using an NP40 based buffer (50mM HEPES pH7.9, 10% glycerol, 150mM NaCl, 1%NP40, 1mM NaF, 10mM B-Glycerophosphate, protease inhibitors). The following antibodies were used: pRB (Cell Signaling, 9309), normal rabbit IgG (Santa Cruz).
Western Blotting

Samples were loaded in SDS Lysis Buffer (8% SDS, 250mM TrisHCl pH 6.6, 40 % glycerol, 5% 2-Mercaptoethanol, bromophenol blue), separated by SDS-PAGE, transferred to a nitrocellulose membrane, and blocked in 5% nonfat milk. The following antibodies were used in 2.5% nonfat milk: human pRB (Cell Signaling, 9309), rodent pRB (Becton Dickinson, 554136), phospho-pRB (Cell Signaling, 2181,9301,9307,9308), procaspase 7 (Cell Signaling, 9492), cleaved caspase 7 (Cell Signaling, 9491), procaspase 3 (Cell Signaling, 9662), cleaved caspase 3 (Cell Signaling, 9661), actin (Santa Cruz, SC1616 HRP), tubulin (Sigma, T9026), BAX (Cell Signaling, 2772; Santa Cruz, sc-493), BAK (Cell Signaling, 3814), cyclin A (Santa Cruz, sc594), HDAC1 (Upstate, 05-614), PCNA (Abcam, ab29), Lamin A/C (Cell Signaling, 2032), COXIV (Cell Signaling, 4850), Histone H3 (Santa Cruz, sc8654), ATPB (Abcam, ab5432), SirT3 (Cell Signaling, 5490), cytochrome c (Becton Dickinson, 556433), p16 (Santa Cruz, sc74401), HA.11 (Covance) and GAPDH (Ambion, 4300). Secondary HRP-conjugated antibodies (Santa Cruz) were used at 1:5000 in 1% nonfat milk.

RealTime PCR

RNA was isolated using RNAeasy Kit (Qiagen) and reverse transcribed using Superscript III reverse transcriptase (Invitrogen). RealTime PCR reactions were performed with SYBR Green (Applied Biosystems) on the ABI Prism 7000 Sequence Detection System and analyzed using the 7000 SDS software. The following primers were used:

Cdc2 (CTGGCCAGTTCACTGGATTCT, ATCAAACTGCGAGATTTCCG),
Cyclin A2 (GAGAATGTCAACCCCGAAAA, ATAAACGATGACGACGTCC),
Mcm3 (GTAGCGAGGTTCCTACATAG, TCTTCTTAGTAGCAGGACAG),

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Mcm5 (GAGGACCAGGAGATGCTGAG, CTTTACCGCCTCAAGGATGAC),
Mcm6 (CACGATTTGGAGGAAAGAA, TCCACGGCAATGATGAAGTA),
PCNA (TCCACAGAACAATGCTTGA, TTATTTGGCTCCCAAGATCG),
UB (TTCTGGAAGACCTCTGACC, ACTCTTCTTTGATGTTGATGTC),
Casp3 (ACTGTGGATTGAGAGACAG, CGCAAAGTGACTGGATGAAC),
Casp7 (GCTCCACCATCATCTTCATCC, TGCCCATCTTCTCAAGGATCC),
Bcl2 (AGTACCTGAAACCGCATCTG, AGGTCTGCTGACCTTACCTTG),
Apafl (CATGCCCTCCTAGAAGGTG, GAACACGGAGAGGTCTTTG),
Casp9 (GTAGTGAAGCTGGACCCATC, TTCCCTGGAGTACAGACATC),
MAP3K5 (CGTGGCCGAATCTCAAAG, ACTGTAGTGTTGGCTCAGAC),
p21 (TGGCCTTGTGCCTCCTTG, AGACCAATCGGGCTTTGAG),
Casp1 (CTAAGGGGGAGGACATCCTTTC, CAGATAATGAGGGCAAGAC),
Casp4 (AAGAGGAGCTTACGGCTGAG, CCTGCAATGTGCCATGAGAC),
Beclin (TAGCTGAAGACCCCCGTAG, TGCTGCTGGACGCCTTAGAC),
Flip (ATAAAGCAGGGGCTGGAGAC, TTGCCTCGGCCTGTATAATC)

Xenograft Model: All animal procedures followed protocols approved by MIT’s Committee on Animal Care. Nude/SCID mice (Taconic) were injected subcutaneously with 10^7 tumor cells per site, 2 sites per mouse. After euthanasia, tumors were removed, fixed overnight in formalin followed by overnight incubation in 70% ethanol, and subjected to histological processing.

Immunofluorescence and Immunohistochemistry

Cells were plated at low density on coverslips and protein expression was induced for 48 hours. Mitochondria were labeled using MitoTracker Deep Red (Invitrogen) at 100nM for 45 minutes,
37°C. Cells were fixed in 4% Formaldehyde (Thermo Scientific), permeabilized with 0.25% TritonX-100/PBS, and blocked with 5% Goat Serum in 0.2% Tween20/PBS for 30 minutes, 37°C. The following antibodies were used at 1:200 for 1 hour, room temperature: pRB (Cell Signaling, 9309), c-REL (Cell Signaling, 4727). Alexa Fluor 488 (Invitrogen) was used at 1:1000 for 1 hour, room temperature and slides were mounted using SlowFade Gold Antifade Reagent with DAPI (Invitrogen) and observed under a fluorescence microscope (Zeiss).

Ki67 and cleaved caspase 3 immunohistochemistry was performed with a modified citric acid unmasking protocol. Briefly, paraffin was removed from slides, followed by incubation in 0.5% H2O2/methanol for 15 minutes and antigen retrieval using citrate buffer, pH 6.0 in a microwave for 15 minutes. Slides were blocked for 1 hour at room temperature in 2% normal horse serum (Ki67) or 10% goat serum (cc3) in PBS. Ki67 (Becton Dickinson, 550609, 1:50) and cleaved caspase 3 (Cell Signaling, 9661, 1:200) antibodies were used in 0.15% Triton/PBS overnight at 4°C. Secondary antibodies (Vector Laboratories) were used at 1:200 in PBS with 0.4% normal horse serum (Ki67) or 2% goat serum (cc3), detected using a DAB Substrate Kit (Vector Laboratories), and counterstained with haematoxylin.
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References


Chapter Three

Regulation of the mitochondrial function of pRB

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K.I.H. contributed to all experiments and text. J.R. contributed Fig. 3A, B. E.S.L contributed Fig. 5B, C.
Abstract

The retinoblastoma protein (pRB) is an important tumor suppressor and functions as a transcriptional co-regulator of a multitude of cellular processes including proliferation, differentiation, and apoptosis. We have recently extended our appreciation of pRB's tumor suppressive roles to show that a fraction of pRB localizes to mitochondria and directly binds to and activates BAX to induce apoptosis. Thus, pRB can promote apoptosis both via a nuclear, transcriptional mechanism and a mitochondrial, non-transcriptional mechanism. This study has yielded many more questions, including how mitochondrial pRB function is regulated. Here we present our initial attempts toward addressing these follow-up questions. Specifically, we show that ectopic pRB expression induces apoptosis within a few hours. We were unable to identify a BH3 domain, but show that pRB impinges on the mitochondrial apoptotic pathway by interacting with several Bcl2 family members. Finally, we show that mitochondrial pRB function is regulated in a manner that is distinct from pRB's anti-proliferative function. Together, these data reinforce and expand our previous observations about mitochondrial pRB function.
Introduction

There are two general apoptotic pathways: the intrinsic/mitochondrial pathway induced by stimuli like DNA damage and the extrinsic pathway induced by death ligands like TNFα. The extrinsic pathway can feed into the mitochondrial pathway and both activate initiator caspases (caspases 8, 9), which in turn cleave and activate effector caspases (caspases 3, 6, 7) (Chipuk et al., 2010). The Bcl2 protein family controls initiation of the mitochondrial pathway and is divided into three subclasses based on the presence of Bcl2 homology domains (BH1-4), which correspond to α-helices. Anti-apoptotic Bcl2 family members (e.g., Bcl2, BclX) contain all four BH domains, while pro-apoptotic, multidomain family members (BAX, BAK) contain three. Finally, pro-apoptotic BH3-only proteins act as upstream sensors of apoptotic stimuli. Activation of BH3-only proteins results in activation and oligomerization of BAX/BAK, formation of a pore and permeabilization of the mitochondrial outer membrane (MOMP), and release of apoptotic regulators (e.g., cytochrome c) from the intermembrane space into the cytoplasm. This in turn causes initiator caspase activation and apoptosis (Wyllie, 2010). Thus, the mitochondrial pathway is dependent on BAX or BAK (Lindsten et al., 2000; Wei et al., 2001). Inactive BAX is cytoplasmic, while BAK is constitutively localized to the outer mitochondrial membrane. BAX/BAK activation is a multi-step process involving translocation of BAX to the mitochondrial outer membrane and conformational changes in BAX and BAK (Youle and Strasser, 2008). BH3-only proteins can effect these changes in BAX/BAK by either acting as activators and directly binding BAX/BAK or by acting as sensitizers and binding anti-apoptotic Bcl2 family members to relieve their inhibition of pro-apoptotic family members (Letai et al., 2002). In addition, a number of non-Bcl2 family members have been identified that can act as activators or sensitizers to promote activation of BAX/BAK and apoptosis. These include
the tumor suppressor p53, the orphan nuclear steroid receptor Nur77, the nuclear protein histone H1.2, and the nucleolar phosphoprotein nucleophosmin (Chipuk et al., 2004; Chipuk et al., 2003; Dumont et al., 2003; Gine et al., 2008; Kerr et al., 2007; Konishi et al., 2003; Li et al., 2000; Lin et al., 2004; Mihara et al., 2003; Okamura et al., 2008).

We have recently uncovered a novel, non-transcriptional role of the tumor suppressor pRB in promoting apoptosis directly at the mitochondria (Hilgendorf et al., 2013). pRB is a pleiotropic protein and has been most well characterized as a transcriptional co-regulator of proliferation. Specifically, pRB regulates entry into the cell cycle by interacting with and inhibiting the E2F family of transcription factors during the G0/G1 phase of the cell cycle. Upon mitogenic signaling, cyclin-CDK complexes are activated and hyperphosphorylate pRB, resulting in release of E2F transcription factors and expression of genes important for S phase entry (van den Heuvel and Dyson, 2008). While the RB-1 gene itself is found mutated in some tumors (e.g. retinoblastoma, osteosarcoma), most tumors contain alterations upstream of pRB, resulting in constitutive hyperphosphorylation of the pRB protein and inactivation of its cell cycle function (Burkhart and Sage, 2008; Gordon and Du, 2011). Nuclear pRB has also been shown to regulate other cellular processes, including apoptosis. Specifically, pRB can transcriptionally co-activate expression of pro-apoptotic genes in response to various apoptotic stimuli, including genotoxic stress, oncogenic stress, and TGFβ treatment (Carnevale et al., 2012; Ianari et al., 2009; Korah et al., 2012).

We have shown that pRB can also promote apoptosis in the presence of an inhibitor of translation and that a fraction of pRB is localized to mitochondria. Moreover, this mitochondrial pRB can directly bind to and activate BAX to induce MOMP. Thus, in addition to its many nuclear functions, pRB can promote apoptosis directly at the mitochondria. Moreover, pRB that
is targeted directly to mitochondria via fusion to a mitochondrial leader peptide is tumor-suppressive \textit{in vivo}. This shows that mitochondrial pRB function can contribute toward pRB's overall role as a tumor suppressor.

We do not understand yet how pRB localizes to mitochondria (including in untreated cells) and is regulated to bind and activate BAX. Moreover, we do not know how this pRB function fits into overall activation of mitochondrial apoptosis. Here we present our initial forays into answering these questions. We note that much of this data is negative and should therefore be interpreted with caution. Nonetheless, these data are important in shaping how we propose to move our study of mitochondrial pRB function forward. In particular, we present evidence that pRB functions as a general, and rapid, modulator of apoptosis by binding BAX as well as Bcl2. Furthermore, we show that mitochondrial pRB activity is regulated in a manner distinct from nuclear pRB's anti-proliferative function. Taken together, we therefore provide further proof for mitochondrial pRB function and have initiated the process of elucidating mitochondrial pRB's mechanism of action.
Results

**Ectopic pRB expression induces effector caspase cleavage and apoptosis within hours.**

We have previously shown that pRB localizes to mitochondria both in the absence and presence of treatment and can induce mitochondrial apoptosis directly in a transcription-independent manner and in response to various apoptotic stimuli, including TNFα, etoposide, staurosporine, and in tumors (Hilgendorf et al., 2013). Moreover, ectopic expression of pRB resulted in a small, but highly reproducible increase in levels of apoptosis (Hilgendorf et al., 2013). This lead us to hypothesize that pRB acts as a general modulator of mitochondrial apoptosis, which can be activated in response to various cellular damages and stresses. However, the mechanism of how pRB localizes to mitochondria and gets activated to promote apoptosis remains to be determined. Since ectopic pRB expression induces apoptosis, we first wanted to investigate the kinetics of mitochondrial pRB function. To study this, we used an immortalized rat embryonic fibroblast cell line variant, RAT16, which is inducible for pRB expression upon withdrawal of doxy-cycline. We induced pRB expression and analyzed cells for apoptosis and effector caspase protein levels every 4 hours for 24 hours in the absence of any apoptotic treatment (Fig. 1). Ectopic expression of pRB did not affect procaspase 3 and 7 protein levels (Fig. 1A). This is consistent with our prior data showing no upregulation of effector caspase mRNA upon ectopic pRB expression (Hilgendorf et al., 2013). Notably, apoptosis levels, as assessed by cleaved caspase 3 and 7 protein levels and AnnexinV staining, were increased within about 12 hours of ectopic pRB expression (Fig. 1A, B). We note that the overall levels of apoptosis were small, but that this experiment was conducted in the absence of an apoptotic stimulus. There was also a small initial increase in apoptosis at the 4 hour time point. Since we observed this both with and without induction of pRB expression, this is likely a
Figure 1. Ectopic pRB expression promotes apoptosis with a few hours. pRB expression was induced in RAT16 cells and cells were collected every four hours for 24 hours and analyzed by both western blotting and AnnexinV staining. (A) Western blot showing pRB, pro-caspase 3 and 7, and cleaved caspase 3 and 7 protein levels. pRB is maximally expressed within 8 hours of induction. Increased levels of cleaved caspase 3 and 7 are observed after about 12 hours of pRB expression. Pro-caspase 3 and 7 levels are unchanged. (B) Levels of apoptosis as assessed by AnnexinV staining. Induced expression of pRB results in a small increase in apoptosis after about 16 hours of treatment. This is consistent with the western blot data. (C) Western blot showing nuclear and mitochondrial pRB levels after 4 and 24 hours of induced pRB expression. Mitochondrial pRB levels accumulate over time.
result of stress induced by the experimental handling of the cells. Importantly, approximately 8 hours of doxycycline withdrawal were required for maximal ectopic pRB expression and the kinetics of mitochondrial pRB protein accumulation mimicked those of nuclear pRB (Fig. 1A, C). Thus, ectopic pRB expression induces apoptosis within a few hours, consistent with the hypothesis that it can function as a modulator of apoptosis to fine-tune the apoptotic response.

**pRB induces mitochondrial apoptosis in a BH3-independent manner.**

We next wanted to investigate which region of pRB is important to directly trigger MOMP. We have previously shown that pRB’s transcription-independent, pro-apoptotic function maps to the small pocket domain. Moreover, we have shown using both genetic and biochemical approaches that pRB acts as a direct activator of the pro-apoptotic multidomain Bcl2 family member BAX (Hilgendorf et al., 2013). Next, we wanted to investigate whether pRB contains a BH3 domain and is therefore a Bcl2 family member. Analysis of the human pRB sequence identified 8 putative BH3 domains based on the presence of the BH3 consensus sequence (LxxxxD), with none of them highly conserved across species. The Small Pocket contains four of these domains (henceforth referred to as peptide #1-4 based on position within the protein) (Fig 2A). Analysis of the crystal structure of the unliganded pRB pocket (Balog et al., 2011) revealed that all four putative domains are exposed, but only peptide #2 forms an α-helix, the secondary structure adopted by BH3 domains to promote apoptosis (Fig. 2B). However, we note that activation of pRB, perhaps via post-translational modifications, may change the structure of pRB to form additional exposed alpha-helices (Burke et al., 2012; Rubin, 2012). We synthesized peptides containing the putative BH3 domain and surrounding residues (20-mer) and assayed for ability to interact with Bcl2 family members. Specifically, we performed an *in vitro* competitive fluorescence polarization assay and tested for ability of one of
Figure 2. Putative BH3 domain in the Small Pocket of pRB. (A) Schematic showing four putative BH3 domains in the small pocket of pRB based on the consensus sequence LxxxxD and conservation across species. (B) Analysis of the crystal structure of the unliganded pRB pocket reveals that all four putative BH3 domains, in red, are exposed. Only peptide #2 forms an alpha helix.
the peptides to displace a fluorescently labeled BIM BH3 peptide from the hydrophobic pocket of several recombinant anti-apoptotic Bcl2 family members. Peptides #1-3 did not interact with any of the Bcl2 family members tested (Fig. 3A). Remarkably, peptide #4 was able to interact with, and specifically displace a BIM BH3 peptide from, all five anti-apoptotic Bcl2 family members in vitro (Fig. 3A). The efficiency of this ability ranged depending on the Bcl2 family member tested, with binding to Mcl1 being the strongest and comparable to that of the BH3 peptide from PUMA, a physiological trigger of MOMP. Importantly, a mutant PUMA peptide (PUMA2A, L and D residues of LxxxxD replaced with alanines) did not show this activity, arguing for the specificity of this assay.

Next, we investigated the ability of peptide #4 to permeabilize mitochondria. We tested both wild-type (AML2 and MOLM13) and BAX/BAK mutant cells (DHL10) and assayed for depolarization of mitochondria using the membrane-permeable JC-1 dye. Remarkably, peptide #4 was able to permeabilize mitochondria of AML2 and MOLM13, but not DHL10 cells in a dose-dependent manner (up to 50uM) (Fig. 3B). This activity was above the threshold permeabilization triggered by the negative control PUMA2A, though not as efficient as the depolarization induced by the BH3 peptides from BIM and PUMA, physiological triggers of MOMP. We note that at higher concentrations, peptide #4 also permeabilizes mitochondria in a BAX, BAK-independent manner (data not shown). Moreover, peptide #1 induces hyperpolarization of mitochondria even at very low concentrations, though the physiological relevance of this finding remains to be elucidated. Regardless, our in vitro assays suggest that pRB peptide#4 may contain a BH3 domain.
Figure 3. pRB does not appear to contain a BH3 domain. (A) Competitive fluorescence polarization assay using peptides containing one of the four putative pRB BH3 domains. Peptide #4 displaces the BIM BH3 peptide from all five recombinant anti-apoptotic Bcl2 family members tested. PUMA BH3 peptide serves as a positive control, mutant PUMA BH3 peptide (PUMA2A) as a negative control. (B) Cells wildtype (AML2, MOLM13) or mutant (DHL10) for BAX and BAK were incubated with peptides and permeabilization of mitochondria was assessed over 3 hours (AUC). Data is normalized to treatment with
We wanted to test this BH3 domain in vivo. Further analysis of the sequence surrounding this domain revealed that the residues immediately following the aspartic acid of the BH3 consensus sequence are serine and proline (Fig. 3C). It has been shown that p38 phosphorylates this serine (Ser567) in response to genotoxic stress and that this event may play a role in induction of apoptosis (Delston et al., 2011). As mentioned above, post-translational modifications can affect the structure of the protein and it is therefore conceivable that Ser567 phosphorylation would result in the formation of an exposed alpha-helix. Moreover, many but not all BH3 sequences contain a glutamic acid residue following the BH3 motif (LxxxxDE) (Lomonosova and Chinnadurai, 2008). This is intriguing, since phosphorylation of serine would mimic a glutamic acid residue and may therefore regulate formation of a more efficient BH3 domain to activate pRB’s mitochondrial, pro-apoptotic function. Finally, mutation analysis of retinoblastoma cases has previously identified a bilateral retinoblastoma case with a missense mutation at residue 567 resulting in a serine to leucine substitution, suggesting that this residue confers a tumor suppressive function to pRB (Yandell et al., 1989). We were intrigued by these observations and wanted to both validate this BH3 domain in vivo as well as investigate the role of Ser567 phosphorylation in transcription-independent induction of apoptosis. We therefore generated RAT16 variants that upon doxycycline withdrawal express wildtype pRB or one of three mutant pRB forms (Fig. 3C). Specifically, we made a non-BH3 mutant in which the leucine and aspartic acid of the putative BH3 domain were mutated to alanine (RB2A). In addition, we generated two mutants in which Ser567 was substituted with leucine to recapitulate DMSO (0%) and treatment with mitochondrial uncoupler FCCP (100%). 50uM of Peptide #4 permeabilizes mitochondria in a BAX/BAK-dependent manner. (C) Schematic of pRB mutants generated in vivo. (D) RAT16 cells expressing wildtype or mutant pRB for 24 hours were treated with TNFα and CHX. None of the mutations tested inactivated pRB’s mitochondrial, pro-apoptotic function. Graph bars represent average of three experiments (±SD).
the naturally occurring mutation (S567L) or glutamic acid to mimic the phosphorylated serine residue (S567E). RAT16 cells with basal or induced wildtype or mutant pRB expression were treated with TNFα and CHX and levels of apoptosis were assessed by AnnexinV staining. However, all three pRB mutants sensitized to TNFα treatment and to a similar extent as wildtype pRB (Fig. 3D). Thus, we conclude that this specific sequence is not required for mitochondrial pRB function. Considering the in vitro and in vivo data together, we suspect that pRB does not contain a BH3 domain. We note that we cannot formally exclude that peptide #1-3 contain a BH3 domain, since they may not be folded correctly in the in vitro assay, either because they are not in the proper structural context of the full-length protein or because they have not been modified correctly as a result of an activating event. Further analysis, either using hydrocarbon-stapled peptides which have greater α-helicity in solution (Walensky et al., 2006) or in vivo by mutation analysis, may be required to draw a more rigorous conclusion. However, these data considered as a whole suggest that while pRB is a direct activator of BAX, it is not a Bcl2 family member. The domains within the small pocket of pRB involved in triggering MOMP as well as the nature of regulation of this function therefore remain to be elucidated.

**pRB also acts as a sensitizer of Bcl2 to induce transcription-independent apoptosis.**

We also wanted to investigate whether pRB can trigger MOMP via multiple mechanisms. Our genetic data strongly argue that mitochondrial pRB function is dependent on BAX since pRB promotes TNFα-induced apoptosis in wildtype and Bak−/−, but not Bax−/− and DKO MEFs. Furthermore, we’ve shown in vitro and in vivo that pRB is a direct activator of BAX (Hilgendorf et al., 2013). We next asked whether pRB can also act as a sensitizer to trigger BAX-dependent MOMP. Sensitizers promote mitochondrial apoptosis by binding anti-apoptotic Bcl2 family members and relieving inhibition of BAX/BAK and/or activator BH3-only proteins. This
Figure 4. pRB interacts with Bcl2. (A) Western blot assessing pRB binding to Flag-tagged Bcl2 or BclXL transiently expressed in IMR90 cells treated with TNFα and MG132. pRB co-immunoprecipitates with Flag-tagged Bcl2. (B, C) Immunoprecipitation of Bcl2 from IMR90 cells that were (B) treated with TNFα and MG132 (C) or also left untreated. pRB binds Bcl2 only upon treated TNFα/MG132 treatment (D) Immunoprecipitation of pRB from untreated and TNFα/MG132-treated IMR90 cells. Bcl2 only binds pRB upon TNFα/MG132 treatment.
approach was in part informed by our \textit{in vitro} competitive fluorescence polarization data (Fig. 3C), which showed some binding of a pRB peptide to anti-apoptotic Bcl2 proteins. We therefore performed co-immunoprecipitation experiments in primary human fibroblasts (IMR90) treated with TNF\(\alpha\) and MG132 and transiently overexpressing flag-tagged Bcl2 or BclXL, two major anti-apoptotic Bcl2 family members. Notably, we observed some pRB co-immunoprecipitating with Bcl2 (Fig. 4A). We also wanted to confirm this interaction with endogenous proteins. We immunoprecipitated endogenous Bcl2 from TNF\(\alpha\)/MG132 treated IMR90 cells and assessed endogenous pRB binding by western blotting. Importantly, pRB again co-immunoprecipitated with Bcl2 (Fig. 4B). Remarkably, this interaction was only observed upon treatment with TNF\(\alpha\) and MG132 (Fig. 4C). We also confirmed the endogenous interaction with the reciprocal co-immunoprecipitation experiment; Bcl2 co-immunoprecipitates with pRB in IMR90 cells and this interaction is only observed upon treatment with TNF\(\alpha\) and MG132 (Fig. 4D). Given pRB’s localization at mitochondria even in the absence of treatment, these data argue that the regulation of apoptosis induction by pRB at the mitochondria may occur at the level of Bcl2 interaction.

Other potential mitochondrial pRB functions.

We also wanted to address the possibility that mitochondrial pRB function extends beyond BAX-dependent induction of MOMP. This question was spurred by an unexpected observation we made during our \textit{in vitro} analysis of pRB action at isolated mitochondria. We have previously shown that recombinant pRB permeabilizes mitochondria and liposomes in a BAX-dependent manner at concentrations of less than 500nM (Hilgendorf et al., 2013). However, at higher concentrations, we observed that addition of recombinant pRB to isolated mouse liver mitochondria also resulted in some cytochrome \(c\) release even in the absence of monomeric BAX addition (Fig. 5A). We note that this permeabilization was less efficient than
Figure 5. Mitochondrial pRB function may extend beyond BAX-dependent induction of MOMP. (A) Isolated mouse liver mitochondria were incubated with 0, 10, or 500nM recombinant pRB in the absence or presence of monomeric BAX. Recombinant pRB induces cytochrome c release efficiently and in a dose-dependent manner in the presence of BAX and to a lesser extent in the absence of BAX. (B) Mouse liver mitochondria null for BAX and BAK were incubated with 0-1000nM pRB in the absence and presence of monomeric BAX. While pRB induces cytochrome c release in a BAX-dependent manner at low concentrations, 1000nM pRB permeabilizes mitochondria independently of BAX. (C) Recombinant pRB was added to ANTS/DPX-loaded liposomes in the absence or presence of BAX. pRB permeabilizes membranes in a BAX-independent manner at very high concentrations.
in the presence of monomeric BAX and that isolated (untreated) mitochondria already contain some Bcl2 family members including BAK, which may substitute for BAX to mediate pRB's ability to trigger MOMP. Therefore we repeated the assay using mitochondria from Albcrepos;Bax−/−;Bak−/− mouse livers in the absence and presence of recombinant BAX (Walensky et al., 2006). As shown previously, pRB triggers MOMP in an entirely BAX-dependent manner at concentrations of up to 250nM. At 500nM, recombinant pRB promotes a small amount of cytochrome c release even in the absence of BAX, though to a much smaller extent than in the presence of BAX or with wildtype mitochondria (and therefore in the presence of BAK) (Fig. 5A, B). Finally, at a concentration of 1μM, recombinant pRB permeabilizes mitochondria completely in a BAX/BAK-independent manner (Fig. 5B).

We sought to validate these data and performed an in vitro liposome release assay, in which recombinant pRB and/or BAX were added to freshly prepared, ANTS/DPX-loaded liposomes. We have previously shown that at concentrations of up to 250nM, pRB permeabilizes liposomes in an entirely BAX-dependent manner. However, consistent with the cytochrome c release data, we saw some BAX-independence with 500nM of recombinant pRB and complete BAX-independence at a concentration of 1μM (Fig. 5C). Thus, while pRB acts as a direct activator of BAX at low concentrations, our in vitro data show that pRB is also capable of permeabilizing membranes in a BAX/BAK-independent manner, albeit only at very high concentrations. The significance of this result remains to be determined. We note that we only see this BAX-independence in vitro and with high pRB concentrations. Thus, it is entirely possible that this is an artifact of the in vitro assays used. Additionally, only a small fraction of endogenous pRB localizes to mitochondria (Hilgendorf et al., 2013). Hence, we do not know whether this pRB activity, only apparent at high concentrations in vitro, has any significance in a
physiological context. Nonetheless, these data raise the possibility that pRB function may extend beyond BAX-dependent induction of MOMP.

**Mitochondrial pRB function is regulated differently than pRB’s anti-proliferative function.**

pRB is a pleiotropic protein that functions in both proliferation and apoptosis. Nuclear pRB’s ability to regulate cell cycle entry is regulated by CDK-phosphorylation. We note that pRB’s cell cycle function is inactivated in most if not all tumors, either by mutation of the RB-1 gene itself or, in approximately two thirds of human tumors, alterations in the upstream regulatory pathway resulting in constitutive pRB phosphorylation. We have previously shown that CDK-phosphorylated pRB localizes to mitochondria and can promote apoptosis in a transcription-independent manner (Hilgendorf et al., 2013). However, it is not clear whether CDK-phosphorylation is required for mitochondrial pRB function. We therefore sought to investigate whether the mitochondrial function of a pRB mutant deficient for regulation by CDK-phosphorylation is affected. We used a RAT16 variant that upon doxycycline withdrawal expresses a pRB mutant with substitutions in 7 CDK- phosphorylation sites in the Large Pocket (PSM-RB) (Fig. 6A). Importantly, consistent with previous observations (Chew et al., 1998; Knudsen et al., 1998; Knudsen and Wang, 1997; Sever-Chroneos et al., 2001), this pRB mutant is constitutively active for pRB’s anti-proliferative function (Fig. 6C). RAT16 cells with basal or induced PSM-RB expression were treated with TNFα and MG132 and levels of apoptosis were assessed by AnnexinV staining. Induced expression of PSM-RB sensitized to apoptosis induced by TNFα treatment (Fig. 6B). This argues that CDK-phosphorylations involved in the regulation of nuclear pRB’s cell cycle function do not affect pRB’s ability to promote TNFα-induced apoptosis. We note that cells were treated with TNFα and MG132, not CHX, and that we can
Figure 6. Mitochondrial pRB activity is regulated in a manner distinct from pRB’s nuclear cell cycle function. (A) Schematic of PSM-RB mutant. Large Pocket of pRB with CDK-phosphorylation sites S780, S788, S795, S807, S811, T821, T826 mutated to alanines. (B) Left: RAT16 cells with and without induced expression of phosphorylation site mutant pRB (PSM-RB) for 24 hours were treated with TNFα and MG132 for 48 hours. Levels of apoptosis were assessed by AnnexinV staining. Graph bars represent average of two experiments (±SD). Induced expression of PSM-RB promotes TNFα-induced apoptosis. Western blot showing induced PSM-RB expression in inset. Right: Levels of apoptosis with and without induced expression of wildtype pRB. Graph bars represent average of three experiments (±SD). (C) Cell cycle phasing of RAT16 cells with and without induced expression of wildtype or phosphorylation site mutant pRB. Induced expression of PSM-RB results in an accumulation of cells in G1. Graph bars represent average of two experiments (±SD).
therefore not exclude the possibility that PSM-RB solely promotes apoptosis in a transcription-dependent manner. However, another study has previously shown that PSM-RB also promotes TNFα/CHX-induced apoptosis (Masselli and Wang, 2006). We also note that we cannot exclude the possibility that mitochondrial pRB function is regulated by one of the CDK-phosphorylation sites not mutated in the PSM-RB mutant. However, our data clearly argue that in general pRB’s pro-apoptotic function is regulated in a manner that is distinct from the one regulating pRB’s anti-proliferative function.
Discussion

We have previously shown that in addition to its many nuclear roles, pRB can also act directly at the mitochondria to activate BAX and trigger mitochondrial outer membrane permeabilization. Here we have expanded these findings to show that ectopic pRB expression promotes low levels of apoptosis within a few hours of induction even in the absence of an apoptotic stimulus. This supports the hypothesis that mitochondrial pRB is a general modulator of apoptosis in response to various apoptotic stimuli and acts to fine-tune the apoptotic threshold. Moreover, we show that in addition to directly activating BAX, pRB can also bind to the anti-apoptotic protein Bcl2. Thus, pRB can impinge on mitochondrial apoptosis as both an activator and sensitizer-like protein. Importantly, we only observe the pRB:Bcl2 complex upon TNFα treatment, reconciling the facts that endogenous pRB is always localized to mitochondria, but only modulates the apoptotic effect upon treatment. However, the mechanism through which apoptotic stimuli activate endogenous pRB to trigger MOMP remains to be fully elucidated. Our data presented here have extended our understanding of how mitochondrial pRB function is regulated. Specifically, we show that pRB’s pro-apoptotic function is regulated independently of pRB’s anti-proliferative function, since both hyperphosphorylated pRB and phosphorylation-site mutant pRB are capable of triggering MOMP. Moreover, we present data arguing that pRB is a non-Bcl2 family activator of BAX. In particular, we show using both in vitro and in vivo assays that the Small Pocket of pRB does not appear to contain a functional BH3 domain. Thus, we have been able to eliminate some possible mechanisms of mitochondrial pRB regulation. Finally, we have also addressed the possibility that mitochondrial pRB has roles beyond activating BAX to trigger MOMP. Specifically, we show that recombinant pRB can also permeabilize mitochondria independent of Bcl2 family members. We note, that the
physiological relevance and function of this observation remains to be elucidated. Taken together, our observations further validate the existence of mitochondrial pRB and extend our understanding of how mitochondrial pRB function is regulated to promote apoptosis directly.

As alluded to above, we have not yet identified the post-translational modifications necessary to activate mitochondrial pRB. Indeed, we do not yet know if pRB activity is regulated by posttranslational modifications. In fact, since BAX is cytoplasmic and sequestered in untreated cells, it would not interact with mitochondrial pRB under normal (untreated) conditions. Consistent with the hypothesis that mitochondrial pRB function is not actively regulated, we show here that ectopic pRB expression promotes some apoptosis even in the absence of treatment. Moreover, we have previously shown that recombinant (and thus likely unmodified) pRB interacts with BAX in vitro. We note that BAX activation is a multistep process that involves both translocation to the mitochondria and profound conformational changes (Youle and Strasser, 2008). Moreover, one of the major physiologic activators of BAX, tBID, is mitochondrial (though only upon treatment with an apoptotic stimulus) and requires prior changes in BAX to occur in order to activate it (including exposure of the N-terminal region of BAX to reveal the tBID interaction site) (Ghibelli and Diederich, 2011). We envision that, consistent with its mitochondrial localization, pRB may act in a later step in the BAX activation pathway and therefore require prior steps of activation to occur first. Whether there is an additional layer of regulation of mitochondrial pRB function via post-translational modifications is still unknown. However, here we have presented data arguing that we can exclude the importance of 7 CDK-phosphorylation sites in this regulation. We note that pRB can also be phosphorylated at other sites, including by kinases other than CDK-cyclins and in response to genotoxic stress (Inoue et al., 2007). The role of these phosphorylation events in
regulating mitochondrial pRB function remains to be determined. Moreover, pRB is also subject
to other posttranslational modifications including acetylation and sumoylation (Chan et al., 2001;
Ledl et al., 2005; Markham et al., 2006). Thus, it remains unclear whether and how pRB posttranslational modifications affect its mitochondrial, pro-apoptotic function.

As described above, we have shown here that mitochondrial pRB not only acts as an activator of BAX, but also as a sensitizer of apoptosis by binding Bcl2. Intriguingly, pRB only interacts with Bcl2 upon TNFa/MG132 treatment despite its localization to mitochondria even in the absence of treatment. Thus, mitochondrial pRB activity may also be regulated at the level of Bcl2 interaction. We do not yet know how the formation of this pRB:Bcl2 complex is regulated and whether this involves a similar mechanism as that regulating pRB binding to BAX. We also note that it is unclear whether this pRB:Bcl2 interaction even has physiological relevance, since pRB does not promote transcription-independent apoptosis in BAX-null MEFs, which are wild-type for Bcl2. Thus, we suspect that pRB’s mitochondrial apoptotic function is primarily mediated by its ability to act as an activator of BAX. However, this function may be supported by pRB’s ability to act as a sensitizer and bind Bcl2, perhaps as a consequence of availability upon treatment, when Bcl2 no longer binds and inhibits BAX.

We have also been able to show that pRB is likely a non-Bcl2 family, direct activator of mitochondrial apoptosis. This adds to a growing list of non-Bcl2 family proteins that can promote apoptosis via both nuclear and mitochondrial mechanisms, including the tumor suppressor p53, the orphan nuclear steroid receptor Nur77, the nuclear protein histone H1.2, and the nucleolar phosphoprotein nucleophosmin (Chipuk et al., 2004; Chipuk et al., 2003; Dumont et al., 2003; Gine et al., 2008; Kerr et al., 2007; Konishi et al., 2003; Li et al., 2000; Lin et al., 2004; Mihara et al., 2003; Okamura et al., 2008). Further characterization of this new class of
pro-apoptotic proteins, including identifying the domains involved in mediating interactions with Bcl2 family members, is necessary to understand how and when these proteins contribute to the apoptotic response in vivo.

Taken together, we have presented data that extend our understanding of mitochondrial pRB function and regulation. Further analysis is necessary to draw a more complete picture of this pRB role. However, since pRB is a pleiotropic protein with both anti- and pro-tumorigenic functions, we strongly believe that further investigation of a pRB function that is both rapid and solely tumor-suppressive is very important and may inform novel therapeutic approaches.
Materials and Methods

Cell Culture and Drug Treatment

RAT16 and IMR90 cells were grown in MEM with Earle’s Salts and 10% FBS, Pen-Strep, L-Glutamine, Sodium Pyruvate, and NEAA. For TNFα treatments, 50ng/ml of recombinant mouse TNFα (Sigma) was used with 0.1μM MG132 (Calbiochem) for 48 hours or 0.5μg/ml CHX (Sigma). TET System approved FBS (Clontech) and 1μg/ml doxycycline (Clontech) were used for inducible cell lines.

FACS Apoptosis Analysis

Cell suspensions were stained with AnnexinV-FITC or APC (Becton Dickinson) and Propidium Iodide (Sigma) or 7AAD (Becton Dickinson). Total apoptotic cells were assessed by gating for AnnexinV positive using a FACScan or FACSCalibur System (Becton Dickinson). Similar trends were observed for all experiments when only early apoptotic (AnnexinV+;PI/7AAD-) cells were considered (data not shown). For cell cycle profiling, cell suspensions were processed as previously described (Ianari et al., 2009) and analyzed by FACScan and FlowJo.

Plasmid and Stable Cell Line Generation

Rat16 tet-off wildtype pRB cells were generated as previously described (Hilgendorf et al., 2013). pRB mutants were generated by site-directed mutagenesis (Agilent) of human pRB using the following primers:

RB2A - gaaatcggaaatcagctgaagctctacatggatatattttttg, caaataaaggttaagcttgatgagccatgcatgattccatgtcatgc
RBS567L - gaaatcggaaatcagctgaagctctacatggatatattttttg, caaataaaggttaatcttgatgagccatgcatgattccatgtcatgc
RBS567E - gaaatcggaaatcagctgaagctctacatggatatattttttg, caaataaaggttaatcttgatgagccatgcatgattccatgtcatgc
Competitive Fluorescence Polarization Assay and BH3 profiling

Competitive FPA and mitochondrial depolarization assays were performed as previously described (Brunelle and Letai, 2009; Letai et al., 2002). The following peptides were synthesized with N-terminus acetylated, the C-terminus amidated, and HPLC purified (KI Swanson Biotechnology Center, Biopolymers & Proteomics Facility):

Peptide #1: mntiqqlmmlhsasdpse
Peptide #2: nctvnpkeilkrvkdigyi
Peptide #3: atyszstqnlsgtldlsfp
Peptide #4: ercehrimeslawlsslfp

Immunoprecipitation and Western Blotting

Proteins were extracted using an NP40 based buffer (50mM HEPES pH7.9, 10% glycerol, 150mM NaCl, 1%NP40, 1mM NaF, 10mM B-Glycerophosphate, protease inhibitors) and quantified using BCA Protein Assay reagent (Pierce). The following antibodies were used: Bcl2 (Cell Signaling), normal rabbit IgG (Santa Cruz), RB (hybridoma supernatant XZ91, crosslinked to Protein A Agarose).

Samples were loaded in SDS Lysis Buffer (8% SDS, 250mM TrisHCl pH 6.6, 40 % glycerol, 5% 2-Mercaptoethanol, bromophenol blue), separated by SDS-PAGE, transferred to a nitrocellulose membrane, and blocked in 5% nonfat milk. The following antibodies were used in 2.5% nonfat milk: RB for human pRB (Cell Signaling, 9309), procaspase 7 (Cell Signaling, 9492), cleaved caspase 7 (Cell Signaling, 9491), procaspase 3 (Cell Signaling, 9662), cleaved caspase 3 (Cell Signaling, 9661), actin (Santa Cruz, SC1616 HRP), Lamin A/C (Cell Signaling, 2032), ATPB (Abcam, ab5432), SirT3 (Cell Signaling, 5490), cytochrome c (Becton Dickinson, 556433), Bcl2
(Cell Signaling, 2870), Flag (Sigma, F1804). Secondary HRP-conjugated antibodies (Santa Cruz) were used at 1:5000 in 1% nonfat milk.

**Cytochrome c release and liposome permeabilization assay**

Mitochondrial isolation and *in vitro* cytochrome c and liposome release assays were performed as previously described (Hilgendorf et al., 2013).

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Chapter Four

Discussion
Initial studies of the retinoblastoma protein tumor suppressor focused on elucidating its role in regulating entry into the cell cycle. Subsequently, it became apparent that pRB also functions in many other cellular processes including senescence, differentiation, and apoptosis. These early studies showed that loss of pRB resulted in increased levels of apoptosis, which led to the characterization of pRB as an anti-apoptotic protein (Almasan et al., 1995; Bosco et al., 2004; Jacks et al., 1992; Knudsen et al., 2000). It was subsequently shown that pRB can also act in a pro-apoptotic manner by functioning as a transcriptional co-activator of pro-apoptotic genes in response to apoptotic stimuli such as DNA damage (Carnevale et al., 2012; Ianari et al., 2009; Korah et al., 2012). The work described in this thesis derived from our efforts to characterize and expand our understanding of this pro-apoptotic role of pRB (Appendix A). Initially, we wanted to know if pRB’s transcriptional, pro-apoptotic function also extended to apoptotic stimuli other than genotoxic stress, including death ligand binding. Thus, we investigated the role of pRB in TNFα-induced apoptosis. Our analysis not only revealed that pRB can promote TNFα-induced apoptosis, but also uncovered a novel, non-nuclear and non-transcriptional function of pRB (Chapter 2). Specifically, we showed that pRB can directly bind to and activate BAX at the mitochondria, resulting in mitochondrial outer membrane permeabilization and induction of apoptosis. Moreover, by specifically targeting pRB to the mitochondria, we generated a separation of function mutant that is deficient for nuclear pRB functions, but capable of promoting transcription-independent apoptosis. Remarkably, this pRB mutant promotes apoptosis in vivo and is tumor suppressive. Thus, mitochondrial pRB function can contribute to pRB’s overall role as a tumor suppressor.

Given the novelty and potential importance of this pRB activity, we are very interested in further understanding how mitochondrial pRB is regulated and the specific context and extent to
which mitochondrial pRB contributes to tumor suppression. Importantly, we have initiated this analysis. Thus, we have shown that mitochondrial pRB localization and activity is regulated in a manner that is distinct from that of pRB's anti-proliferative function, since phosphorylated pRB can localize to mitochondria and promote apoptosis (Chapter 2) and a phosphorylation-site mutant pRB is capable of doing the same (Chapter 3). This is significant, because the retinoblastoma protein gene is mutated in about one-third of human tumors, but pRB is constitutively phosphorylated and thus inactive for its cell cycle function in most, if not all, remaining human tumors. Thus, mitochondrial pRB function appears to be unaffected in the majority of human tumors and can potentially be exploited for therapeutic purposes. We have also mapped this pRB function to the small pocket domain, known to be important for pRB's tumor suppressive function (Chapter 2). We anticipate that the BAX interaction site is therefore localized in this part of the protein. However, our explorative analyses of putative BH3 domains have not yielded any positive results, suggesting that pRB is a non-Bcl2 activator of BAX (Chapter 3).

Thus, we have uncovered a novel role of pRB in promoting apoptosis, but are only just beginning to understand how this mitochondrial pRB function fits into the overall picture of pRB tumor suppression and apoptosis. Our findings have lead to many more intriguing questions, including how the localization of pRB to mitochondria is regulated, how the activity of mitochondrial pRB is regulated, and what the extent is to which mitochondrial pRB contributes toward tumor suppression. I will outline potential experimental approaches below and would like to point out that several of these experiments will address multiple questions and that the results obtained will inform future approaches. First however, I will start by discussing the remarkable parallels between the tumor suppressor proteins pRB and p53. Importantly, our
findings and a comparison to mitochondrial p53 function may inform possible future directions for both fields of study. Moreover, the existence of this non-canonical mechanism of action for both of these major tumor suppressor proteins supports the notion that direct induction of apoptosis is an important tumor suppressive mechanism.

**Part I: Parallels between pRB and p53**

p53 is a key tumor suppressor and considered to be the "guardian of the genome" (Lane, 1992). It is mutated in the majority of human tumors and functions to protect the cell from insults by inducing cell cycle arrest or promoting apoptosis. Thus, p53 and pRB both play an integral role in deciding the cellular fate of the cell, though the mechanism underlying the decision to arrest versus undergo apoptosis is still poorly understood. The two tumor suppressors also differ in that pRB is a transcriptional co-factor, while p53 contains a DNA-binding motif. Moreover, while both pRB and p53 are regulated by post-translational modifications, pRB is stable in normal, untreated cells and p53 is only stabilized in response to cellular stresses.

It was shown many years ago that in addition to transcriptionally activating pro-apoptotic genes (e.g. PUMA) in response to DNA damage (Yu and Zhang, 2003), p53 can also promote apoptosis in the absence of translation (Caelles et al., 1994; Haupt et al., 1995). Furthermore, a fraction of p53 translocates to mitochondria in response to cellular stress (Marchenko et al., 2000; Sansome et al., 2001). Finally, a series of papers showed that p53 can act directly at the mitochondria to induce MOMP (Chipuk et al., 2004; Dumont et al., 2003; Mihara et al., 2003). However, the exact mechanism of mitochondrial p53 action is still unclear and findings are
partly contradictory. For instance, both the DNA-binding domain and the proline-rich domain were shown to be important for mitochondrial p53 function, and the data is contradictory as to whether these domains affect mitochondrial localization or interaction with Bcl2 family members (Chipuk et al., 2004; Dumont et al., 2003; Leu et al., 2004b; Marchenko et al., 2000; Mihara et al., 2003; Petros et al., 2004; Sot et al., 2007; Tomita et al., 2006). Furthermore, p53 can sometimes act in a BAX-dependent and other times in a BAK-dependent manner, both in vitro and in vivo (Chipuk et al., 2004; Leu et al., 2004a; Mihara et al., 2003). Finally, p53 has been shown to interact directly with BAX, BAK, Bcl2, BclXL, and BAD and it is unclear which of these interactions has the greatest physiological importance (Chipuk et al., 2004; Jiang et al., 2006; Leu et al., 2004a; Mihara et al., 2003). It is also not known what regulates p53 localization to mitochondria and interaction with Bcl2 family proteins, though some studies have implicated mono-ubiquitination, acetylation, and phosphorylation as being important (Geyer et al., 2000; Kawaguchi et al., 2006; Park et al., 2005; Sykes et al., 2009). Thus, there are still many remaining questions regarding the mechanism of action of mitochondrial p53.

Regardless, the parallels between p53 and pRB, both in terms of nuclear roles and mitochondrial function are remarkable. Moreover, generation of separation of function mutants of p53 and pRB that only promote transcription-independent apoptosis has revealed that direct induction of apoptosis is an efficient mechanism of tumor suppression (Erster et al., 2004; Hilgendorf et al., 2013; Palacios et al., 2008; Palacios and Moll, 2006; Talos et al., 2005). Finally, small molecule screens have identified compounds that either inhibit or potentiate mitochondrial p53 function. For instance, a small molecule screen to identify compounds that specifically inhibit p53-mediated apoptosis, but not arrest, identified the small molecule pifithrin-μ (Hilgendorf et al., 2013; Strom et al., 2006). Further analysis revealed that pifithrin-μ
does not affect p53’s transactivation function, but instead inhibits p53-mediated apoptosis by specifically affecting mitochondrial association of p53 and reducing p53 binding to Bcl2 and BclXL (Strom et al., 2006). Remarkably, there was no effect on cytoplasmic p53 protein levels (Strom et al., 2006). Another small molecule screen identified CP-31398 as an activator of p53 activity (Foster et al., 1999). Subsequent analysis revealed that this compound blocked UVB-induced skin carcinoma both by increasing p53 transactivation function as well as by increasing p53 mitochondrial localization and function (Tang et al., 2007). Thus, mitochondrial pRB and p53 function may be exploited for cancer therapeutics.

Taken together, mitochondrial pRB function is reminiscent of that of mitochondrial p53 function. The mechanism of action of both of these functions, including the domains involved and regulation of localization and activity, remains to be fully elucidated. However most importantly, this mitochondrial function has been shown to be tumor suppressive in vivo, and further characterization of these two functions may therefore inform novel therapeutic approaches.

Part II: Characterization of mitochondrial pRB function

A: How is mitochondrial localization of pRB regulated?

We have shown that a fraction of pRB localizes to mitochondria both in the absence and presence of treatment with an apoptotic stimulus. We do not yet know if this mitochondrial pRB in normal, untreated cells differs from nuclear pRB in terms of post-translational modifications. Moreover, we do not know if localization of pRB to mitochondria is an active process and therefore regulated by post-translational modifications or rather a consequence of interaction
with mitochondrial proteins. Importantly, addressing these questions would not only expand our understanding of pRB biology, but may also uncover novel mechanisms of pRB functional inactivation in tumors.

It would be interesting to investigate whether any posttranslational modifications are enriched in the mitochondrial pRB population and may therefore function in targeting pRB to mitochondria. This question can be addressed by immunoprecipitating pRB from mitochondrial fractions and analyzing it by mass spectrometry. I anticipate that mouse liver mitochondria would be a good source of pRB for this analysis, since we can isolate milligram amounts of mitochondria from just one adult mouse liver. While this approach may help identify modifications present on mitochondrial pRB, any importance in regulating localization (or perhaps activity) would need to be evaluated using both in vivo and in vitro assays.

pRB localization to mitochondria may also be mediated by interaction with mitochondrial proteins. This question can be addressed using two parallel approaches. We have already shown that pRB can interact with BAX (Chapter 2) and Bcl2 (Chapter 3) upon treatment with an apoptotic stimulus. However, we do not know if pRB also binds Bcl2 family proteins in the absence of treatment and whether these interactions tether pRB to the mitochondria. We can address this hypothesis by analyzing the amount of mitochondrial pRB protein in cells that are deficient for various Bcl2 family members. We note, however, that there may be several mitochondrial pRB interactors and that they may compensate for each other. Therefore, we can also screen for mitochondrial pRB interactors by mass spectrometry. We would subsequently validate their importance in regulating mitochondrial pRB localization in vivo.

Finally, mitochondrial pRB localization could be regulated at the level of nuclear export. While pRB is primarily nuclear, it has long been known that a fraction of pRB localizes to the
cytoplasm and that the phosphorylation status of pRB correlates with nucleo-cytoplasmic localization. However, while this existence of cytoplasmic pRB was previously thought to be a consequence of loss of nuclear tethering of hyperphosphorylated pRB (Mittnacht and Weinberg, 1991; Templeton, 1992), a series of recent papers have shown that nuclear export of pRB is actively regulated and that hyperphosphorylated pRB directly interacts with Exportin1 (Fulcher et al., 2010; Jiao et al., 2006; Jiao et al., 2008; Roth et al., 2009). It is, however, unclear whether the amount of cytoplasmic pRB correlates with the amount of mitochondrial pRB. Specifically, it would be interesting to investigate how the relative amounts of nuclear, cytoplasmic, and mitochondrial pRB change as the cell proceeds through the cell cycle as well as in response to tumorigenic events that result in constitutive hyperphosphorylation of pRB. Alternatively, we can also investigate the mitochondrial activity of constitutively hyperphosphorylated pRB. We have already shown that hyperphosphorylated pRB can promote transcription-independent apoptosis (Chapter 2). Further investigation is necessary to determine if, perhaps as a result of increased mitochondrial localization, phospho-pRB promotes more apoptosis when compared to equal cellular amounts of non-phosphorylated pRB.

B: How is mitochondrial pRB activity regulated?

Another important question that emerges from our studies is how mitochondrial pRB activity is regulated, in particular given that pRB is always localized to mitochondria and the amount of pRB is unchanged with apoptotic treatment. In other words, if pRB binding to BAX (and Bcl2) directly causes MOMP, then how is this interaction regulated so that pRB only binds BAX (and Bcl2) upon treatment. As discussed above (Chapter 3), we do not yet know if formation of these complexes is simply a function of Bcl2 family protein availability upon
treatment, or whether there is an active process of regulation and mitochondrial pRB is post-translationally modified upon treatment.

Given that nuclear pRB function is regulated by post-translational modifications, the latter hypothesis warrants further investigation. We have already shown that mitochondrial pRB activity is not regulated by any of at least 7 CDK-phosphorylation sites. However, it has previously been shown that pRB is both phosphorylated and acetylated in response to genotoxic stress (Chan et al., 2001; Inoue et al., 2007; Markham et al., 2006). We are interested in testing whether mutation of these residues (non-modifiable and mimetic) affects mitochondrial pRB function both \textit{in vitro} and \textit{in vivo}. As a parallel approach, we can also screen for changes in pRB post-translational modifications in response to treatment by mass spectrometry.

Regardless of the importance of post-translational modifications in regulating mitochondrial pRB activity, it is essential to better understand the nature of the pRB:BAX interaction. We have shown using both genetic and biochemical approaches (Chapter 2) that mitochondrial pRB function is dependent on BAX. I anticipate that the BAX interaction site is localized in the Small Pocket of pRB, since it is sufficient for mitochondrial function. Moreover, since pRB is specifically dependent on BAX, not BAK, the pRB binding site on BAX is either masked in BAK or unique to BAX. Intriguingly, an activation site that is unique to BAX was recently identified at the N-terminal side of the protein and therefore distinct from the C-terminal canonical BH3-binding site (Gavathiotis et al., 2010; Gavathiotis et al., 2008). Moreover, this site was shown to be important for a BIM BH3 domain peptide to trigger BAX to adopt its active conformation (Gavathiotis et al., 2010; Gavathiotis et al., 2008).

We have been unable to identify specific sequences on pRB, including BH3 consensus sequences, that mediate this pRB:BAX interaction (Chapter 3). We can potentially identify the
pRB:BAX binding domains using two approaches. Specifically, we can take advantage of the fact that pRB shares a high degree of homology with the pocket proteins p107 and p130 in the A and B folds of the pocket domain. We do not yet know if pRB is the only pocket protein capable of promoting apoptosis directly at the mitochondria. However, our preliminary study using shRNAs against individual pocket proteins in human primary fibroblasts suggests that this mitochondrial function is unique to pRB (Appendix A). Further analysis, including overexpression, mitochondrial targeting, and in vitro analysis, will help us confirm whether this mitochondrial function is specific to pRB. We can subsequently conduct domain-swapping experiments and generate chimeric proteins to determine the region of pRB necessary for mitochondrial function and/or sufficient to impart this function to other pocket proteins. As a parallel approach, we can also identify the pRB:BAX interaction domain by cross-linking the complex and performing mass spectrometry. This approach would potentially identify the interaction domains in both pRB and BAX. This interaction would subsequently have to be validated by mutational analysis.

I would like to note that both approaches would generate a non-BAX binding mutant of pRB and that this pRB mutant would be very valuable in assessing the extent to which mitochondrial pRB contributes to induction of apoptosis and the specific contexts in which this mitochondrial function is most important for tumor suppression. Thus, our investigation of how mitochondrial pRB activity is regulated would also contribute to our understanding of the physiological importance of mitochondrial pRB function. I will discuss the approaches used to address this question in more detail below.

Finally, characterization of the pRB:BAX interaction domain would not only increase our understanding of mitochondrial pRB function, but it may also contribute to other field of studies.
For instance, it may allow us to identify a novel interaction domain used by non-Bcl2 family protein capable of promoting apoptosis both via a nuclear mechanism and directly at the mitochondria. It would be very interesting to compare and contrast this domain to Bcl2 homology domains.

C: Conclusion

We have previously shown that pRB is localized to mitochondria and can bind to and activate BAX directly upon treatment with apoptotic stimuli. However, we do not know how mitochondrial pRB localization and function is regulated to induce MOMP only upon treatment with an apoptotic stimulus. Importantly, we have initiated a number of experiments to address these follow-up questions. These studies will not only further our understanding of pRB biology, but also generate tools to investigate the relative importance of this mitochondrial pRB function compared to other pRB functions in a physiological context. I will elaborate on this below.

Part III: Contribution of mitochondrial pRB function to tumor suppression

We have identified a novel, non-nuclear and non-canonical role of pRB. We next need to determine if this activity has physiological importance. Importantly, we already know that mitochondrial pRB can function in physiological settings. In particular, we have shown that pRB promotes TNFα-induced apoptosis not only using overexpression studies, but also by knockdown studies in two different cell lines, including a primary human fibroblast cell line (Chapter 2). Furthermore, our localization studies showed mitochondrial association of the
endogenous pRB protein both in cell lines and in normal mouse tissue (Chapter 2). Finally, we have shown in vivo (using endogenous proteins) that pRB can interact with BAX and Bcl2 (Chapter 2, 3). Further investigation will identify the specific contexts in which mitochondrial pRB function is most important and I will discuss potential approaches below.

A: Contribution of mitochondrial pRB function to development and tumor suppression

pRB is a pleiotropic protein that functions in both development and tumor suppression by impinging on several cellular processes. However, the relative contribution of these many cellular functions to pRB's overall role in development and tumor suppression is still unclear. This is partly due to the fact that most pRB functions map to the Small Pocket domain and it is therefore difficult to generate pRB mutants either deficient for only one function or capable of performing only one function.

A number of pRB knock-in mouse models have been generated. In particular, a pRB mutant (R654W) has been generated to model a naturally occurring human tumor mutation (R661W) known to cause partially penetrant retinoblastoma. Expression of this R654W mutant in mice has profound effects on both development (Sun et al., 2006) and tumor suppression (Sun et al., 2011). Since this mouse model recapitulates a naturally-occurring mutation, this is not unexpected. However, while this pRB mutant is impaired for E2F binding, it is unclear what other pRB functions are affected, complicating the analysis of the relative contributions of pRB functions to development and tumor suppression. Similarly, another knock-in mouse has also been generated in which the caspase cleavage site on pRB was mutated (Chau et al., 2002). This non-cleavable pRB knock-in mouse model has a subtle phenotype, showing a decreased apoptotic response to endotoxic stress in the intestine, but no effect in other tissues, in response to other stresses, and no general developmental or tumor suppression defects (Chau et al., 2002).
Since it is unclear what pRB functions are affected by caspase cleavage, this study also does not further our understanding of the relative contribution of pRB functions to development and tumor suppression. Finally, an LxCxE binding cleft deficient pRB mutant mouse has been generated (Francis et al., 2009; Talluri et al., 2010). Again, this mutation affects many pRB functions, since the LxCxE binding cleft mediates interaction with many proteins, including chromatin-remodeling factors such as HDAC1. This knock-in mouse model has no defect on tumor suppression and is viable, with only a defect in mammary gland development (Francis et al., 2009; Talluri et al., 2010). Thus, a number of knock-in mouse models have been generated, but we do not yet know the specific contexts in which (and extent to which) any pRB function contributes to normal development and tumor suppression. This is due to the fact that it is very difficult to generate separation-of-function mutants of pRB.

In contrast, the fact that mitochondrial pRB function localizes to a different cellular compartment than the canonical pRB functions has allowed us to generate a pRB mutant that can only induce transcription-independent apoptosis. This was done by specifically targeting pRB to the mitochondria (Hilgendorf et al., 2013). Importantly, expression of this mutant in xenografted tumors resulted in significant tumor suppression, showing that mitochondrial pRB can contribute to tumor suppression. Moreover, analysis of the pRB:BAX complex may allow us to generate a pRB mutant that specifically lacks mitochondrial pRB function (Amito-pRB). It would be interesting to generate knock-in mouse models of these two pRB mutants and analyze the effect of mutant pRB expression during development and in tumorigenesis. There are of course numerous caveats to this approach. In particular, I anticipate that a mito-tagged pRB mouse, like the Rb-null mouse, would be embryonic lethal since it would lack pRB’s anti-proliferative function. Conditional expression may therefore be necessary for this analysis. However, we do
not know which tissues and settings, if any, are most affected by mitochondrial pRB function, making this a very time-consuming approach. Finally, both the non-cleavable and LxCxE binding cleft deficient pRB mutant mouse showed no tumor-suppressive defect, suggesting that pRB is indeed a pleiotropic protein with an arsenal of tumor suppressive capabilities, and that loss of one function does not result in loss of overall tumor suppression. Thus, I anticipate that analysis of the Δmito-pRB mutant mouse will show no defect in tumor suppression. Since this does not mean that mitochondrial pRB cannot contribute to tumor suppression, merely that this is not the only tumor-suppressive pRB function, this approach may not be very informative.

Alternatively, we hypothesize that if mitochondrial pRB function contributes to tumor suppression, this function must be affected in some human tumors harboring point mutations in the RB-1 gene. Thus, it would be very interesting to analyze how naturally-occurring mutations of pRB affect mitochondrial pRB function. This can be done using two parallel approaches. It is possible to analyze patient tumor samples and determine if mitochondrial localization is affected and the amount of mitochondrial pRB relative to nuclear pRB is therefore altered. We note that this analysis would not be trivial, since there are technical difficulties to pRB immunohistochemistry and the relatively larger amount of nuclear pRB may make quantification of mitochondrial pRB protein levels difficult. Furthermore, localization to mitochondria is not the only way to affect mitochondrial pRB function, so that not all point mutations will likely impinge on mitochondrial pRB activity via this mechanism. Nevertheless, in addition to confirming that mitochondrial pRB function is affected by some point mutations and thus contributes to tumor suppression in vivo, this analysis could also help us understand the mechanism of mitochondrial pRB localization. Alternatively, we can generate pRB mutants that recapitulate tumor-derived pRB mutations and assay for mitochondrial pRB function in vitro and
in vivo. This approach can be informed by the results we obtain from our analyses of what regulates mitochondrial pRB localization as well as identification of the pRB:BAX interaction domain. We note that our mutant pRB analysis so far (Chapter 2, 3) has required us to generate stable pools of cells with inducible expression, since constitutive pRB expression proved to be highly toxic. This approach would not be amendable to testing many tumor-derived mutations. However, we have already initiated the generation of Bak;Bax-deficient MEFs inducible for BAX or BAK and anticipate that transient expression of pRB may be possible in these cells, allowing us to more rapidly screen pRB mutants.

B: Therapeutic Potential

The RB-1 gene is mutated in about one-third of human tumors and our above analysis may determine if mitochondrial pRB function is affected by these mutations and tumor suppressive in a physiological context. Regardless, pRB is wild-type, though constitutively CDK-phosphorylated, in the majority of human tumors. We have already shown that this CDK-phosphorylated pRB can promote transcription-independent apoptosis. Thus, characterization of mitochondrial pRB function may inform novel therapeutic approaches to exploit mitochondrial phospho-pRB already present and active in human tumors to promote apoptosis. This approach is purely speculative at this point, but we note that small molecule screens have identified drugs that can impair or potentiate mitochondrial p53 function (Strom et al., 2006; Tang et al., 2007). A similar approach may be taken to identify modulators of mitochondrial pRB. Since mitochondrial pRB (and p53), unlike nuclear pRB (and p53), is purely anti-tumorigenic, we believe that this approach merits consideration.

C: Conclusion
Our analysis has identified a novel, tumor suppressive mechanism of pRB. Further characterization is necessary to confirm that mitochondrial pRB function is affected in human tumors harboring RB-1 mutations and to identify to what extent and in what context mitochondrial pRB contributes to pRB's overall developmental and tumor suppressive function. However, pRB is only mutated in one-third of human tumors, but constitutively phosphorylated in most if not all remaining ones. It may be possible to exploit this mitochondrial pRB for cancer therapeutics.
References


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Appendix A

The Role of the Pocket Protein Family and the E2F Transcription Factor Family in Apoptosis

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K.I.H. contributed to all figures and text.
Abstract

The family of pocket proteins and the family of E2F transcription factors regulate entry into the cell cycle. In addition, several family members have been implicated in induction of apoptosis. Specifically, E2F1 and E2F3 are known to promote apoptosis and pRB can induce apoptosis both by forming a transcriptionally-active complex with E2F1 on promoters of apoptotic genes, and by directly binding BAX and inducing mitochondrial outer membrane permeabilization. The role of other family members in these pro-apoptotic mechanisms of action has not been established. Here we have initiated our investigation of the role of E2F1-4 and the pocket proteins pRB, p107, and p130 in apoptosis induction. We show that pRB specifically interacts with E2F1 upon treatment with a genotoxic agent and that only knockdown of pRB, not p107 and p130, impairs TNFα/cycloheximide-induced apoptosis. Thus, we propose that the ability to promote apoptosis is specific to pRB and E2F1.
Introduction

The pocket protein family, composed of pRB, p107, and p130, derives its name from the presence of a highly conserved pocket domain (Mulligan and Jacks, 1998). Pocket proteins are key regulators of the cell cycle and function via interaction with and inhibition of the E2F family of transcription factors. Upon mitogenic signaling, cyclin-CDK complexes phosphorylate the pocket proteins, resulting in release of E2Fs and transcriptional activation of genes important for S phase entry (van den Heuvel and Dyson, 2008). This phosphorylation event demarks the Restriction Point, the point of the cell cycle after which the cell is committed to enter the cell cycle (Trimarchi and Lees, 2002). While pRB is constitutively expressed, p130 is most highly expressed in G0 and in quiescent cells and p107 is most highly expressed in G1/S (Baldi et al., 1995; Beijersbergen et al., 1994; Chen et al., 1989). Pocket protein functions are at least partly overlapping, in particular with regard to cell cycle regulation. However, only RB-I is commonly found mutated in human tumors, suggesting that pRB also has some unique functions.

The E2F family functions in the regulation of proliferation and can be subdivided into three functional groups based on their affinity for specific pocket proteins, ability to activate transcription, and temporal association with cell cycle progression promoters. Activating E2Fs (E2F1, 2, and 3) are bound to and inhibited by pRB under physiological conditions and occupy cell cycle progression gene promoters during G1/S (Lees et al., 1993). Overexpression of any activating E2Fs is sufficient to induce quiescent cells to re-enter the cell cycle and mouse embryonic fibroblasts deficient for all three activating E2Fs are blocked in their ability to proliferate (Johnson et al., 1993; Lukas et al., 1996; Qin et al., 1994; Wu et al., 2001). In contrast, repressive E2Fs (E2F4, 5) cannot drive quiescent cells to re-enter the cell cycle and mouse embryonic fibroblasts deficient for repressive E2Fs proliferate, but fail to cell cycle arrest.
in response to p16\textsuperscript{INK4A} (Humbert et al., 2000; Lindeman et al., 1998; Lukas et al., 1996; Mann and Jones, 1996). Moreover, repressive E2Fs occupy cell cycle progression gene promoters during the G0/G1 phase of the cell cycle, with E2F5 interacting with p130, and E2F4 binding to all three pocket proteins (Hijmans et al., 1995; Moberg et al., 1996). Thus, repressive E2Fs are thought to function in transcriptional repression of cell cycle progression genes via interaction with pocket proteins, while activating E2Fs function in activation of cell cycle progression genes. A third group functions independently of pocket proteins and generally in a repressive manner (Trimarchi and Lees, 2002).

Both activating and repressive E2Fs have an obligate heterodimerization partner, DP, and are structurally similar in that they contain a DNA binding domain, a DP dimerization domain, and a transactivation domain. The pocket protein binding site is localized within the transactivation domain and pocket proteins inhibit E2Fs by physically blocking the transactivation domain and by recruiting transcriptional co-repressors. While activating E2Fs contain a nuclear localization signal, repressive E2Fs do not, are predominantly cytoplasmic and require pocket protein binding for nuclear localization (Muller et al., 1997; Verona et al., 1997).

In addition to regulating cell cycle entry, some E2Fs also function in other cellular processes including apoptosis. Thus, loss of either E2F1 or E2F3 suppresses the apoptotic phenotype of the \textit{Rb}-null embryo (Tsai et al., 1998; Ziebold et al., 2001). Moreover, ectopic expression of E2F1 promotes apoptosis both in cultured cells and transgenic mice (Stanelle and Putzer, 2006). This occurs in both a p53-dependent and -independent manner, by transactivation of p14\textsuperscript{ARF} and consequently stabilization of p53 (Bates et al., 1998), as well as transcription of pro-apoptotic proteins including Apaf-1, caspase 7, and p73 (Ginsberg, 2002; Moroni et al., 2001; Muller et al., 2001). E2F3 has also been shown to promote apoptosis in some settings,
though this may be a consequence of its ability to transactivate E2F1 (Lazzerini Denchi and Helin, 2005; Ziebold et al., 2001).

We and others have previously shown that in response to various apoptotic stimuli including DNA damage, pRB and E2F1 form a transcriptionally-active complex on promoters of pro-apoptotic genes such as p73 and caspase 7 (Ianari et al., 2009; Korah et al., 2012). Thus, both free E2F1 and pRB-bound E2F1 can promote apoptosis (Carnevale et al., 2012). The role of other pocket proteins and other E2F family members has not been investigated to date.

In addition to functioning as a transcriptional co-activator of E2F1, we have also recently shown that pRB can promote apoptosis directly at the mitochondria and in a transcription-independent manner by directly binding to and activating BAX (Hilgendorf et al., 2013). In vitro binding assays show that this pro-apoptotic function of pRB does not require E2F transcription factors, though we do not know if E2F transcription factors potentiate or regulate mitochondrial pRB function. Moreover, we do not know if this mitochondrial function is unique to pRB or instead is conserved to other pocket proteins. Since RB-1 is the only pocket protein gene commonly mutated in human tumors and pocket proteins have overlapping functions in cell cycle regulation, we are intrigued by the possibility that this mitochondrial function is specific to pRB.

We have initiated experiments to address some of the questions outlined above. Here we show that DNA damage stabilizes specifically the pRB:E2F1 complex as well as p107:activating E2F complexes. We do not yet know the function of these p107 complexes, but propose that pRB’s transcriptional, pro-apoptotic function is mediated specifically by E2F1. Moreover, we present data arguing that only pRB can promote transcription-independent apoptosis. Further analysis is necessary to further confirm and extend these data.
Results

In response to genotoxic stress, pRB interacts specifically with E2F1, not E2F2, E2F3, and E2F4.

We have previously shown that genotoxic stress results in stabilization of a pRB:E2F1 complex in a human glioblastoma cell line, T98G (LANARI et al., 2009). We next wanted to investigate the effect of DNA damage on other pRB:E2F family protein complexes. We note that it was previously shown that both E2F1 and E2F3 can promote apoptosis, though E2F1’s pro-apoptotic function is more established (POLAGER and GINSBERG, 2008). We treated T98G cells with the genotoxic agent doxorubicin for 24 hours and assayed for association of pRB with E2F family members by immunoprecipitation of endogenous pRB. As expected, doxorubicin treatment resulted in increased association between pRB and E2F1 (Fig. 1A). Remarkably, we observed a decreased association between pRB and E2F2, E2F3, and E2F4 (Fig. 1A). Thus, pRB specifically associates with E2F1 upon treatment, suggesting that pRB’s pro-apoptotic function is mediated via association with E2F1 and not the other E2F transcription factors. Importantly, we also wanted to confirm this observation by reciprocal immunoprecipitation. Thus, we treated T98G cells with doxorubicin for 24 hours and immunoprecipitated endogenous E2F1, E2F2, E2F3, or E2F4. Two independent experiments are shown (Fig. 1B). Consistent with our previous observation, we observe an increased association between pRB and E2F1, but not the other E2F transcription factors. We note that for all E2F immunoprecipitations, we pulled-down more protein in the treated condition. Thus, we cannot exclude that the increased association between pRB and E2F1 is a consequence of increased pull-down. However, this cannot explain the pRB pull-down data and increased cellular E2F1 protein levels would also allow for more pRB binding upon treatment in vivo. Regardless, the fact that we see less pRB
Figure 1. DNA damage specifically stabilizes a pRB:E2F1 complex. (A) Western blot showing pRB immunoprecipitation and association with E2F1-4 in the presence and absence of DNA damage. Only the pRB:E2F1 complex is stabilized upon genotoxic stress. (B) Two western blots showing E2F1-4 immunoprecipitates and association with pRB in the presence and absence of DNA damage. Genotoxic stress specifically stabilizes the pRB:E2F1 complex.
binding to E2F2, E2F3, and E2F4 upon treatment is more significant when considering that there is more E2F protein available for binding. The functional consequence and mechanism underlying this differential association between pRB and E2F1 versus the other E2F proteins investigated here remain to be determined. We anticipate that this reflects pRB’s ability to specifically activate E2F1-mediated apoptosis while relieving inhibition of cell cycle progression genes.

**In response to genotoxic stress, p107 interacts with E2F1, E2F2, and E2F3.**

We also wanted to investigate whether other pocket proteins bind E2F transcription factors upon treatment with a genotoxic agent. Thus, we treated T98G cells with doxorubicin for 24 hours, immunoprecipitated endogenous E2F1, E2F2, E2F3, or E2F4, and assayed for p107 association by western blotting. Remarkably, doxorubicin treatment stabilized p107:E2F1, E2F2, and E2F3 complexes, and destabilized p107:E2F4 complexes (Fig. 2A). Once again, this correlates with increased E2F protein immunoprecipitation upon treatment. Thus, doxorubicin treatment specifically results in formation of a pRB:E2F1 complex as well as various p107:activator E2F complexes. We do not yet know the function of these p107 complexes.

We have previously shown that in addition to transcriptionally co-activating pro-apoptotic E2F1 target genes like p73 and caspase 7 (Ianari et al., 2009), pRB can also directly induce mitochondrial outer membrane permeabilization at the mitochondria by directly binding BAX and activating it in response to various apoptotic stimuli (Hilgendorf et al., 2013). We therefore wanted to investigate the cellular localization of the various pocket protein:E2F family protein complexes. We fractionated T98G cells left untreated or treated with doxorubicin for 24 hours into nuclear and cytoplasmic fractions and assayed for complex formation by immunoprecipitating E2F1, E2F3, and E2F4. In response to DNA damage, the pRB:E2F1
Figure 2. DNA damage stabilizes p107:activating E2F complexes both in the nucleus and in the cytoplasm. (A) Two western blots showing E2F1-4 immunoprecipitates and association with p107 in the presence and absence of DNA damage. All p107:activating E2F complexes are stabilized in response to genotoxic stress. (B) T98G cells left untreated or treated with doxorubicin were fractionated into nuclear and cytoplasmic fraction and E2F1-4 were immunoprecipitated. The stabilized pRB:E2F1 complex is primarily nuclear, while the p107:activating E2F complexes are both nuclear and cytoplasmic.
complex formed is mostly nuclear, though some is cytoplasmic (Fig. 2B). The pRB:E2F3 and E2F4 complexes are primarily nuclear. In contrast, the p107:E2F complexes stabilized upon DNA damage are localized to both the nucleus and cytoplasm (Fig. 2B). Again, the function of any nuclear or cytoplasmic p107 complex remains to be determined.

Numerous pocket protein:E2F complexes are formed upon genotoxic treatment. While we know that pRB:E2F1 functions in promoting apoptosis, we do not know the role of p107:activator E2F complexes. However, since some of these complexes are nuclear, we anticipate that they may function in transcriptional co-activation. It is unclear whether this is to promote cell cycle arrest or apoptosis.

Only pRB, not p107 and p130, promotes TNFα-induced apoptosis.

We have previously shown that pRB promotes TNFα-induced apoptosis at least in part via a novel non-nuclear and non-transcriptional mechanism (Hilgendorf et al., 2013). Since p107:E2F complexes form in response to treatment with a DNA damaging agent and at least some of these complexes are cytoplasmic, we wanted to investigate whether any of the other pocket proteins can function in the cytoplasm to promote apoptosis. TNFα synergizes with the translation inhibitor cycloheximide (CHX) to promote apoptosis, allowing us to use TNFα/CHX treatment to rapidly identify proteins with functions that do not require changes in gene expression. Thus, we generated variants of human primary fibroblasts (IMR90 cells) with stable knockdown of pRB, p107, p130, or a control hairpin and treated these IMR90 variants with TNFα used in conjunction with CHX or the proteasome inhibitor MG-132. Use of either factor in concert with TNFα prevents TNFα-induced activation of NF-κB and induction of a pro-inflammatory, anti-apoptotic response. Consistent with prior observations, knockdown of pRB resulted in an impaired apoptotic response to TNFα used in conjunction with either.
Figure 3. Only pRB promotes TNFα-induced apoptosis, including in the presence of CHX. IMR90 cells stably expressing shRNA against Luc, pRB, p107, and p130 were left untreated or treated with TNFα and CHX or MG132. Knockdown of pRB impaired TNFα-induced apoptosis, even in the presence of CHX. Knockdown of p107 did not have this effect.
MG132 or CHX (Fig. 3). In contrast, cells with a stable p107 knockdown displayed similar levels of apoptosis in response to TNFα treatment as cells with control hairpin (Fig. 3). This argues that p107 does not function in TNFα-induced apoptosis and cannot promote apoptosis in a transcription-independent manner. It was difficult to discern the role of p130 in TNFα-induced apoptosis since levels of apoptosis were increased even in the absence of treatment (Fig. 3). Thus, p130 appears to act in an anti-apoptotic manner in the absence of treatment and its role in TNFα- induced apoptosis remain to be determined. Taken together, our preliminary analysis of other pocket proteins in TNFα-induced apoptosis, including in the presence of cycloheximide, suggests that only pRB functions to promote apoptosis, including directly at the mitochondria. Further investigation, including in other cell lines and with overexpression, is necessary to confirm this.
Discussion

We and others have previously shown that in addition to its anti-apoptotic function, pRB can also transcriptionally co-activate expression of pro-apoptotic genes (Carnevale et al., 2012; Ianari et al., 2009; Korah et al., 2012). Specifically, apoptotic stimuli result in stabilization of a RB:E2F1 complex. We show here that formation of this pRB complex in response to treatment is unique to E2F1 and not E2F2, E2F3, and E2F4. Moreover, this complex localizes primarily to the nucleus. This suggests that only E2F1 mediates pRB-induced, transcription-dependent apoptosis. We also show that DNA damage induces the formation of complexes containing p107 and any of the activating E2Fs. In contrast to the pRB:E2F1 complex, a significant fraction of p107:activating E2Fs complexes are localized to the cytoplasm. We do not know the function of these p107 complexes, in the nucleus or cytoplasm. However, preliminary investigation of the role of pRB, p107, and p130 shows that only pRB acts in a pro-apoptotic manner both in untreated cells and TNFα-treated cells. We therefore hypothesize that the p107 containing E2F complexes are not pro-apoptotic and instead propose that pRB and E2F1 are unique amongst their family proteins in being able to form a pro-apoptotic, transcriptionally active complex. Moreover, our TNFα experiments were also conducted in the presence of cycloheximide, suggesting that pRB’s ability to activate apoptosis directly at the mitochondria by activating BAX is not conserved to other pocket proteins. Taken together, our data argues that only pRB and E2F1 can promote apoptosis in response to various apoptotic stimuli and via both transcriptional and, in the case of pRB, non-transcriptional mechanisms.

We do not yet know the nature of the pRB:E2F1 complex including how the interaction sites and regulation of formation differ from that of the canonical, anti-proliferative pRB:E2F1 complex. We note that there is a second E2F1 specific binding site on pRB distinct from the one
used by all other E2Fs as well as a second pRB binding site on E2F1. It remains to be seen if this specific binding site mediates the pro-apoptotic response. Alternatively, the pro-apoptotic pRB:E2F1 complex does not differ from the anti-proliferative one and induction of apoptosis is regulated by binding to pro-apoptotic gene promoters via an as of yet unknown mechanism. Since pRB blocks the E2F1 transactivation domain in this scenario, promoters would need to be occupied by both free E2F1 and pRB-bound E2F1, with the latter functioning in recruiting transcriptional co-factors like P/CAF. Intriguingly, a recent paper showed that both free E2F1 and pRB-bound E2F1 are required for maximal induction of apoptosis in response to DNA damage (Carnevale et al., 2012). Thus, we have shown here that pRB’s transcriptional, pro-apoptotic function is mediated specifically by E2F1, but we do not yet understand the nature of this complex.

We are also intrigued by our observation that p107 forms a complex with all three activating E2Fs upon treatment with doxorubicin. However, our data argue that only pRB, and not p107, can promote apoptosis, at least in response to TNFα. We note that our analysis of p107 function is preliminary and that these data need to be extended to other apoptotic stimuli, including DNA damage. However, we hypothesize that the p107:E2F complexes formed in response to apoptotic stimuli are located on promoters of cell cycle progression genes and function to induce cell cycle arrest. Further investigation is needed to test this hypothesis. We note that this does not explain our observation that a significant amount of these p107 containing complexes were also found in the cytoplasm.

Finally, we have shown here that knockdown of pRB and not the other pocket proteins sensitizes to TNFα-induced apoptosis in the presence of an inhibitor of translation. We have previously shown that pRB can activate BAX directly and induce mitochondrial outer membrane
permeabilization (Hilgendorf et al., 2013). The data presented here extend these observations by suggesting that this mitochondrial function is specific to pRB. We note that pRB is affected in human tumors by either direct mutation of the RB-1 gene or by constitutive CDK-phosphorylation as a result of alterations in the upstream regulatory pathways. The latter mechanism also affects the other pocket proteins. Pocket proteins show redundancy in their cellular functions. However, our data suggest that pRB differs from p107 and p130 in its ability to promote apoptosis, both transcriptionally and non-transcriptionally. Moreover, pRB’s pro-apoptotic function via either mechanism is not inactivated by CDK-phosphorylation (Hilgendorf et al., 2013; Ianari et al., 2009). Thus, we propose that in settings were pRB’s tumor suppressive function is mediated to a significant extent by its ability to promote apoptosis, tumors will select for direct inactivation via mutation in the RB-1 gene. In other tumors, constitutive phosphorylation of pocket proteins is sufficient to inhibit tumor suppression. Further investigation is necessary to test this hypothesis.
Materials and Methods

Cell Culture and Drug Treatment
T98G cells were grown in DMEM with 10% FBS and Pen-Strep, IMR90 cells were grown in MEM with Earle’s Salts and 10% FBS, Pen-Strep, L-Glutamine, Sodium Pyruvate, and NEAA. For doxorubicin treatment, 2μM Doxorubicin hydrochloride (Sigma) was used for 24 hours. For TNFα treatments, 50ng/ml of recombinant mouse TNFα (Sigma) was used with 0.1μM MG132 (Calbiochem) for 48 hours or 0.5μg/ml CHX (Sigma) for 24 hours.

FACS Apoptosis Analysis
Cell suspensions were stained with AnnexinV-FITC or APC (Becton Dickinson) and Propidium Iodide (Sigma) or 7AAD (Becton Dickinson). Total apoptotic cells were assessed by gating for AnnexinV positive using a FACScan or FACSCalibur System (Becton Dickinson). Similar trends were observed for all experiments when only early apoptotic (AnnexinV+;PI/7AAD-) cells were considered (data not shown). For cell cycle profiling, cell suspensions were processed as previously described (Ianari et al., 2009) and analyzed by FACScan and FlowJo.

Immunoprecipitation and Western Blotting
Proteins were extracted using an NP40 based buffer (50mM HEPES pH7.9, 10% glycerol, 150mM NaCl, 1%NP40, 1mM NaF, 10mM B-Glycerophosphate, protease inhibitors) or RIPA buffer (50 mM Tris-HCl pH 7.6, 1% NP40, 140 mM NaCl, 0.1% SDS, 0.5% Sodium Deoxycholate, 5mM EDTA, 100mM NaF, 2mM NaPPi) and quantified using BCA Protein Assay reagent (Pierce). The following antibodies were used: pRB (Cell Signaling, 9309), E2F1 (Santa Cruz, sc193), E2F2 (Santa Cruz, sc633x), E2F3 (Santa Cruz, sc878x), E2F4 (Santa Cruz, sc866), normal rabbit IgG (Santa Cruz).
Samples were loaded in SDS Lysis Buffer (8% SDS, 250mM TrisHCl pH 6.6, 40% glycerol, 5% 2-Mercaptoethanol, bromophenol blue), separated by SDS-PAGE, transferred to a nitrocellulose membrane, and blocked in 5% nonfat milk. The following antibodies were used in 2.5% nonfat milk: pRB (Cell Signaling, 9309), p107 (Santa Cruz, sc318), E2F1 (Santa Cruz, sc193), E2F2 (Santa Cruz, sc633x), E2F3 (Santa Cruz, sc878x), E2F4 (Santa Cruz, sc866), Lamin A/C (Cell Signaling, 2032), tubulin (Sigma, T9026).

Secondary HRP-conjugated antibodies (Santa Cruz) were used at 1:5000 in 1% nonfat milk.

**Cytoplasmic Fractionation**

Cell pellets were resuspended in Buffer A (19mM Hepes pH 7.9, 10mM KCl, 1.5mM MgCl₂, 0.34M Sucrose, 10% Glycerol, 1mM DTT, protease inhibitors, 0.1% Triton X-100) and incubated on ice for 8 minutes. Cytoplasmic fraction was obtained by pelleting twice at 1300g, 4°C, 5 minutes and clarified by high-speed centrifugation at 20000g, 4°C, 5 minutes. Nuclear fraction was obtained from first 1300g pellet, washed with Buffer A, and lysed in RIPA or NP40 buffer.
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References


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