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Proapoptotic Function of the Retinoblastoma Tumor Suppressor Protein

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SUMMARY

The retinoblastoma protein (pRB) tumor suppressor blocks cell proliferation by repressing the E2F transcription factors. This inhibition is relieved through mitogen-induced phosphorylation of pRB, triggering E2F release and activation of cell-cycle genes. E2F1 can also activate proapoptotic genes in response to genotoxic or oncogenic stress. However, pRB's role in this context has not been established. Here we show that DNA damage and E1A-induced oncogenic stress promote formation of a pRB-E2F1 complex even in proliferating cells. Moreover, pRB is bound to proapoptotic promoters that are transcriptionally active, and pRB is required for maximal apoptotic response in vitro and in vivo. Together, these data reveal a direct role for pRB in the induction of apoptosis in response to genotoxic or oncogenic stress.

INTRODUCTION

The retinoblastoma gene (*RB1*), a member of the pocket protein family with *p107* and *p130*, was the first known tumor suppressor. The retinoblastoma protein (pRB) is targeted by the transforming proteins of the DNA tumor viruses (e.g., adenoviral E1A), and it is functionally inactivated in a large proportion of human tumor cells due to mutations of either the *RB1* gene itself or its upstream regulators (Trimarchi and Lees, 2002). pRB's tumor-suppressive activity is thought to be largely dependent upon its ability to directly bind members of the E2F family of transcription factors and prevent them from promoting transcription of genes required for cell proliferation (Trimarchi and Lees, 2002). This inhibition can occur via two distinct mechanisms: pRB binds to sequences within E2F's transactivation domain and inhibits its function, and the resulting pRB-E2F complex recruits a number of transcriptional corepressors, including histone deacetylases (HDACs), methyltransferases, and polycomb group proteins to actively repress the promoters of E2F target genes.

In normal cells, pRB's repressive activity is controlled by its cell-cycle-dependent phosphorylation (Trimarchi and Lees, 2002). In response to mitogenic signaling, pRB is sequentially phosphorylated by the Cdk complexes cyclin D-Cdk4/6 and cyclin E-Cdk2. This phosphorylation is sufficient to induce pRB to release E2F, thereby allowing activation of E2F-responsive genes in late G1. However, phosphorylated pRB (ppRB) persists in the nucleus through the remainder of the cell cycle until it is dephosphorylated by protein phosphatase 1 at the end of mitosis (Ludlow et al., 1993). It is widely assumed that ppRB is functionally inactive and that dephosphorylation restores pRB to the active state. The majority of human tumors carry mutations that disable pRB-mediated repression of E2F (Sherr and McCormick, 2002). These mutations either inactivate the *RB1* gene itself or promote pRB phosphorylation in the absence of normal mitogenic signals through activation of the cyclin D-Cdk4/6 kinases or inactivation of the Cdk inhibitor *p16*. These changes result in the inappropriate release of E2F, thereby inducing transcriptional activation of E2F target genes and consequently cell proliferation.

SIGNIFICANCE

Retinoblastoma protein (pRB) function is disrupted in many human tumors through either inactivation of the *RB1* gene or alterations in its upstream regulators. pRB's tumor-suppressive activity is at least partially dependent upon its ability to arrest cells through E2F inhibition. Our data here now establish a second role for pRB as a stress-induced activator of apoptosis. Notably, pRB's ability to promote either arrest or apoptosis seems to be context dependent, with apoptosis being favored in proliferating cells. This finding has the potential to explain why cells are typically more resistant to apoptosis when in the arrested state. Most importantly, our observations suggest that *RB1* status will influence tumor response to chemotherapy by impairing both the arrest and apoptotic checkpoint responses.

It is well established that E2F1, among other E2F family members, also contributes to the induction of apoptosis in response to either DNA damage or oncogenic stress (Iaquinta and Lees, 2007). This is thought to be a critical event in suppressing the formation of tumors. Work from many laboratories has shown that genotoxic stress induces E2F1 recruitment to the promoters of proapoptotic genes including *p73* and *Caspase 7*, coincident with their transcriptional activation, even in cells that retain wild-type pRB (Pediconi et al., 2003). This led us to consider how pRB influences DNA damage-induced apoptosis. The prevailing view is that pRB is an antiapoptotic regulator. Early support for this model came from the finding that several tissues in *Rb* mutant mice display both ectopic proliferation and apoptosis (Jacks et al., 1992). However, it is now clear that much of this apoptosis is non-cell autonomous, resulting from a proliferation defect in the extraembryonic tissues (de Bruin et al., 2003; Wenzel et al., 2007; Wu et al., 2003). Analysis of tissue-specific *Rb* mutant models reinforces the notion that pRB plays a much more nuanced role in apoptosis. Loss of pRB in neuronal tissue (MacPherson et al., 2003), lung (Mason-Richie et al., 2008; Wikenheiser-Brokamp, 2004), skin (Ruiz et al., 2004), and intestine (Haigis et al., 2006; Wang et al., 2007) drives ectopic proliferation but has no effect on apoptosis. In contrast, *Rb* inactivation in the lens (de Bruin et al., 2003) and myoblasts (Huh et al., 2004) does induce apoptosis, but this is specifically observed in the differentiating cells. Thus, taken together, these mouse studies support two general conclusions: first, in many different settings, *Rb* inactivation can induce inappropriate proliferation without triggering apoptosis, and second, when apoptosis is observed, it seems to result from an inability to cease proliferation and undergo terminal differentiation.

pRB's apoptotic role has also been analyzed in established tissue culture cell lines. However, these studies have yielded conflicting results: some conclude that pRB suppresses apoptosis (Almasan et al., 1995; Bosco et al., 2004; Knudsen et al., 2000), whereas others suggest that it is proapoptotic (Araki et al., 2008; Bowen et al., 1998, 2002; Knudsen et al., 1999). None of these studies addresses the molecular basis for the observed role of pRB. In this study, we investigated how pRB influences the ability of E2F to induce apoptosis in response to genotoxic stress.

RESULTS

Stabilization of the pRB-E2F1 Complex in Response to DNA Damage

In cells committed to die by apoptosis in response to either DNA damage or oncogenic stress, E2F1 transcriptional activity is directed toward promoters of a subset of apoptotic genes that include *Caspase 7*, *p73*, and *Apaf1* (Iaquinta and Lees, 2007). Thus, we hypothesized that DNA damage must somehow inactivate pRB's repressive function to allow the release of transcriptionally active E2F1. To test this hypothesis, we assessed the binding of pRB to E2F1 both before and after doxorubicin treatment of human T98G cells. However, contrary to our expectations, we found that the pRB-E2F1 complex was stabilized by this genotoxic stress (Figure 1A). Notably, T98G cells are p53 defective and therefore treatment with doxorubicin causes them to accumulate in G2/M (Figure 1B). Thus, we can conclude that the observed DNA damage-induced formation of the pRB-E2F1 complex is neither dependent on p53 nor simply an indirect consequence of a G1 arrest.

pRB's ability to bind to E2F is normally limited to the early stages of the cell cycle when pRB exists in the hypophosphorylated form. Therefore, it was surprising to observe pRB-E2F1 complexes in a population highly enriched for G2/M phase cells. To more directly address the influence of cell-cycle phasing, we used serum deprivation and readdition to generate two populations of T98G cells that were greatly enriched for either G0/G1 (70%) or proliferating (95% S or G2/M phase) cells (Figure 1C) and then treated these with doxorubicin. Consistent with the known cell-cycle-dependent phosphorylation of pRB, the pRB protein was present in its slower mobility form in untreated proliferating cells, and it bound little E2F1 (Figure 1C). Notably, doxorubicin treatment was still able to induce formation of the pRB-E2F1 complex in this proliferating population (Figure 1C). This occurred independently of any change in total levels of E2F1 protein (Figure 1C), and it correlated with full activation of the apoptotic program as judged by PARP-p85 induction (data not shown). Importantly, the G0/G1 cells within the proliferating population cannot fully account for this DNA damage-induced pRB-E2F1 complex because the treated proliferating cells and untreated G0/G1 cells had comparable levels of pRB-associated E2F1 (Figure 1C), but the fraction of G0/G1 cells in the enriched populations differed by 14-fold (5% versus 70%). Thus, these data show that pRB-E2F1 complexes can form in S/G2/M phase cells in response to DNA damage.

pRB is sequentially phosphorylated by the cyclin D-Cdk4/6 and cyclin E-Cdk2 complexes, and this is thought to disrupt the interaction between pRB and E2F. Since DNA damage causes pRB to bind to E2F1 irrespective of cell-cycle phase, we wished to determine whether ppRB could participate in this complex. To this end, we generated enriched populations of G0/G1 and proliferating T98G cells, exposed them to either ionizing radiation (IR) or doxorubicin (see schema in Figure 1D, left panel), and then assessed both the levels and E2F1-binding properties of ppRB using antibodies that specifically recognize known Cdk phosphorylation sites within pRB, pSer780, pSer795, and pSer807–811. Fluorescence-activated cell sorting (FACS) analysis confirmed the high degree of enrichment of the G0/G1 and proliferating populations both before and after IR or doxorubicin treatment (Figure 1D, right panel). As expected, ppRB was present at much higher levels in the proliferating versus the G0/G1 cells as judged by both the immunoprecipitation (IP) of ppRB and the mobility shift of the total pRB (Figure 1E). We found that a small subset of the E2F1 coimmunoprecipitated with ppRB before treatment, and IR and doxorubicin both increased this level (Figure 1E, left panel). This increased binding occurred independently of any change in the total levels of E2F1 (Figure 1E, right panel). Interestingly, IR and doxorubicin increased the levels of ppRB in the G0/G1 population (Figure 1E, compare lane 1 with lanes 2 and 3), even though neither treatment altered the cell-cycle distribution of these cells (Figure 1D, right panel).

The increased level of ppRB in the treated G0/G1 cells clearly contributes to, but seems insufficient to fully account for, the increased level of E2F1 in the ppRB immunoprecipitates. Indeed, for all of the cell-cycle fractions, we clearly recovered

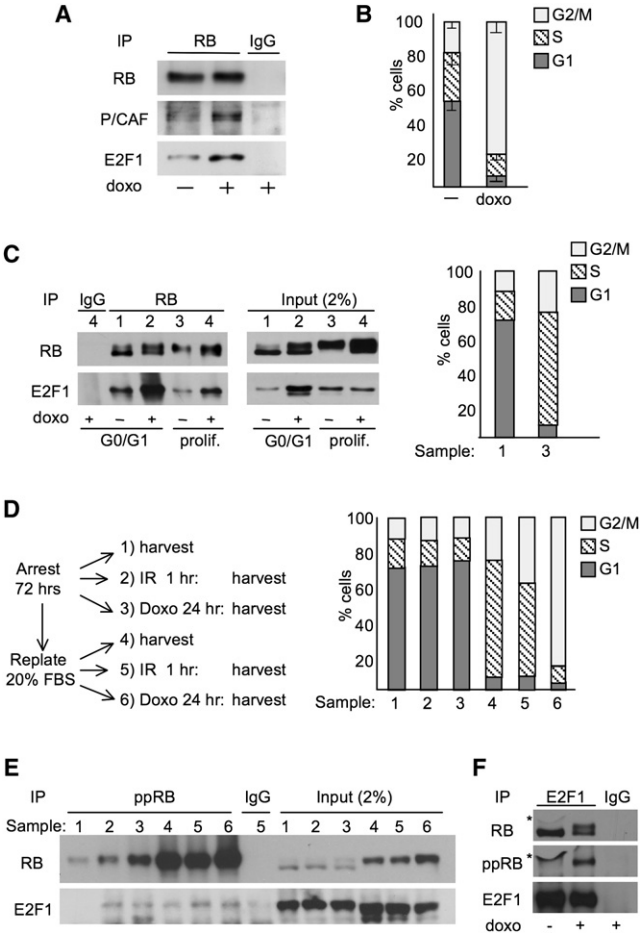

Figure 1. DNA Damage Promotes Formation of a pRB-E2F1 Complex in Proliferating Cells

(A and B) Asynchronous T98G cells untreated (−) or treated with 2 μM doxorubicin (doxo) for 24 hr (+) were screened by immunoprecipitation (IP) using an antibody against pRB followed by western blotting (WB) to assess levels of pRB and associated E2F1 and P/CAF (A) or assayed for cell-cycle distribution by FACS analysis (B). Bars in (B) represent the mean of three independent experiments ± SD.

(C) T98G cells were highly enriched for G0/G1 or S/G2/M (prolif.) cells as determined by FACS (right panel) by culturing in 0.1% FBS for 72 hr and then maintaining in 0.1% FBS or replating in 20% FBS for 16 hr. The cells were collected (samples 1 and 3) or treated with 2 μM doxo for additional 48 hr (samples 2 and 4) and then assayed in parallel for pRB-E2F1 complexes by IP-WB (left panel).

(D and E) Enriched populations of G0/G1 or proliferating T98G cells were untreated (samples 1 and 4), irradiated (IR, 10 Gy) for 1 hr (samples 2 and 5), or treated with 2 μM doxo for 24 hr (samples 3 and 6) as indicated (D, left panel). These samples were analyzed for cell-cycle phasing by FACS (D, right panel), the presence of ppRB-E2F1 complexes by IP with either IgG control or ppRB antibodies and then WB for pRB or associated E2F1 (E, left panel), or the levels of total pRB and E2F1 of the input lysates by WB (E, right panel).

(F) Asynchronous T98G cells, untreated (−) or treated with 2 μM doxo for 24 hr (+), were screened for the presence of ppRB-E2F1 complexes by IP with either IgG control or E2F1 antibodies and then WB, first for pRB and subsequently (after stripping the blot) for ppRB. A bubble in the blot yielded the nonspecific signal (*).

a smaller fraction of total E2F1 in ppRB immunoprecipitates (Figure 1E) versus total pRB immunoprecipitates (Figure 1C). It was unclear whether the phosphospecific pRB antibody cocktail disrupts the ppRB-E2F1 complex, leading to an underestimation of its levels, or whether hypophosphorylated or other phosphorylated pRB species are the main constituent of this complex. To distinguish between these possibilities, we conducted a reciprocal IP. Specifically, we immunoprecipitated E2F1 from T98G cells before or after doxorubicin treatment and then screened for associated pRB by western blotting with an antibody that recognizes all forms of pRB (Figure 1F). In the untreated cells, the E2F1 immunoprecipitate contained a single pRB species. In contrast, doxorubicin caused E2F1 to bind two distinct pRB bands that were present at approximately equal levels. One of these comigrated with the single pRB species seen in the untreated cells, while the other had a slower mobility characteristic of ppRB. To verify this, we stripped and reprobed the blot with the anti-ppRB antibody cocktail. This recognized only the slower migrating pRB species specific to the doxorubicin-treated cells, confirming that this was ppRB. Based on the relative levels of the two bands in the anti-pRB blot, we conclude that ppRB accounts for at least half of the E2F1-associated pRB activity in the doxorubicin-treated T98G cells. Taken together, these experiments show that DNA damage induces formation of pRB-E2F1 complexes in both arrested and proliferating cells and that ppRB is able to participate in this complex.

pRB Participates in Transcriptional Activation of Proapoptotic Genes in Response to Stress

Histone acetyltransferases (HATs) and HDACs are known to play key roles in mediating the transcriptional properties of pRB and the E2F proteins (Frolov and Dyson, 2004). The pRB-E2F complex is thought to act as a repressor of classic E2F target genes through recruitment of HDACs, while HATs acetylate E2F1 and promote its transcriptional activity. Importantly, DNA damage triggers the HAT P/CAF to bind and acetylate E2F1 (Ianari et al., 2004), and this modification is required for E2F1 association with proapoptotic promoters (Pediconi et al., 2003). Given these observations, we screened for the presence of P/CAF in pRB immunoprecipitates in untreated versus doxorubicin-treated cells (Figure 1A). We found that DNA damage promotes P/CAF-pRB complex formation (Figure 1A). This raised the possibility that pRB might participate in a transcriptionally active complex under proapoptotic conditions.

To understand the transcriptional relevance of the DNA damage-induced pRB-E2F1 complex, we examined the regulation of representative cell-cycle control and proapoptotic E2F1-responsive genes in DNA-damaged T98G cells. This analysis revealed that doxorubicin caused a differential response of these two target gene classes: activation of the proapoptotic genes *Caspase 7* and *p73* and repression of the cell-cycle regulator *Cyclin A2* (Figure 2A). To further understand this differential response, we conducted chromatin immunoprecipitation (ChIP) assays. Notably, doxorubicin treatment induced pRB recruitment to both the cell-cycle and proapoptotic promoters (Figure 2B). Quantitative analysis of these results (see Figure S1 available online) showed that the increase of pRB levels was slightly higher at *Caspase 7* (2-fold) and *p73* (2.2-fold) than at *Cyclin A2* (1.5-fold) gene promoters. For all of the other proteins that we assayed, doxorubicin treatment caused differential changes at cell-cycle versus proapoptotic promoters

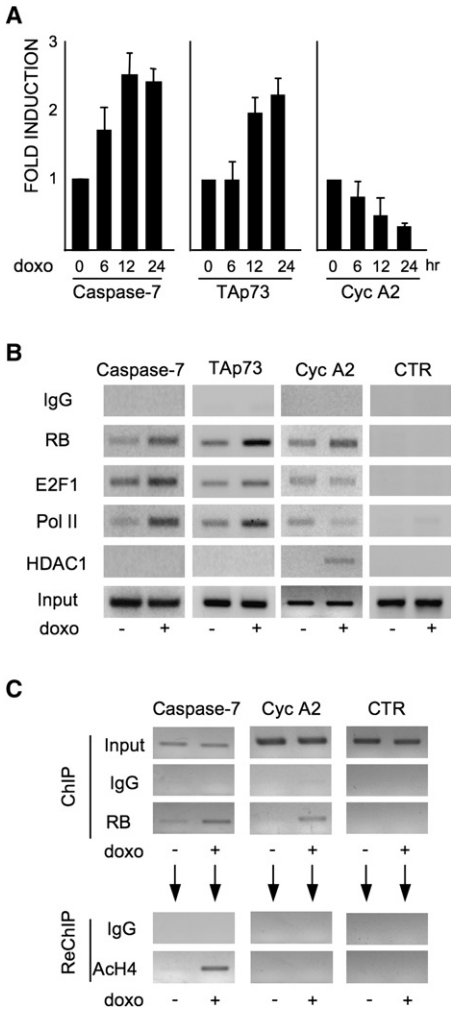

Figure 2. DNA Damage Induces pRB-Dependent Activation of Proapoptotic Gene Promoters

(A) Untreated and doxo-treated asynchronously growing T98G cells were assessed for levels of *Caspase 7*, *p73*, and *Cyclin A2* mRNAs by real-time RT-PCR analysis. Results are normalized to *GAPDH* and shown relative to the levels observed in untreated cells (set to 1). Bars represent the mean of three independent experiments ± SD.

(B) T98G cells were untreated (−) or treated with 2 μM doxo for 16 hr (+), and the binding of pRB, E2F1, RNA polymerase II (Pol II), and HDAC1 to the *Caspase 7*, *p73*, and *Cyclin A2* gene promoters was determined by ChIP.

(C) Acetyl-H4 (AcH4) reChIP analysis of the pRB ChIP shows that pRB is bound to the transcriptionally active *Caspase 7* gene promoter following doxo treatment.

(Figure 2B). At the *Cyclin A2* gene promoter, we found a reduction in the binding of both E2F1 and RNA polymerase II. In addition, the transcriptional corepressor HDAC1 was specifically recruited to the *Cyclin A2* promoter in treated, but not untreated, cells (Figure 2B). These changes are consistent with the observed downregulation of *Cyclin A2* mRNA and the prevailing view that pRB mediates the transcriptional repression of cell-cycle promoters. At the same time, doxorubicin treatment induced the recruitment of both E2F1 (1.4- and 2.7-fold) and RNA polymerase II (2.3- and 2-fold) to the *Caspase 7* and *p73* promoters. Importantly, we did not detect any recruitment of HDAC1 to the proapoptotic promoters in either the damaged or undamaged cells (Figure 2B).

The coordinated enrichment of pRB, E2F1, and RNA polymerase II at the *Caspase 7* and *p73* promoters fits with the hypothesis that pRB contributes to activation of proapoptotic genes. However, we could not rule out the possibility that there are two distinct populations of cells in which these promoters are either bound by pRB and repressed or associated with RNA polymerase II and activated. To address this possibility, we performed ChIP-reChIP experiments in which immunoprecipitated pRB-chromatin complexes were eluted and then subjected to a second round of immunoprecipitation with either control IgG or an antibody against acetyl-H4 (AcH4), a marker of transcriptional activation (Figure 2C). As with our previous experiment, the primary ChIP showed that doxorubicin promoted recruitment of pRB to both the *Caspase 7* and *Cyclin A2* promoters (Figure 2C). However, when we analyzed the eluate from the pRB immunoprecipitates, we found that acetyl-H4 was specifically detected at the *Caspase 7*, but not the *Cyclin A2*, gene promoter (Figure 2C). This analysis showed unequivocally that pRB was bound to the transcriptionally active *Caspase 7* gene promoter, presumably via its participation in the pRB-E2F1-P/CAF complex that is promoted by DNA damage. At the same time, pRB binds to cell-cycle promoters and recruits HDAC1 to mediate their repression.

pRB Is Required for Maximal Induction of the Apoptotic Response In Vitro and In Vivo

These data show that pRB is associated with proapoptotic promoters that are transcriptionally active in DNA-damaged cells. However, they do not establish whether pRB contributes to transcriptional activation of these proapoptotic genes or to the apoptotic response. To address these questions, we took advantage of the lentivirus pPRIME-GFP-shRB and its control pPRIME-GFP producing respectively either a short hairpin against human pRB (shRB) or a hairpin targeting the luciferase gene, in the context of miR30 (Stegmeier et al., 2005; http://elledgelab.bwh.harvard.edu/protocols/pPRIME/pPRIME_vectors.doc). We used these viruses to infect T98G cells and selected parallel populations of GFP-positive cells. The shRB reduced pRB levels to less than 50% of those seen in the control cells (Figure 3A). Importantly, this partial knockdown had no effect on cell-cycle phasing (Figure 3A). This allowed us to assess pRB's contribution to apoptosis independent of its role in the cell cycle. We found that this partial pRB knockdown significantly reduced the fraction of cells undergoing apoptosis in response to either doxorubicin or another topoisomerase II inhibitor, etoposide (50% and 30% reduction, respectively; Figure 3C). This correlated with a reduction in the levels of *p73* (>50%) and *Caspase 7* (~20%) mRNA in the shRB-expressing cells (Figure 3D). Thus, we conclude that pRB loss can impair the apoptotic response in the absence of any cell-cycle defects.

All of the previous experiments were conducted in the p53-deficient tumor cell line T98G. To determine whether our findings were more broadly relevant, we repeated this analysis in a second tumor cell line, U2OS (Figure S2). These cells express wild-type p53, and pRB is constitutively hyperphosphorylated due to hypermethylation and silencing of the $p16^{INK4a}$ gene promoter (Park et al., 2002). In accordance with our prior results,

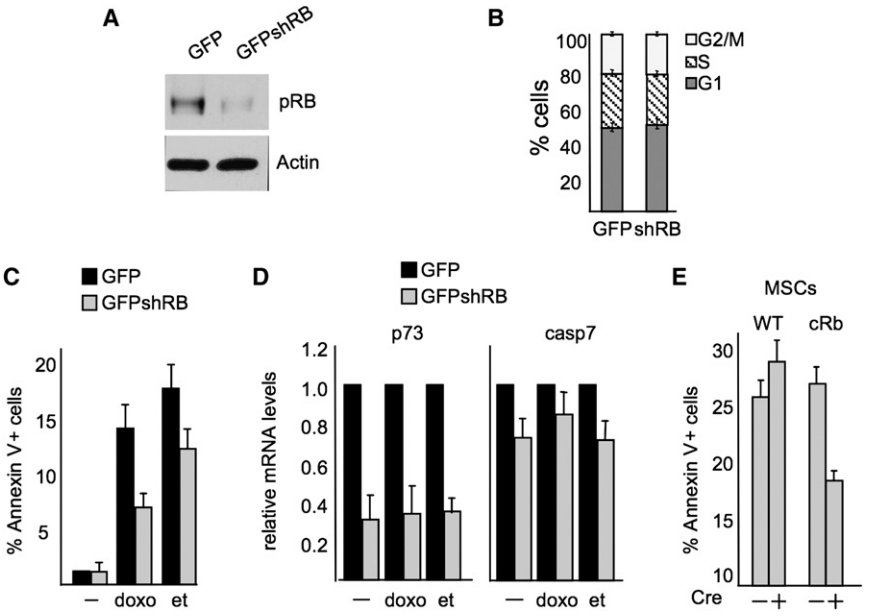

Figure 3. pRB Loss Impairs the Apoptotic Response to DNA Damage

(A–D) T98G cells were infected with pPRIME-GFP (GFP) or pPRIME-GFP-shRB (GFPshRB) lentivirus and sorted for >20% GFP-positive cells.

(A–C) Sorted GFP and GFPshRB cells were screened for the level of pRB by WB using actin as a loading control (A), cell-cycle phasing by FACS analysis (B), or the percentage of early apoptotic cells by FACS (annexin V^+, $7AAD^-$) after culturing for 48 hr in either the absence (–) or presence of 2 μM doxo or 25 μM etoposide (et) (C).

(D) *Caspase 7* and *p73* mRNA levels measured by real-time RT-PCR analysis. Results are normalized to *GAPDH* and expressed relative to levels observed in GFP-infected cells (set as 1).

(E) Wild-type (WT) or $Rb^{2lox/2lox}$ (cRb) mesenchymal stem cells (MSCs) were infected with GFP- or GFP-Cre-expressing adenoviruses. The percentage of GFP^+ apoptotic cells was measured by FACS (annexin V^+, $7AAD^-$).

Bars in (B)–(E) represent the mean of three independent experiments ± SD.

we found that numerous genotoxic agents (including doxorubicin, etoposide, and camptothecin) triggered pRB to bind to E2F1 and promoted both activation of proapoptotic and repression of cell-cycle-related E2F target genes (Figures S2A–S2C). Moreover, pRB knockdown using either the pPRIME-GFP-shRB lentivirus or a tTA-inducible RB hairpin impaired the apoptotic response and the transcriptional activation of the proapoptotic gene *p73* (Figures S2D–S2G). Thus, pRB can play a positive role in DNA damage-induced apoptosis in both the absence and the presence of p53.

It has previously been reported that *Rb* inactivation renders mouse embryonic fibroblasts (MEFs) more, not less, sensitive to DNA damage-induced apoptosis (Almasan et al., 1995; Knudsen et al., 2000). We have repeated these experiments in both germline and conditional *Rb* mutant MEFs and obtained similar results (A.I. and J.A.L., unpublished data). Thus, in different settings, pRB can either promote or inhibit apoptosis. It seemed possible that the differential consequences of pRB loss reflect fundamental differences between tumor versus normal, or mouse versus human, cells. To address these possibilities, we examined a second source of primary murine cells, mesenchymal stem cells (MSCs). We generated these MSCs from mice carrying either wild-type (WT) or conditional *Rb* (cRb) alleles, infected them with adenoviruses expressing either the Cre recombinase gene (+Cre) or a GFP control (–Cre), and confirmed recombination of the cRb alleles by PCR (data not shown). The four cell populations were then treated with ionizing radiation (Figure 3E) or doxorubicin (data not shown), and the fraction of apoptotic cells was quantified by FACS analysis. Cre expression had no significant effect on the level of apoptosis in the WT cells, but it reduced apoptosis in the cRb cells by more than 30% (Figure 3E). Thus, pRB loss also impairs the apoptotic response of these primary murine cells.

To further extend this analysis, we next examined pRB's role in the DNA damage response in vivo. For this, we used mice carrying conditional *Rb* alleles and a *Villin-Cre* transgene, which is expressed in the adult intestinal epithelium. To eliminate any potential contribution of Cre-mediated deletion to the DNA damage response, we selected $Rb^{+/2lox}$;*Villin-Cre*$^+$ mice (effectively *Rb* heterozygous) as controls for our analysis of $Rb^{2lox/2lox}$;*Villin-Cre*$^+$ (*Rb* mutant) mice. $Rb^{2lox/2lox}$;*Villin-Cre*$^+$ mice are known to have histologically normal intestinal crypts, but these contain proliferating cells at ectopic locations (Kucherlapati et al., 2006). Immunohistochemical staining confirmed that pRB was expressed in the intestinal epithelium of the $Rb^{+/2lox}$;*Villin-Cre*$^+$ controls, but not in the $Rb^{2lox/2lox}$;*Villin-Cre*$^+$ animals (Figure 4A, left). We then assessed the level of proliferating cells by screening for the cell-cycle marker Ki-67 (Figure 4A, middle). Consistent with prior studies (Haigis et al., 2006; Kucherlapati et al., 2006), the proliferating cells were restricted to the intestinal crypts of the $Rb^{+/2lox}$;*Villin-Cre*$^+$ controls, but they existed in both the crypts and throughout the height of the villi of *Rb*-deficient intestinal epithelium. We then subjected these $Rb^{2lox/2lox}$;*Villin-Cre*$^+$ mice and their $Rb^{+/2lox}$;*Villin-Cre*$^+$ littermate controls to intraperitoneal (i.p.) injections of doxorubicin and examined the levels of apoptosis in the proximal small intestines by staining for cleaved caspase-3 (Figure 4A, right). As expected, apoptosis was essentially absent in the untreated intestinal epithelia of both genotypes (Figure 4A, right). In response to treatment, we observed high levels of cleaved caspase-3 in the $Rb^{+/2lox}$;*Villin-Cre*$^+$ control tissues. Interestingly, these apoptotic cells were localized exclusively within the intestinal crypts, clearly correlating with the proliferative region of the intestinal epithelium. This is consistent with the prevailing view that the cycling cells are more predisposed to undergo apoptosis than their arrested counterparts. Notably, quantification of 60 villi from each genotype showed that the fraction of proliferating cells undergoing apoptosis was reduced in the *Rb* mutant (1.9% ± 0.88%) versus control (3.8% ± 0.72%) tissue. This effect was most striking in the height of the villi, where we observed few, if any, apoptotic cells in the $Rb^{2lox/2lox}$;*Villin-Cre*$^+$ mice even though this zone was highly proliferative (Figure 4A). However, we did not observe

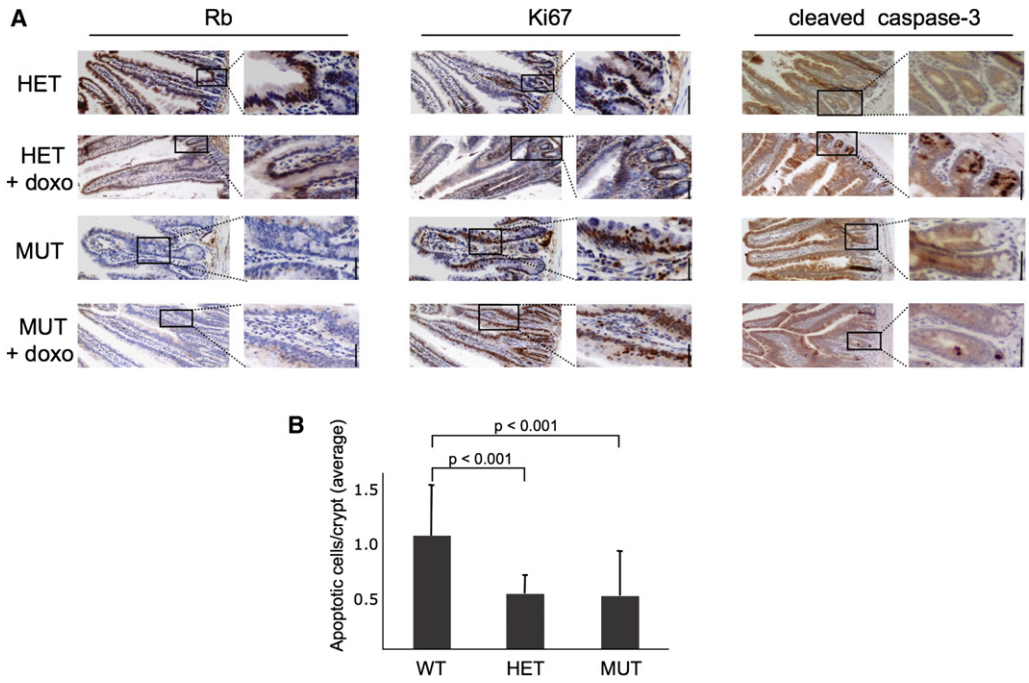

Figure 4. Conditional pRB Knockout Mice Are Less Sensitive to Genotoxic Stress

(A) Analysis of proximal small intestines of $Rb^{+/2lox}$;*Villin-Cre*$^{+}$ (HET) and $Rb^{2lox/2lox}$;*Villin-Cre*$^{+}$ (MUT) mice treated with vehicle control or doxo. Left: immunostaining confirms Cre-mediated loss of pRb in the proximal small intestine of MUT animals. Middle panel: analysis of Ki-67, a marker of proliferating cells, shows that pRB loss causes proliferation throughout the villi. Ki-67 levels were similar in the absence and presence of doxorubicin treatment. Right: staining for cleaved caspase-3 shows high levels of apoptotic cells specifically in the base of the crypts in doxo-treated, but not untreated, WT and MUT mice. Scale bars = 50 μm in all panels.

(B) The average number of apoptotic cells per intestinal crypt (±SEM) in the proximal small intestines of doxo-treated $Rb^{+/+}$;*Villin-Cre*$^{+}$ (WT; n = 5), $Rb^{+/2lox}$;*Villin-Cre*$^{+}$ (HET; n = 5), and $Rb^{2lox/2lox}$;*Villin-Cre*$^{+}$ (MUT; n = 6) mice was determined by counting cleaved caspase-3-positive cells in 21 crypts for each animal.

a significant difference in the level of apoptotic cells in the intestinal crypts of $Rb^{2lox/2lox}$;*Villin-Cre*$^{+}$ mice versus $Rb^{+/2lox}$;*Villin-Cre*$^{+}$ controls (data not shown).

Since our cell studies had shown that a partial knockdown of pRB was sufficient to impair the apoptotic response, we wondered whether there might be a heterozygous mutant phenotype in the $Rb^{+/2lox}$;*Villin-Cre*$^{+}$ intestinal crypts. To address this possibility, we subjected a second cohort of $Rb^{+/+}$;*Villin-Cre*$^{+}$ (n = 5), $Rb^{+/2lox}$;*Villin-Cre*$^{+}$ (n = 5), and $Rb^{2lox/2lox}$;*Villin-Cre*$^{+}$ (n = 6) mice to i.p. injections of doxorubicin, stained for cleaved caspase-3, and quantified the level of apoptosis in the intestinal crypts. Consistent with our prior analysis, doxorubicin treatment induced a similar apoptotic response in the intestinal crypts of $Rb^{+/2lox}$;*Villin-Cre*$^{+}$ (HET) and $Rb^{2lox/2lox}$;*Villin-Cre*$^{+}$ (MUT) mice (Figure 4B). However, these two genotypes had a 2-fold lower level of apoptosis than the $Rb^{+/+}$;*Villin-Cre*$^{+}$ (WT) controls (Figure 4B). In both cases, this difference was statistically significant ($p < 0.001$). Thus, in this tissue, pRB loss promotes inappropriate proliferation while reducing the ability of these cells to undergo DNA damage-induced apoptosis. Moreover, mutation of a single *Rb* allele is sufficient to impair the apoptotic response without altering proliferation. These observations show that *Rb* influences the apoptotic response to DNA damage in vivo. They also raise the possibility that this impaired response could occur in patients carrying germline *RB1* mutations.

E1A Promotes the Formation of Transcriptionally Active pRB-E2F1 Complexes

Given our findings, we decided to extend our analysis to examine the mechanism of action of adenoviral E1A. *E1A* is a potent oncogene that induces uncontrolled proliferation and also sensitizes cells to apoptosis. It binds to pRB with high affinity, and this requires the LXCXE motif that is essential for E1A's transforming activity (Helt and Galloway, 2003). The prevailing view is that E1A acts to sequester pRB, allowing release of transcriptionally active E2Fs. This model can explain the increased proliferation rate seen for E1A-infected cells. However, prior studies have shown that the interaction between E1A and pRB is insufficient to promote apoptosis in response to doxorubicin (Samuelson et al., 2005). Indeed, the previous mutant analysis suggests that this requires E1A binding to both pRB and p400, a component of the TRAAP/Tip60 HAT chromatin-remodeling complex. Given our observations, we hypothesized that E1A's proapoptotic function might reflect its ability to promote formation of transcriptionally active pRB-E2F1 complexes, in a manner analogous to genotoxic stress. To test this notion, we investigated the interplay between E1A and the pRB-E2F1 complex using primary IMR-90 human diploid fibroblasts that express the murine ecotropic receptor. We selected these cells because they apoptose in response to genotoxic agents or E1A but are more resistant to apoptosis than many other cell lines and thus are better able to tolerate E1A expression. Importantly, in

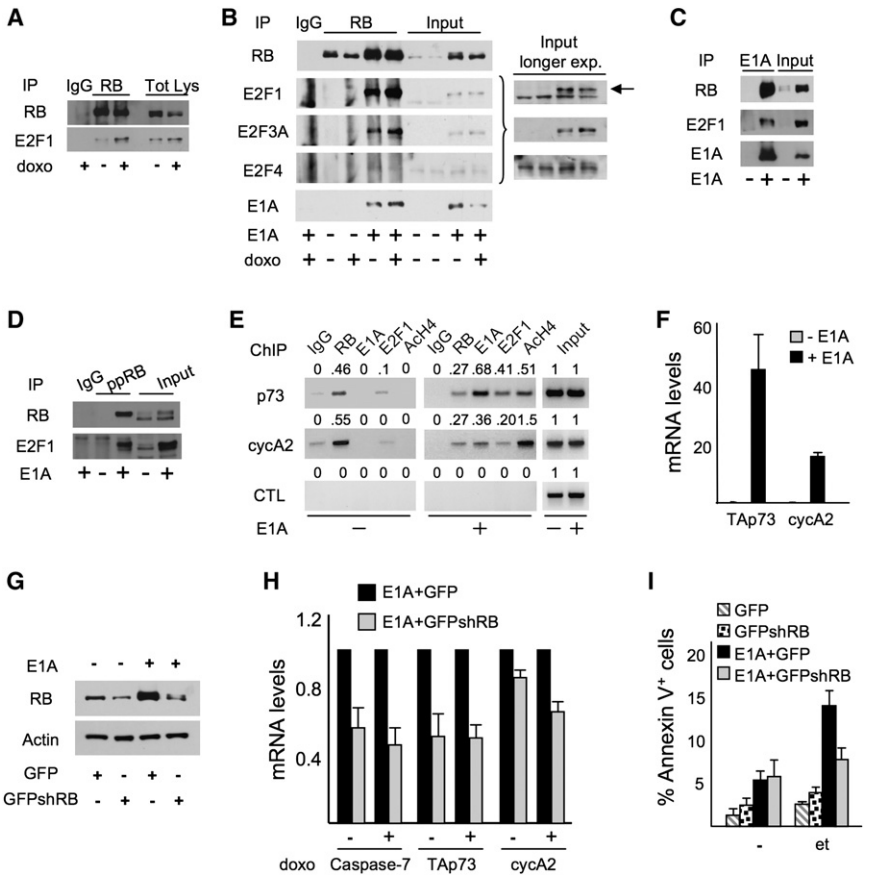

Figure 5. pRB Facilitates E1A's Ability to Promote Apoptosis

(A) In the absence of E1A, a pRB-E2F1 complex was induced by treatment of IMR-90 cells with doxo as judged by IP for pRB and WB for pRB and E2F1.

(B–I) IMR-90 cells were retrovirally transduced with either pBabe-hygro vector (−E1A) or pBabe-hygro-E1A 12S (+E1A), selected with 75 μg/ml hygromycin for 4 days, and then assayed as follows.

(B) Cells were treated with 2 μM doxo for 16 hr, and WB was used to determine the levels of pRB, E2F1, E2F3A, E2F4, and E1A in pRB IPs (left) and total lysates (right). A longer exposure of the key input lanes is shown at far right. E1A expression strongly increased both the total levels of pRB, E2F1, and E2F3A and the level of pRB-E2F1/E2F3A complexes. At the exposure shown, the pRB-E2F1 interaction is not visible in the −E1A +doxo cells.

(C) The binding of E1A to pRB and E2F1 is also detected via IP of E1A.

(D) IPs with antibodies against ppRB and WB for pRB and E2F1.

(E) The levels of pRB, E1A, E2F1, and AcH4 associated with the *p73* and *Cyclin A2* gene promoters were determined by ChIP. Densitometric quantification of each signal relative to the input is shown.

(F) Real-time RT-PCR analysis of *p73* and *Cyclin A2* mRNA levels. Results are expressed as arbitrary units normalized to *GAPDH* and show the mean of three independent experiments ± SD.

(G–I) Control or E1A-transduced cells were infected with either GFP or GFPshRB lentiviruses.

(G) WB confirmed a reduction in pRB levels after shRB infection, using actin as a loading control.

(H) *Caspase 7*, *p73*, and *Cyclin A2* mRNA levels were determined by real-time RT-PCR analysis in cells cultured in the absence (−) or presence of 2 μM doxo for 12 hr (+). Results are normalized to *GAPDH* and show the mean of three independent experiments ± SD, expressed relative to levels observed in the untreated cells (set as 1).

(I) FACS analysis of early apoptotic cells (annexin V^+, $7AAD^-$) after culture in the absence (−) or presence of 1 μM etoposide (et) for 16 hr. Values represent the percentage of apoptotic GFP^+ cells and represent the mean of three independent experiments ± SD.

a manner similar to T98G and U2OS cells, doxorubicin promotes formation of pRB-E2F1 complexes in IMR-90 cells in the absence of E1A (Figure 5A). To study the role of E1A, IMR-90 cells were retrovirally transduced with either pBabe-puro vector or pBabe-puro expressing the 12S form of E1A. We found that E1A and E2F1 both coimmunoprecipitated with pRB (Figure 5B). Doxorubicin treatment caused a modest additional increase (1.6-fold) in the level of E2F1 coimmunoprecipitating with pRB in the E1A-expressing IMR-90 cells (Figure 5B). These observations show that E1A potently induces formation of pRB-E2F1-E1A complexes and that exogenous DNA damage reinforces this response.

In the presence of E1A, pRB also associated with E2F3A, another activating E2F that is known to participate in the oncogenic stress response, but not with the repressive E2F E2F4 (Figure 5B). Importantly, the reciprocal immunoprecipitation using antibodies against E1A also recovered both pRB and E2F1 (Figure 5C). Since it is well established that a pocket protein, such as pRB, is required to bridge the interaction between E1A and E2F (Fattaey et al., 1993), we can infer that these three proteins must be part of the same complex. Similar to our analysis of the DNA damage response, E1A also enhanced the formation of ppRB species and their binding to E2F1 (Figure 5D). Additionally, E1A yielded a dramatic increase in the intracellular levels of pRB, E2F1, and E2F3A, but not E2F4 (Figure 5B). DNA binding is known to protect E2F-pocket protein complexes from degradation (Hofmann et al., 1996). Given the observed stabilization of both pRB and the activating E2Fs in E1A-expressing cells, we hypothesized that E1A induces the formation of pRB-E2F1-E1A complexes that bind to DNA and activate transcription. Thus, we used ChIP assays to assess binding to the *p73* and *Cyclin A2* promoters (Figure 5E). In the absence of E1A, we observed a significant pRB ChIP signal at both promoters, but little or no binding of either E2F1 or acetyl-H4 (Figure 5E). Since the uninfected IMR-90 cells are predominantly in G0/G1 phase, we speculate that pRB contributes to the repression of both *p73* and *Cyclin A2* in this setting. Accordingly, these genes appeared to be transcriptionally silent, as judged by the lack of *p73* and *Cyclin A2* mRNA (Figure 5F). In contrast, in the E1A-infected cells, pRB, E2F1, E1A, and acetyl-H4 all associated with both the *Cyclin A2* and *p73* promoters, coincident with the dramatic induction of both of

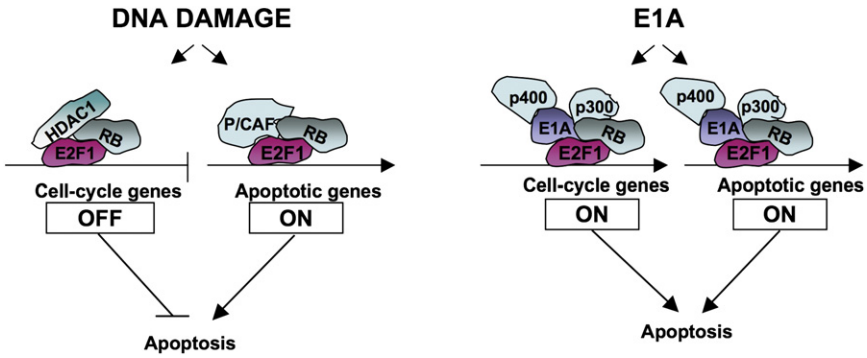

Figure 6. Model of pRB-E2F1 Complexes Involved in the Regulation of Proliferation and Proapoptotic Genes in Response to DNA Damage and E1A-Induced Oncogenic Stress

See text for details.

these mRNAs (Figures 5E and 5F). Given the existence of a pRB-E2F1-E1A complex and the positive ChIP signals for E1A, pRB, and E2F1, we speculate that E1A's ability to activate apoptosis and cell-cycle genes reflects, at least in part, its direct action at their promoters.

As noted above, the current view of E1A action is that it acts to sequester pRB and thereby release transcriptionally active E2F. If this model fully explains the relationship between E1A and pRB, E1A's ability to induce apoptosis should not be impaired by pRB loss. To test this, we performed a pRB knockdown in E1A-infected IMR-90 cells. The shRB yielded significant, but not complete, pRB knockdown in both uninfected and E1A-infected IMR-90 cells (Figure 5G). Strikingly, the shRB caused a 2-fold reduction in the levels of *Caspase 7* and *p73* mRNAs in either the absence or presence of DNA damage (Figure 5H). Consistent with previous studies (Samuelson et al., 2005), expression of E1A alone induced only low levels of apoptosis in these cells, and this was unaffected by pRB levels (Figure 5I). However, when combined with genotoxic stress, E1A induced programmed cell death at 2-fold higher levels in control versus shRB-expressing cells (Figure 5I). Thus, pRB plays a positive role in E1A-induced apoptosis. Taken together, our findings suggest that E1A associates with both pRB and E2F1, stabilizing these proteins, and the resulting complex associates with both proapoptotic promoters to promote their transcription.

DISCUSSION

The E2F transcription factors play a key role in promoting cellular proliferation under the control of the pRB tumor suppressor. It is well established that E2F1, among other E2F family members, also contributes to the induction of apoptosis in response to either DNA damage or oncogenic stress (Iaquinta and Lees, 2007). However, the role of pRB in this process is poorly understood. We anticipated that DNA damage would have to release E2F1 from pRB to allow it to activate proapoptotic genes. Instead, our data suggest an unexpected mechanism of pRB action—that DNA damage induces pRB to participate in a transcriptionally active complex that drives expression of proapoptotic genes. This model is supported by three central observations: (1) pRB is induced to bind both E2F1 and the histone acetylase P/CAF in DNA-damaged cells; (2) ChIP-reChIP assays show unequivocally that pRB is bound to the promoters of proapoptotic genes that are transcriptionally active; and (3) knockdown and genetic ablation experiments confirm that pRB loss typically reduces the apoptotic response to DNA damage by 34%–50%. Since the apoptotic threshold is determined by many different factors, the degree of impairment is striking. Moreover, this is observed in many different settings including both primary and tumor cells derived from either mice or humans, and it is independent of p53. Thus, our finding that pRB has proapoptotic activity has broad relevance.

The concept that pRB can participate in transcriptionally active complexes is not without precedent, since pRB has been shown to cooperate with differentiation-specific transcription factors in the activation of key target genes (Charles et al., 2001; Gery et al., 2004; Thomas et al., 2001). However, this proapoptotic function of pRB is not observed in all situations. As we outlined in the introduction, the analysis of *Rb* mutant embryos led to the prevailing view that pRB is antiapoptotic. Although much of this apoptosis is non-cell autonomous (de Bruin et al., 2003; Wenzel et al., 2007; Wu et al., 2003), pRB loss does promote apoptosis in a cell-autonomous manner in some tissues of the developing embryo, and this seems to reflect an inability to undergo terminal differentiation (de Bruin et al., 2003; Haigis et al., 2006; Huh et al., 2004; MacPherson et al., 2003; Mason-Richie et al., 2008; Ruiz et al., 2004; Wang et al., 2007; Wikenheiser-Brokamp, 2004). Moreover, some cell types, such as MEFs, have a heightened sensitivity to DNA damage in the absence of pRB. Thus, taken together, the existing literature and the present study indicate that pRB can either suppress or promote apoptosis, depending on the cellular context. We note that there is a strong correlation between the proliferative properties of the cell and the observed role of pRB in apoptosis. Thus, we propose the following model of pRB action (Figure 6). In G0/G1 cells, genotoxic stress induces pRB recruitment into the classic repressive pRB-E2F-HDAC complex. This prevents cell-cycle entry and thus acts indirectly to protect cells from apoptosis. Thus, in G0/G1 cells, pRB loss would impair arrest and thereby promote apoptosis. In contrast, in proliferating cells, genotoxic stress favors formation of the transcriptionally active pRB-E2F1-P/CAF complexes because the hyperphosphorylation of pRB inhibits its participation in the repressive complexes. Consequently, in this setting, pRB is proapoptotic. Interestingly, this context-dependent model of pRB function has the potential to explain the well-established phenomenon that proliferating cells have a greater predisposition to undergo apoptosis compared to their quiescent counterparts. Importantly, both our cell line and in vivo studies show that a reduction in pRB levels is sufficient to impair pRB's proapoptotic function without any disruption of proliferation control. This was particularly striking in our animal experiments, where *Rb* haploinsufficiency

reduced the apoptotic response to doxorubicin as efficiently as the complete inactivation of *Rb.* This dose-dependent effect raises the possibility that mutation of a single *Rb* allele might increase the probability of cellular transformation by impairing the apoptotic response to DNA damage and thereby enabling the acquisition of mutations within other genes. If this is true, individuals carrying germline *RB1* mutations would be particularly at risk.

Clearly, additional questions remain about how genotoxic stress triggers formation of the pRB-E2F1-P/CAF complex and directs it specifically to proapoptotic promoters. Our data show that ppRB can participate in the DNA damage-induced pRB-E2F1 complexes. However, it is still unclear whether pRB phosphorylation actively promotes or is merely permissive for formation of the proapoptotic pRB-E2F1-P/CAF complex. Moreover, although we conducted these studies using antibodies against known Cdk phosphorylation sites (Ser780, Ser795, and Ser807–811), we cannot be sure whether cyclin/Cdk complexes or other kinases are responsible for this modification in the DNA-damaged cells. Furthermore, it is entirely possible that additional posttranslational modifications of pRB and/or E2F1 may facilitate formation of the proapoptotic complex. We note that pRB has been shown to contain a second E2F binding site that does not interfere with E2F1's transactivation domain (Dick and Dyson, 2003). Thus, it is intriguing to speculate that DNA damage somehow induces pRB and E2F1 to adopt this alternate structure. If this model is true, this conformation must also enable recruitment of P/CAF, which is known to be critical for E2F1-dependent activation of proapoptotic target genes in response to DNA damage (Ianari et al., 2004; Pediconi et al., 2003).

Our data also cause us to revise our view of E1A's mechanism of action. The prevailing view holds that E1A acts to disrupt pRB-E2F complexes, releasing free E2F1 to induce transcription of its target genes. However, our data show that E1A forms a stable complex with both pRB and the activating E2Fs. Moreover, pRB, E2F1, and E1A are all recruited to the promoters of both apoptosis and cell-cycle genes, coincident with their transcriptional activation. Interestingly, mutant analysis has shown that E1A must interact with both pRB and p400 in order to promote apoptosis in response to doxorubicin (Samuelson et al., 2005). Thus, we now propose that E1A's ability to promote both apoptosis and proliferation reflects at least in part its direct action at proapoptotic and cell-cycle gene promoters through its association with the pRB-E2F1 complex and the concomitant recruitment of p400 and other transcriptional coactivators (Figure 6). Given the recent finding that oncogenic stress activates the DNA damage response (Bartkova et al., 2006; Di Micco et al., 2006), formation of the transcriptionally active pRB-E2F1 complexes may be further reinforced through E1A's activation of the DNA damage-dependent process. Essentially, E1A would trigger the DNA damage response and then piggyback onto the resultant pRB-E2F1 complex to create a stable superactivator. Notably, in contrast to the DNA damage-induced pRB-E2F1 complex, which specifically activates only proapoptotic genes, the E1A-containing species has an expanded target specificity that now includes both apoptosis and cell-cycle targets. Importantly, in agreement with this superactivator model, pRB knockdown impairs E1A's ability to promote the transcription of both apoptosis and cell-cycle-related genes and to induce apoptosis in response to DNA-damaging agents.

The elucidation of the mechanism by which pRB acts as a tumor suppressor has been complicated by various factors. In addition to its role in cell-cycle control, pRB has been implicated in regulating a wide variety of cellular processes, including DNA replication, differentiation, and apoptosis (Classon and Harlow, 2002). Whereas the decreased differentiation potential and the increase in proliferative rate observed in pRB-deficient cells could contribute to tumorigenesis, it is more difficult to reconcile pRB's role as a tumor suppressor with the notion that loss of pRB may lead to increased apoptosis. Our finding that pRB plays a positive role in DNA damage-induced apoptosis widens our understanding of pRB's functions. Notably, the behavior of pRB in the DNA damage response bears strong parallels to that of p53: both of these tumor suppressors appear capable of triggering either cell arrest or apoptosis, depending on the cellular context. Given this model, we propose that *RB1* inactivation in tumor cells promotes tumorigenicity by yielding both a proliferative advantage and resistance to apoptotic stimuli such as chemotherapeutic treatments.

EXPERIMENTAL PROCEDURES

Cell Culture and Infections

T98G, U2OS, and IMR-90 cell lines were cultured in DMEM with 10% heat-inactivated FBS. The IMR-90 cells overexpressed the murine ecotropic receptor. Lentiviral and retroviral preparations and infections were performed as described previously (Samuelson et al., 2005; Stegmeier et al., 2005). Mesenchymal stem cells were generated by mechanically crushing femurs and tibias of 6- to 8-week-old mice and culturing in α-MEM with 10% heat-inactivated FBS. These cells were infected with either Ad5CMVCre-eGFP or Ad5CMVeGFP at about 100 plaque-forming units per cell for 4 hr (University of Iowa Gene Transfer Vector Core) and treated 3 days later with 2 μM doxorubicin or irradiated for 15 min for a total dose of 10 Gy and analyzed by FACS after 24 hr. Hairpins used in this study are shown in Table S1.

FACS Analysis

Suspensions of T98G or IMR90 cells were processed for DNA content as described previously (Pozarowski and Darzynkiewicz, 2004). For apoptosis assays, cell suspensions were stained with annexin V APC and 7AAD (Becton Dickinson). Cells were analyzed using a FACScan system (Becton Dickinson), and the data were analyzed using ModFit LT software (Verity Software).

Immunoprecipitations and Western Blotting

Proteins were extracted with RIPA buffer (Pediconi et al., 2003) and quantified using BCA protein assay reagent (Pierce). Extracts were immunoprecipitated with the indicated antibodies and either protein A or protein G Plus (Santa Cruz Biotechnology). Antibodies were obtained from Santa Cruz Biotechnology (E2F1 [C-20], RNAPol-II [N-20], E1A [M73-HRP], actin [I-19], E2F3 [C-18], and E2F4 [C-20]), Cell Signaling Technology (RB [4-H1], 780-795-807-811 ppRB, and cleaved caspase-3), BD Pharmingen (pRB and Ki-67), Upstate Biotechnology (acetyl-H4 and HDAC1), and P. Nakatani of Dana-Farber Cancer Institute, Harvard Medical School (P/CAF) .

ChIP Assay

Chromatin immunoprecipitation was performed as described previously (Pediconi et al., 2003). For reChIP experiments, RB immunoprecipitates were eluted with DTT and then subjected to a second round of immunoprecipitation with acetyl-H4 antibody or with IgG. Densitometric quantification of ChIP results was performed using the NIH ImageJ 1.4 program. Primer sequences are described in Table S2.

Real-Time RT PCR

Total RNA was extracted with a QIAGEN RNeasy Kit and reverse transcribed with oligo(dT) primers and SuperScript II reverse transcriptase (Invitrogen). Quantitative analysis of *Caspase 7*, *TAp73*, and *Cyclin A2* mRNA expression was performed employing an ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Gene expression values were normalized to *GAPDH*. Results are expressed as mean ± SD. Statistical differences were analyzed by Mann-Whitney nonparametric test. $p < 0.05$ was considered statistically significant.

Animal Maintenance and Tissue Analyses

The *Rb*^{2lox}*;Villin-Cre*$^{+}$ mice were maintained and genotyped as described previously (Haigis et al., 2006). The relevant genotypes were injected intraperitoneally with either saline vehicle (0.9% NaCl) or doxorubicin (10 mg/kg) and sacrificed 3 hr later, and intestines were collected for histology. All animal procedures followed protocols approved by MIT's Committee on Animal Care. pRB immunostaining was conducted using an UltraVision LP Detection System (Lab Vision Corporation) with the primary antibody (G3-245, BD) at a concentration of 1:100. Immunohistochemistry was performed as described previously for cleaved caspase-3 (Haigis et al., 2006) and Ki-67 (Danielian et al., 2007). All samples were counterstained with hematoxylin.

SUPPLEMENTAL DATA

The Supplemental Data include two tables and two figures and can be found with this article online at http://www.cancercell.org/supplemental/S1535-6108(09)00033-6.

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