

Rb function is required for E1A-induced S-phase checkpoint activation

A Nemaierova^{1,2}, F Talos^{1,2}, UM Moll¹ and O Petrenko^{*1}

It is widely accepted that adenoviral E1A exerts its influence on recipient cells through binding to the retinoblastoma (Rb) family proteins, followed by a global release of E2F factors from pocket-protein control. Our study challenges this simple paradigm by demonstrating previously unappreciated complexity. We show that E1A-expressing primary and transformed cells are characterized by the persistence of Rb–E2F1 complexes. We provide evidence that E1A causes Rb stabilization by interfering with its proteasomal degradation. Functional experiments supported by biochemical data reveal not only a dramatic increase in Rb and E2F1 protein levels in E1A-expressing cells but also demonstrate their activation throughout the cell cycle. We further show that E1A activates an Rb- and E2F1-dependent S-phase checkpoint that attenuates the growth of cells that became hyperploid through errors in mitosis and supports the fidelity DNA replication even in the absence of E2F complexes with other Rb family proteins, thereby functionally substituting for the loss of p53. Our results support the essential role of Rb and E2F1 in the regulation of genomic stability and DNA damage checkpoints.

Cell Death and Differentiation (2008) 15, 1440–1449; doi:10.1038/cdd.2008.66; published online 2 May 2008

Adenoviral E1A was originally described as an retinoblastoma (Rb)-binding protein, whose expression induces DNA synthesis in quiescent cells and cooperates with oncogenic ras in the transformation of primary rodent cells *in vitro*.^{1–3} The regions of E1A required for these properties encompass two discrete segments of the protein, termed conserved region 1 (CR1) and 2 (CR2). These same segments, and a small region within the N terminus of E1A, are responsible for the interaction with a plethora of cellular proteins that are critical for normal cell cycle progression, including members of the Rb protein family, p300, CBP, p400, and TRRAP.^{4–6} The correlation between the transforming capacity of E1A and its ability to bind these cellular proteins has led to the suggestion that E1A alters responsive gene expression and thereby reprograms normal cell cycle control.^{4–6}

Given that E1A can activate transcription from cellular promoters containing E2F-binding sites, it is presumed that these effects are mediated, alongside other mechanisms, through inactivation of Rb and upregulation of endogenous E2F activity.^{4,6} Paradoxically, however, E1A suppresses the growth of primary human tumors and reverses the transformed phenotype of many human cancer cells.⁶ The mechanism of tumor suppression by E1A is not understood. While a number of previous studies have used exogenous expression assays to address the effects of E1A in transformed cells, little is known about which endogenous Rb–E2F complexes are specifically targeted by E1A. Furthermore, the effect of E1A expression on Rb association with E2F family proteins in primary cells has hitherto not been studied. Here, we examined the composition of specific complexes between pocket proteins (Rb, p107, and p130) and the E2F family

members in primary and transformed E1A-expressing cells, and assessed the functional consequences of their interaction with E1A.

Results

Rb forms stable complexes with E2F1 in adenovirus-transformed 293 cells. Current models imply that E1A's activity is primarily mediated by binding to the Rb family proteins, followed by the release of free E2F factors.^{4–6} To explore the efficacy of these E1A-regulated processes, we analyzed the composition of protein complexes containing Rb and E2F in human 293 cells, which express the integrated adenovirus genes. Whole cell lysates from continuously growing 293 cells were immunoprecipitated with E1A-, Rb-, or E2F-specific antibodies and then probed for the presence or absence of complexes composed of Rb and E2Fs (Figure 1a). The previously characterized E1A-binding proteins (Rb, p107, and p130) were readily detected in anti-E1A immunoprecipitations (Figure 1a, lane 4). However, in contrast to earlier reports that suggested that E1A dissociates E2F-containing complexes in gel mobility shift assays,^{7–10} we did not observe a complete disruption of E2F complexes with Rb. Thus, complexes containing both Rb and E2F1 were present in either anti-Rb or anti-E2F1-immunoprecipitations (Figure 1a, lanes 1 and 3). Likewise, Rb formed complexes with E2F3 (Figure 1a, lane 1). On the basis of the relative intensity of bands, we estimate that up to 40% of DP1-bound E2F1 and 10% of E2F3 form stable complexes with Rb in 293 cells, despite high levels of E1A

¹Department of Pathology, Health Science Center, State University of New York at Stony Brook, Stony Brook, NY 11794, USA

*Corresponding author: O Petrenko, Department of Pathology, Health Science Center, State University of New York at Stony Brook, Stony Brook, NY 11794, USA. Tel: 631 444 3520; Fax: 631 444 3420; E-mail: apetrenko@notes.cc.sunysb.edu

²These authors contributed equally to this work.

Keywords: E1A; Rb protein; E2F; checkpoint; DNA replication

Abbreviations: Rb, retinoblastoma protein; CR1, conserved region 1; CR2, conserved region 2; WT, wild type; MEF, mouse embryo fibroblast

Received 07.3.08; accepted 30.3.08; Edited by M Blagosklonny; published online 2.5.08

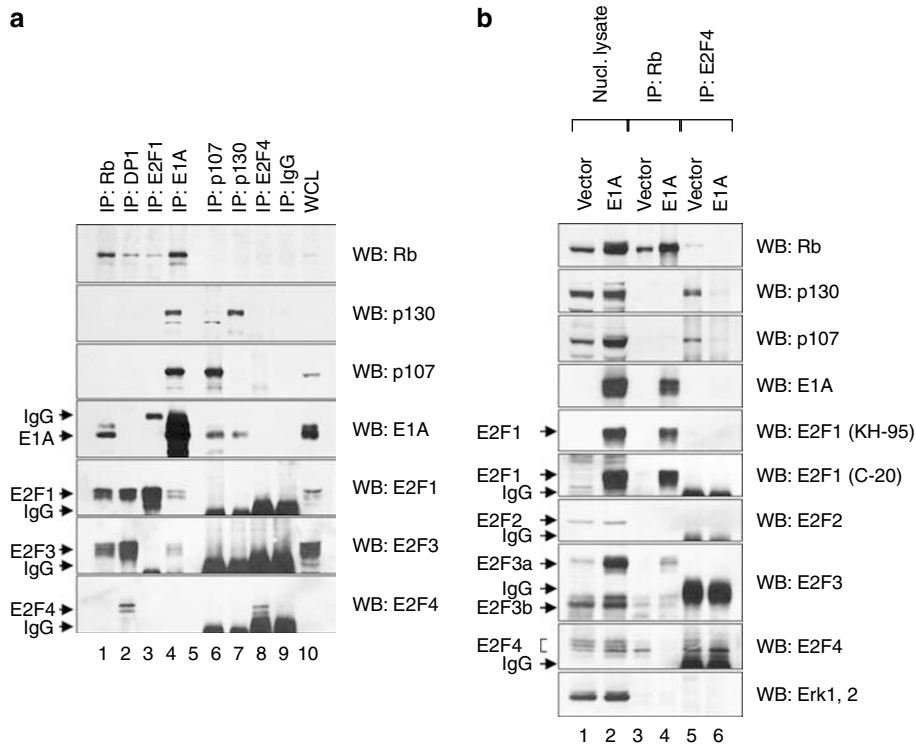


Figure 1 Rb forms stable complexes with E2F1 in E1A-expressing cells. **(a)** Composition of Rb–E2F complexes in whole cell lysates of continuously growing 293 cells. Whole cell lysate (WCL, 5% of the IP input) is shown for control. Note low endogenous levels of p130 and E2F4 in 293 cells. **(b)** Composition of Rb–E2F complexes in whole cell lysates from IMR90 normal human fibroblasts transduced with vector alone or E1A-expressing retroviruses. Nuclear lysates are shown for control. KH-95 and C-20 are E2F1-specific mAb and polyclonal Ab, respectively

expression (Figure 1a, lanes 1 and 2). On the other hand, we were unable to detect complexes of Rb, p107, or p130 with E2F4 (Figure 1a, lanes 1, 6–8). A small fraction of E2F1 and E2F3 was also present in anti-E1A immunoprecipitations (Figure 1a, lane 4). Consistent with the latter data, Fattaey *et al.*¹¹ demonstrated that E1A can be found in intermediate complexes with Rb and E2F, contingent on the presence of its Rb-binding conserved region 2 (CR2). We next examined 293 cells that were synchronized at different stages of the cell cycle by incubation with pharmacological inhibitors L-mimosine (G1 arrest), aphidicolin (S-phase arrest), VM26 (G2 arrest), or nocodazole (microtubule inhibitor) (Supplementary Figure 1A). Alternatively, 293 cells were serum-starved for 48 h and then released into serum-containing medium (Supplementary Figure 1B). The immunoprecipitation analyses showed that the association of Rb with E2F1 was particularly strong in G1- or G2-phase-arrested 293 cells (Supplementary Figure 1A). Moreover, Rb–E2F1 complexes were more abundant in cycling than in serum-starved 293 cells (Supplementary Figure 1B). These results indicate that Rb–E1A and Rb–E2F1 complexes coexist in 293 cells at different stages of the cell cycle.

E1A-expressing primary cells are characterized by the persistence of Rb–E2F1 complexes. To learn if E1A affects the abundance of Rb–E2F complexes, we analyzed IMR90 normal diploid human fibroblasts and mouse embryonic fibroblasts (MEFs) infected with control- or E1A-expressing retroviruses. The expression of E1A in these two

cell types resulted in markedly elevated levels of Rb and E2F1 (Figure 1b and Supplementary Figure 1C and D). In addition, E1A caused a profound rearrangement of endogenous Rb–E2F complexes (Figure 1b). Thus, Rb–E2F1 complexes were more abundant in E1A-expressing IMR90 cells than in control-infected cells (Figure 1b, lanes 3 and 4). On the other hand, the binding of Rb to E2F2 and E2F3 was barely detectable (Figure 1b, lanes 3 and 4), despite the fact that total levels of E2F3 were also significantly increased in E1A-expressing cells (Figure 1b and Supplementary Figure 1D). Likewise, E1A effectively dissociated E2F4 complexes with Rb, p107, and p130 (Figure 1b, lanes 3–6). Thus, E1A-expressing primary cells are characterized by the persistence of Rb–E2F1 complexes.

E1A leaves chromatin-bound Rb–E2F1 complexes intact. These results prompted us to explore the possibility that E2F1 is unique among other E2Fs in that it can compete with E1A for binding to Rb. To test this possibility, E1A-expressing cells were transduced with wild-type (WT) E2F1 or with a DB-E2F1 mutant which lacks the C-terminal Rb-binding domain.¹² Because overexpressed E2F1 causes a p53-dependent G1 arrest in IMR90 cells, we used E1A-expressing p53^{-/-} MEFs for this purpose. Exogenous E2F1 indeed formed stable complexes with Rb (Figure 2a), reducing its proportion bound to E1A (Figure 2b). By contrast, DB-E2F1 failed to compete with E1A for binding to Rb (Figure 2b). Likewise, ectopically expressed E2F4 had no effect on the binding of E1A to Rb, p107 or p130 (data not

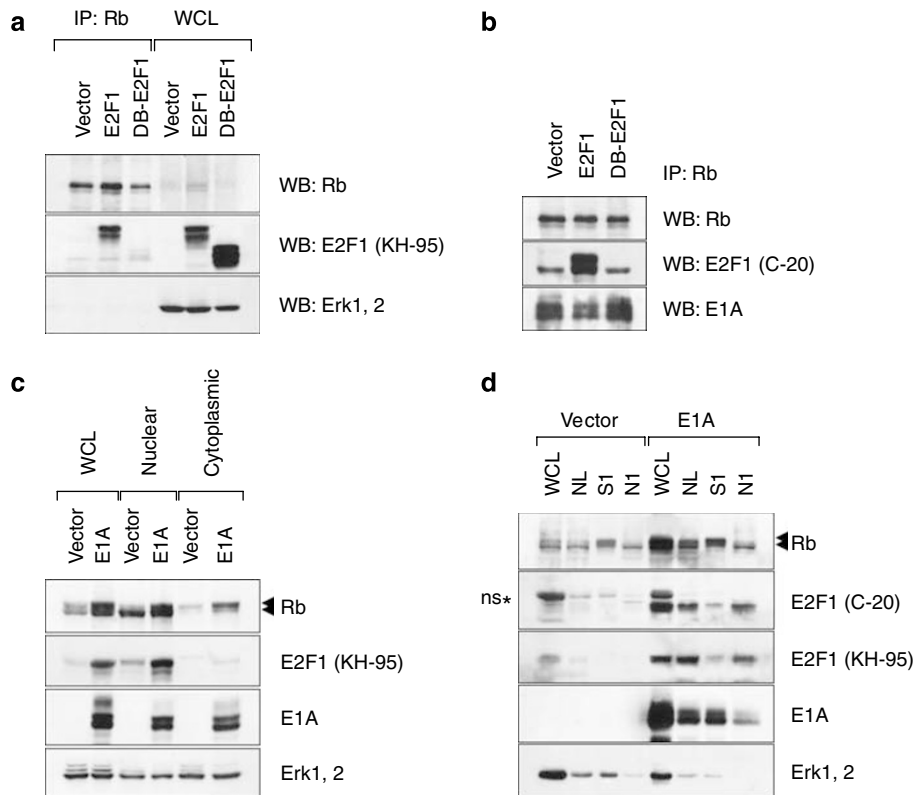


Figure 2 E1A leaves chromatin-bound Rb–E2F1 complexes intact. **(a, b)** E1A-expressing p53^{-/-} MEFs were transduced with vector-, E2F1-, or DB-E2F1-containing retroviral vectors. Immunoprecipitation with Rb-specific mAb, followed by probing with the indicated Abs. Whole cell lysates are shown for equal input. The results are shown before (A) and after normalization for Rb levels (B). **(c)** Immunoblot analysis of whole cell (WCL), nuclear, and cytoplasmic lysates from vector- and E1A-transduced IMR90 cells. Arrows indicate hypo- and hyperphosphorylated Rb. **(d)** Chromatin-rich fraction (non-soluble N1) isolated from nuclei of E1A-expressing IMR90 cells contains higher levels of Rb and E2F1 compared to control-infected cells. Whole cell lysates, nuclear lysates (NL), and soluble nucleoplasmic fractions (S1) are shown for controls. Asterisk indicates a nonspecific band (ns)

shown). Taken together, these data indicate that E2F1 and E1A, depending on their relative abundance, are able to compete with each other for binding to Rb.

Rb associates with E2F1 only when hypophosphorylated.¹³ Immunostaining of E1A-expressing cells revealed that Rb and E2F1 were mainly localized in the nucleus (Supplementary Figure 2A and B). The hypophosphorylated Rb also selectively fractionated with the nuclei of E1A-expressing cells, whereas the phosphorylated forms were present in both the nuclei and cytosol (Figure 2c). Furthermore, chromatin-rich fraction isolated from E1A-expressing cells contained higher levels of both Rb and E2F1 compared to control-infected cells (fraction N1, Figure 2d). By contrast, E1A was predominantly present in the soluble nuclear fraction (Figure 2d and Supplementary Figure 1). Hence, E1A leaves a proportion of chromatin-bound Rb–E2F1 complexes intact.

E1A protects the genomic integrity of primary cells. To assess the functional status of Rb in E1A-expressing cells, we analyzed primary MEFs. Previous studies demonstrated that inactivation of Rb promotes genomic instability of rodent cells.^{14,15} In accord, cultured Rb^{-/-} MEFs showed a gradual shift from diploid to tetraploid populations (4n DNA content; Figure 3a). By contrast, WT MEFs retained diploid status throughout their entire lifespan until the onset of senescence at 9–10 passages (Figure 3b). We next examined E1A-

expressing cells. If E1A were to cause complete Rb inactivation, then E1A-expressing WT MEFs should mimic the phenotype of Rb^{-/-} MEFs also becoming tetraploid. Moreover, E1A expression often selects for cells that have sustained p53 mutations.¹⁶ Because loss of p53 function also impinges on genomic stability,¹⁷ E1A expression should aggravate the defective phenotype of Rb^{-/-} MEFs. However, E1A failed to affect the ploidy of WT or Rb^{-/-} MEFs, even though greatly extending the lifespan of WT cells (Figure 3a and b).

Because both WT and Rb^{-/-} MEFs used in our analyses contained functional p53, as evidenced by its robust induction upon expression of E1A or following exposure of cells to genotoxic drug adriamycin (Supplementary Figure 3A and B), we next examined p53^{-/-} MEFs. As expected, control-infected p53^{-/-} MEFs became hyperploid within 15 ± 2 passages in culture (Figure 3c). In stark contrast, the majority of E1A-expressing p53^{-/-} MEFs maintained their diploid DNA content beyond 15 passages (Figure 3c). These data were confirmed by spectral karyotyping. In control p53^{-/-} MEFs at passage 7, only 20% were diploid, while 60% had chromosome numbers ranging from 75 to 85 corresponding to tetraploid cells (Supplementary Figure 3C). By contrast, more than 60% of E1A-expressing p53^{-/-} MEFs contained diploid chromosome contents (Supplementary Figure 3C). Aneuploidy was also less apparent in E1A-expressing p53^{-/-} MEFs

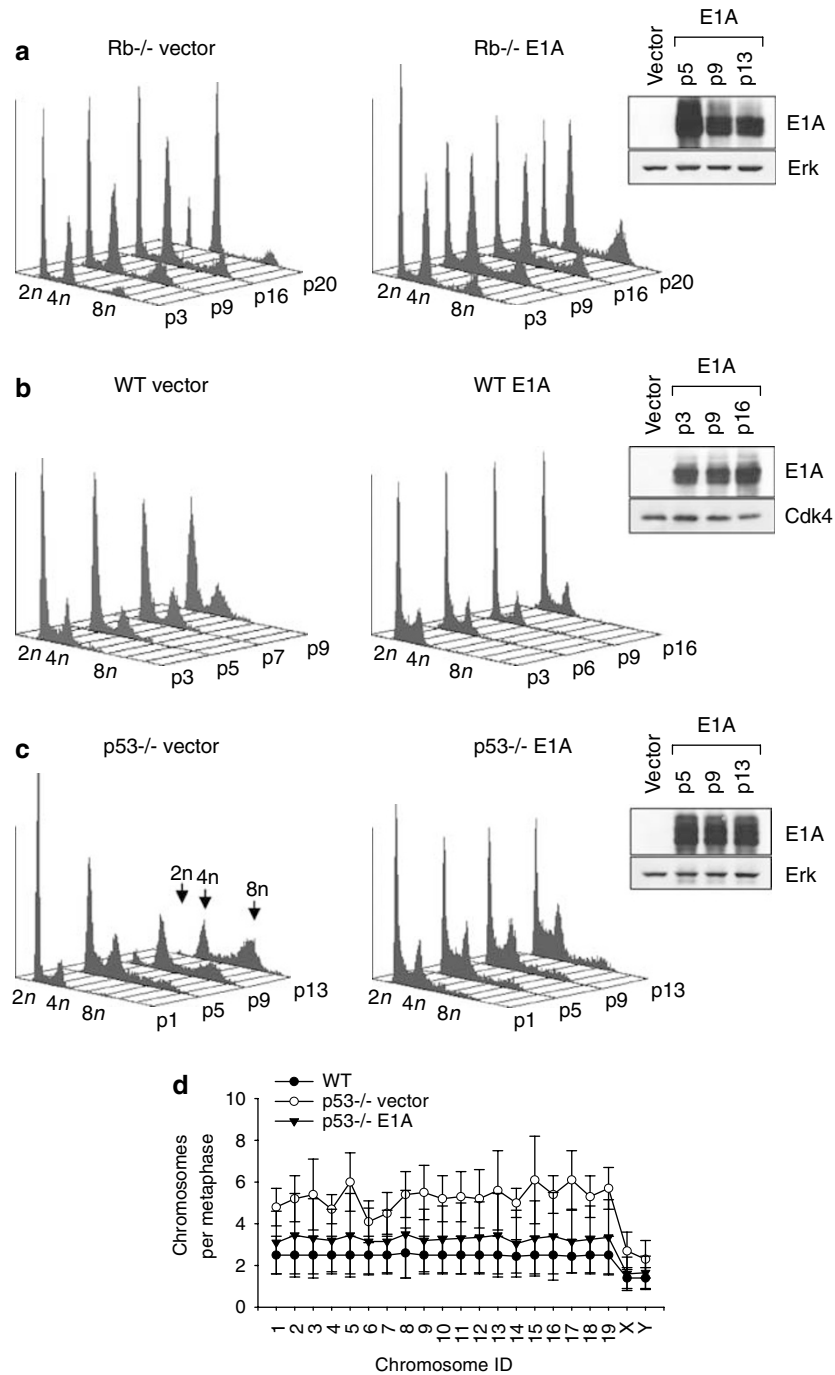


Figure 3 E1A protects the genomic integrity of primary cells. (a-c) DNA histograms of vector- or E1A-transduced Rb^{-/-}, WT, and p53^{-/-} MEFs passed on the 3T3 protocol. 2n, 4n, and 8n DNA content and passage numbers are indicated. Insets show immunoblots of E1A expression in MEFs of the indicated genotypes after 3, 9, and 13 passages in culture. Erk is the loading control. (d) Chromosome content of WT, vector-transduced p53^{-/-}, and E1A-transduced p53^{-/-} MEFs at passage 7. Chromosome identity is indicated. Error bars represent the standard deviation

compared to controls (Figure 3d). In addition, E1A-expressing p53^{-/-} MEFs were predominantly mononucleated and showed no obvious centrosome abnormalities (Supplementary Figure 3D). These data indicate that E1A protects genomic integrity of primary cells. In doing so, E1A engages an Rb-dependent mechanism that partly substitutes for the loss of p53 function.

Rb and E2F1 cooperate in maintaining the diploidy of cells. Rb regulates the cell cycle in part by binding to promoters of E2F-responsive genes and through LxCxE motif-dependent interaction with proteins involved in chromatin remodeling.^{18,19} To assess the role of these interactions in Rb function and genome stabilization, we next examined MEFs in which WT Rb alleles were

substituted by an LxCxE-binding defective Rb Δ L mutant²⁰ or E2F-binding defective Rb R654W mutant.²¹ The former mutation allows Rb to function as a cell cycle regulator via association with E2Fs, while being resistant to interaction with E1A.²⁰ Indeed, WT and Rb Δ L/ Δ L MEFs senesced after 7–8 passages in culture, whereas Rb $^{-/-}$ MEFs showed no features of senescence and grew indefinitely (Figure 4a). Expression of E1A rendered Rb Δ L/ Δ L MEFs effectively immortal, but failed to affect the ploidy of cells (Figure 4a and b). Notably, Rb–E2F1 complexes were more abundant in E1A-expressing Rb Δ L/ Δ L cells than in control-infected cells (Figure 4c). Furthermore, silencing Rb expression by short hairpin RNA (shRNA) impaired the ability of E1A to maintain diploidy of Rb Δ L/ Δ L MEFs (Figure 4d and Supplementary Figure 4A), whereas reducing levels of p53 had no similar effect on ploidy of Rb Δ L/ Δ L cells (Figure 4e and Supplementary Figure 4A). E1A also failed to block accumulation of hyperploid p53 $^{-/-}$ E2F1 $^{-/-}$ MEFs

(Supplementary Figure 4B) and Rb R654W MEFs (Supplementary Figure 4C). These data imply that E1A-induced cellular immortalization does not depend on full Rb inactivation. Moreover, both Rb and E2F1 are required components of E1A-activated checkpoints that protect the ploidy of cells.

E1A causes Rb stabilization by interfering with its proteasomal degradation. We next assessed the mechanisms by which E1A causes Rb stabilization and ploidy maintenance. It was shown that Rb stabilizes E2F1, protecting it from proteasomal degradation,^{22,23} and E1A achieves a similar stabilization of E2F1.²⁴ E1A binds to and interferes with the activity of 26S proteasome.^{25–27} While the loss of CR2 domain disables binding of E1A to both Rb and the S2 proteasomal subunit, point mutations within the LxCxE Rb-binding site of CR2 disable E1A’s binding to Rb but do not affect its capacity to bind S2.²⁷ Consistently,

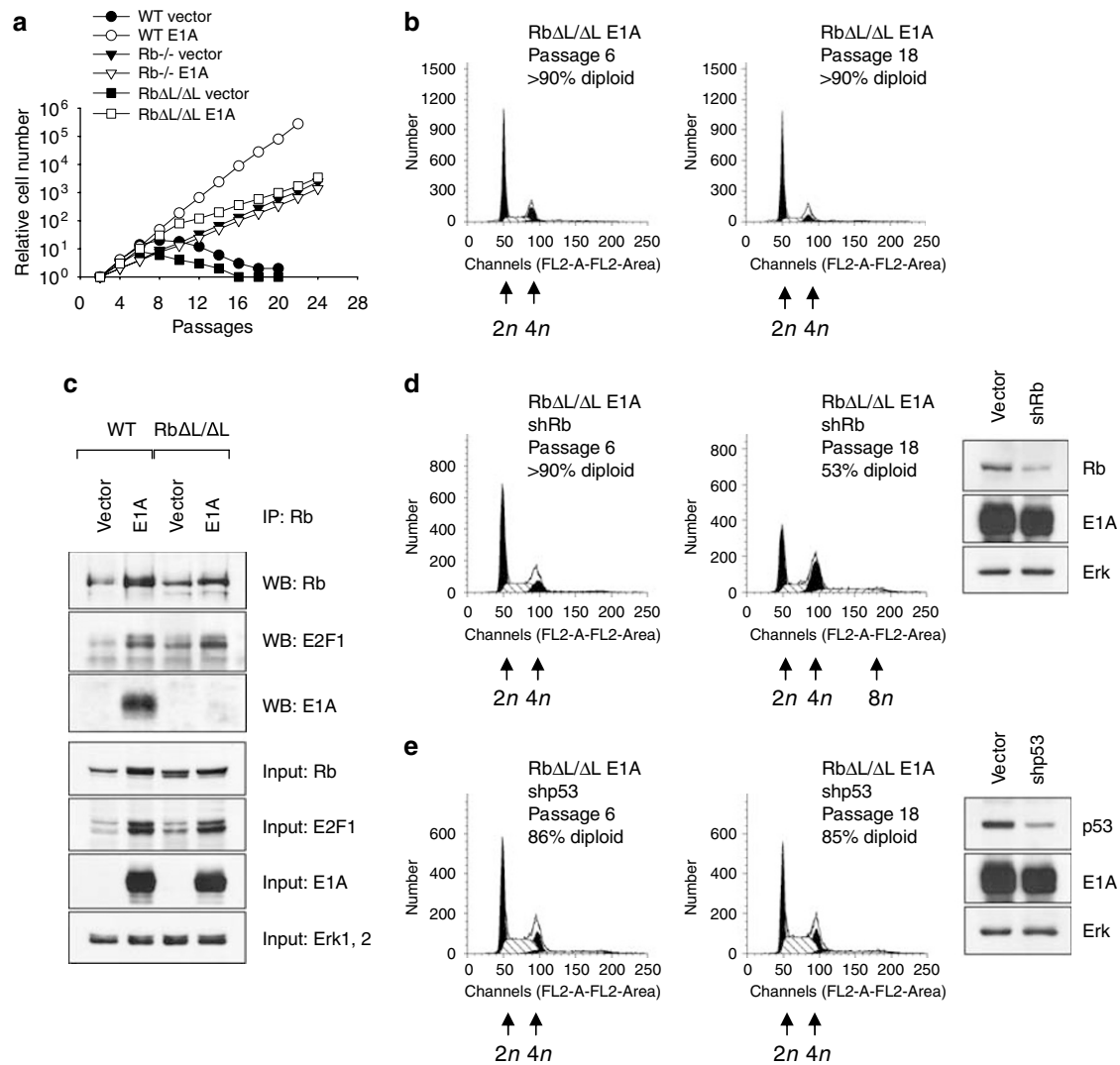


Figure 4 Rb and E2F1 cooperate in maintaining the diploidy of cells. (a) The growth curves of vector- or E1A-transduced MEFs of the indicated genotypes. (b) DNA histograms of E1A-transduced Rb Δ L/ Δ L MEFs passaged on the 3T3 protocol. (c) Composition of Rb–E2F1 complexes in whole cell lysates of vector- or E1A-transduced WT and Rb Δ L/ Δ L MEFs. Immunoblots of Rb, E2F1 and E1A expression are shown for input control. DNA histograms of E1A-expressing Rb Δ L/ Δ L MEFs transduced with Rb-specific (d) or p53-specific shRNAs (e) and then passaged on the 3T3 protocol. Insets show immunoblots of Rb, p53, and E1A expression. Erk is the loading control

expression of shRNAs specific for Rb reduced the levels of both Rb and E2F1 (Supplementary Figure 5A). However, RT-PCR analysis of Rb and E2F1 expression failed to detect any E1A-dependent differences, suggesting a post-transcriptional cause (Supplementary Figure 5B).

To explore the mechanism by which E1A causes Rb stabilization, we used previously characterized E1A deletion mutants (Supplementary Figure 5C). The Δ N E1A mutant carrying a deletion within the N-terminal region was as effective as WT E1A in causing stabilization of Rb (Figure 5a) concurrent with increased accumulation of Rb–E2F1 complexes (Figure 5b). By contrast, the Δ CR1 mutant was only partially able to cause Rb stabilization, while the Δ CR2 mutant was completely devoid of this capacity (Figure 5a and b). On the other hand, the E1A point mutant pm47/124 (which carries a mutation in the LxCxE motif²⁸), while unable to bind Rb, still caused its significant accumulation (Figure 5a). The LxCxE-binding-deficient Rb Δ L mutant was also stabilized by E1A (see Figure 4c). Thus, E1A-mediated Rb stabilization does not require direct binding of E1A to Rb but appears to depend on its binding to proteasomal S2.

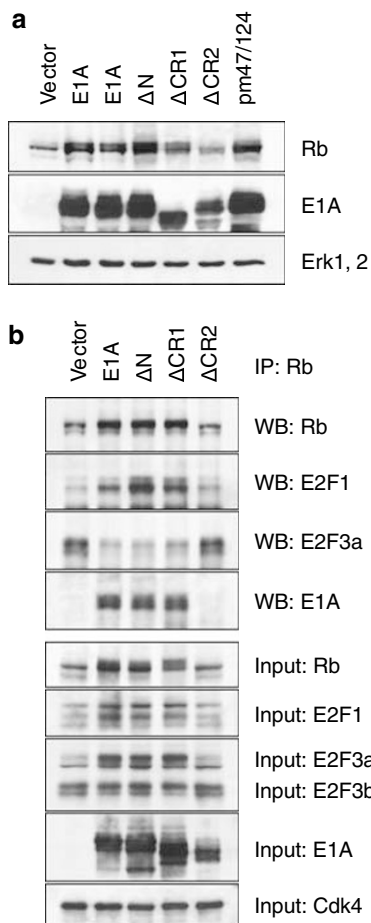


Figure 5 E1A causes Rb stabilization by interfering with its proteasomal degradation. (a) Immunoblot analysis of Rb expression in p53^{-/-} MEFs transduced with E1A or indicated E1A mutants. (b) Immunoprecipitation analysis of p53^{-/-} MEFs transduced with wild-type E1A or E1A deletion mutants. Total cell lysates were immunoprecipitated with Rb-specific mAb and probed with the indicated Abs

Because Rb protects E2F1 from proteasomes,^{22–24} this also explains why Rb and E2F1 are co-stabilized in E1A-expressing cells.

E1A activates an Rb-dependent G1/S-phase checkpoint that protects diploidy.

Polyploidy and subsequent aneuploidy can be achieved by several means that uncouple DNA replication from the cell cycle checkpoint controls.²⁹ Thus, damage to the mitotic spindle activates the mitotic checkpoint and arrests cells transiently in metaphase. However, after prolonged exposure to spindle inhibitors an initial mitotic delay is followed by slippage through mitosis, producing tetraploid cells. At this point, a second p53-dependent checkpoint prevents cells from further replicating their DNA.¹⁷ To assess a possible role of E1A in these processes, control- and E1A-infected MEFs were treated with spindle inhibitor nocodazole for 12 h to allow accumulation of polyploid populations. The cells were then withdrawn from nocodazole for different time periods, and their DNA content was analyzed by flow cytometry.

As expected, nocodazole-treated WT MEFs regained their diploid status within 3 days of drug withdrawal (Supplementary Figure 6A), while a large proportion of similarly treated p53^{-/-} MEFs continued to proliferate as tetraploid populations (Figure 6a). In stark contrast, the majority of E1A-expressing p53^{-/-} MEFs restored their diploid status within 6 h of nocodazole withdrawal (Figure 6a). Rb silencing blocked the ability of E1A to maintain diploid state in nocodazole-treated p53^{-/-} cells (Supplementary Figure 6B), confirming the correlation between E1A expression and Rb function. In all cases examined, nocodazole did not induce cell death (Figure 6a and data not shown). However, BrdU incorporation assays revealed an E1A-dependent reduction in the proportion of p53^{-/-} MEFs that entered S phase following nocodazole treatment (Figure 6b). This was paralleled by a decrease in the levels of G1/S-specific markers cyclins D1 and E, which were consistently lower in E1A-expressing p53^{-/-} MEFs than in controls (Figure 6c).

We next set out to determine if E1A affects mitosis proper. To this end, we used p53^{-/-} MEFs stably expressing GFP-tagged histone H2B, which allows direct visualization of mitotic progression without compromising nuclear or chromosomal structures.³⁰ In these experiments, cells were treated with nocodazole as outlined above and then released into drug-free media or into media containing S-phase inhibitor hydroxyurea (Figure 6d). Scoring mitotic indexes confirmed that only a minority of nocodazole-treated p53^{-/-} MEFs resumed mitosis upon withdrawal from the drug (Figure 6e). On the other hand, most p53^{-/-} cells have undergone mitosis within 2 h of nocodazole withdrawal when released into media containing hydroxyurea (Figure 6e). Furthermore, mitotic progression of E1A-expressing p53^{-/-} MEFs was almost as efficient as that of hydroxyurea-treated controls (Figure 6f). Thus, p53^{-/-} MEFs are fully competent to undergo at least one round of compensatory mitosis upon nocodazole withdrawal. Imposing an S-phase block upon such p53^{-/-} MEFs allows them to restore diploidy and hence to mimic E1A-expressing cells. Therefore, we conclude that E1A does not perturb checkpoint function at mitosis. Instead, E1A activates an Rb-dependent G1/S-phase checkpoint that

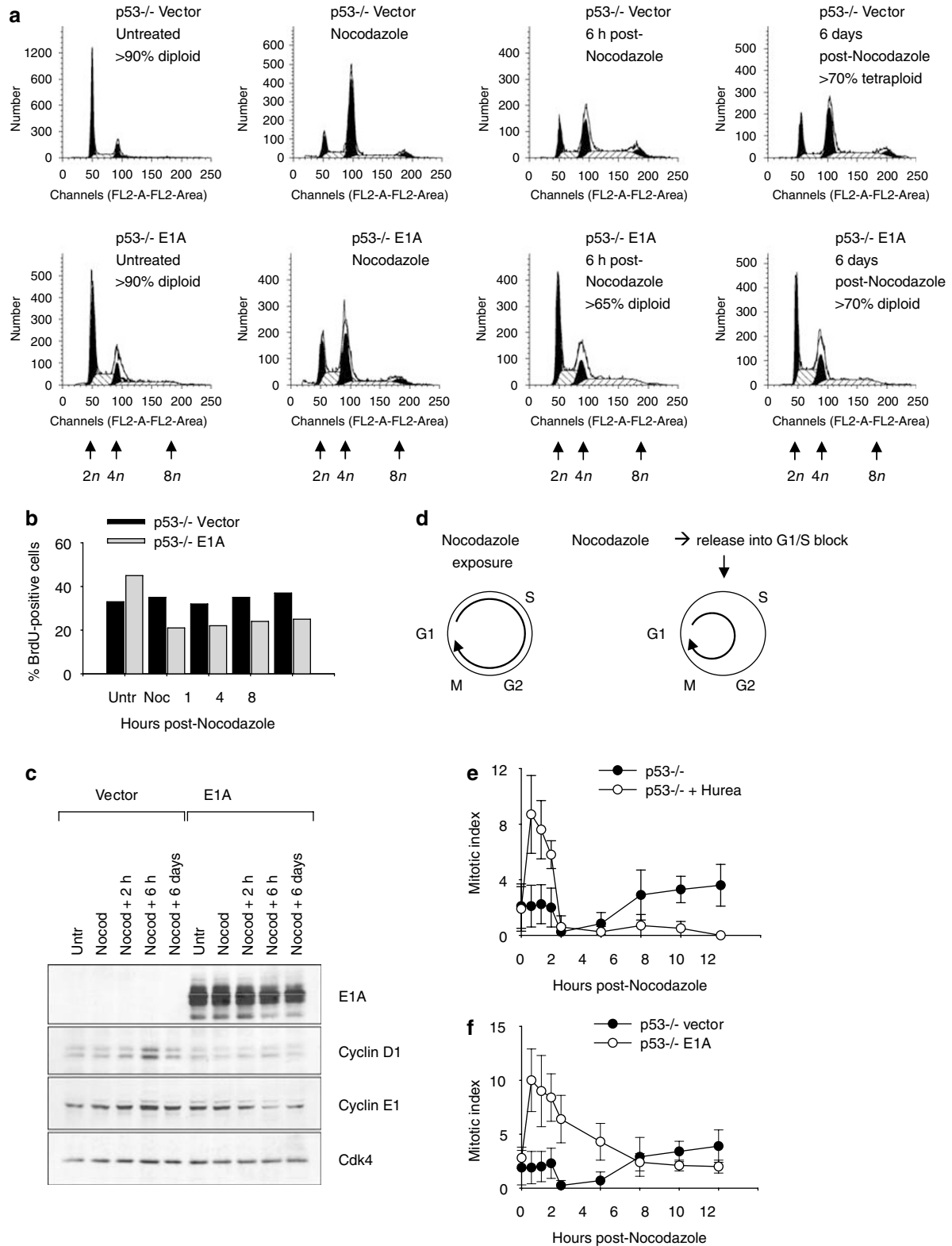


Figure 6 E1A activates an Rb-dependent G1/S-phase checkpoint that protects diploidy. (a) DNA histograms of p53^{-/-} MEFs transduced with vector alone or E1A-expressing retroviruses. Cells were treated with nocodazole for 12 h and then released into nocodazole-free media for 6 h and 6 days. (b) BrdU incorporation assays of vector alone- or E1A-transduced p53^{-/-} MEFs treated as in (a). Cells were pulse-labeled with BrdU for 1 h. (c) Immunoblot analysis of control- or E1A-transduced p53^{-/-} MEFs treated as in (a). (d) Nocodazole-treated tetraploid cells can undergo forced reduction mitosis when released into media containing S-phase inhibitor hydroxyurea. (e) Mitotic indices of GFP-H2B-marked p53^{-/-} MEFs treated for 12 h with nocodazole, and then incubated in the absence or presence of 0.5 mM hydroxyurea for the indicated times. At least 1000 cells were counted for each analysis. (f) Mitotic indices of GFP-H2B-marked p53^{-/-} MEFs transduced with vector alone or E1A. Cells were treated with nocodazole as in (e)

prevents hyperploid p53^{-/-} cells from entering the cell cycle and initiating another round of DNA synthesis.

S-phase DNA damage checkpoint is constitutively active in E1A-expressing cells. Because E2F1 loss abrogated the tetraploidy resistance of E1A-expressing cells, we set out

to show that this phenotypic reversion correlates with the expression of E2F target genes. Initially, we focused our efforts on Cdc6 and the Mcm helicases because of their critical involvement in DNA replication. Analysis of these E2F-responsive genes indeed showed E1A-dependent transcriptional activation at various stages of the cell cycle

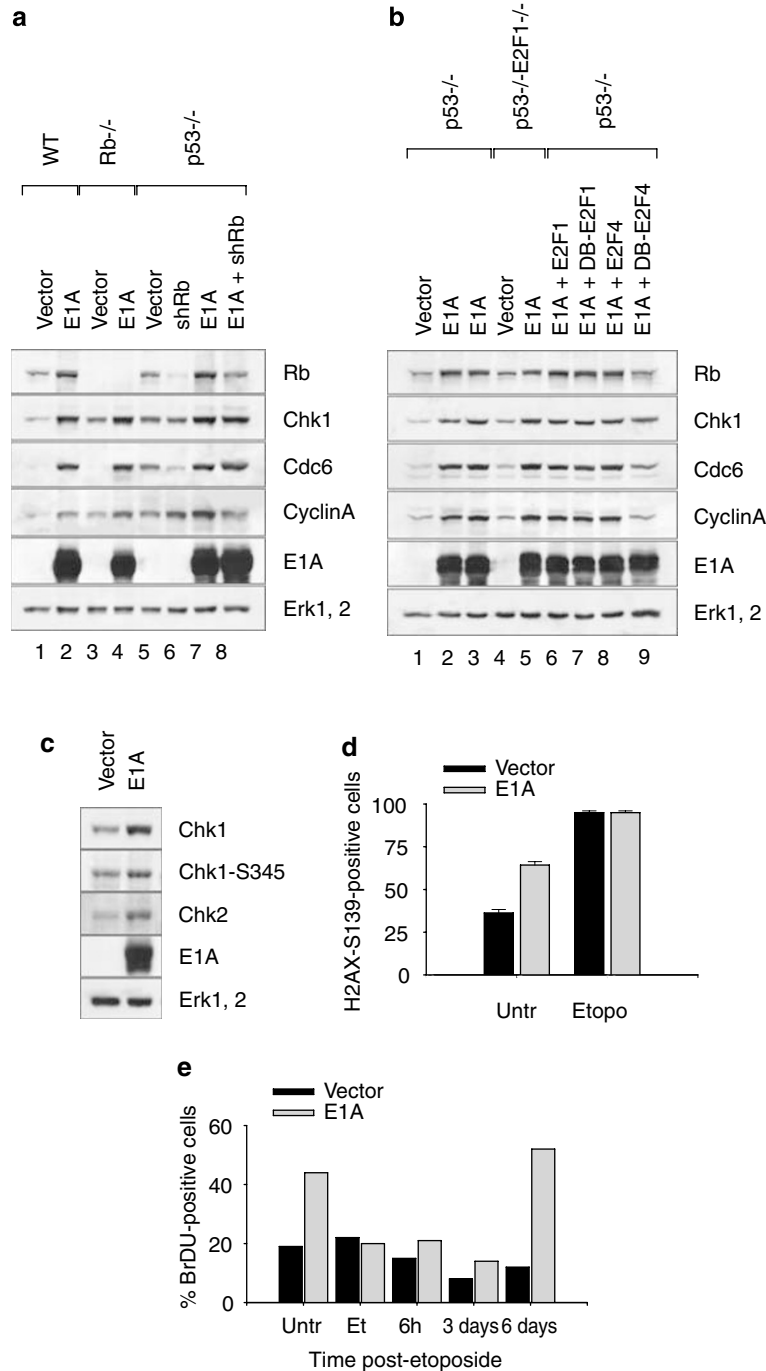


Figure 7 S-phase DNA damage checkpoint is constitutively active in E1A-expressing cells. (a) Immunoblot analysis of WT, Rb^{-/-}, and p53^{-/-} MEFs infected with vector-, E1A-, or shRb-containing retroviruses. (b) Immunoblot analysis of p53^{-/-} and p53^{-/-}E2F1^{-/-} MEFs transduced with vector-, E1A-, E2F1-, DB-E2F1-, E2F4-, or DB-E2F4-containing retroviruses. (c) Immunoblot analysis of Chk1 and Chk2 expression in vector- or E1A-transduced p53^{-/-} MEFs. (d) Induction of histone H2AX-S139 phosphorylation in vector- or E1A-transduced p53^{-/-} MEFs. Cells were incubated in the presence or absence of 2 μM etoposide (4-h treatment), fixed in ethanol, stained with PI and H2AX-S139 Ab, and then analyzed by flow cytometry. Error bars represent the standard error. (e) BrdU incorporation assays of vector alone- or E1A-transduced p53^{-/-} MEFs treated as in (d). Cells were pulse-labeled with BrdU for 2 h

(Figure 7a and b and Supplementary Figure 7A and B). However, we failed to detect any Rb- or E2F1-dependent differences. Thus, all E2F-responsive genes were equally upregulated in E1A-expressing MEFs of different Rb genotypes (Figure 7a). Likewise, E2F target genes were upregulated in E1A-transduced p53^{-/-} MEFs of different E2F1 genotypes (Figure 7b and Supplementary Figure 7A–C). By contrast, co-expression of a dominant-negative DB-E2F4 mutant weakened E1A-dependent upregulation of Rb, Cdc6, and cyclin A (Figure 7b). Consistent with these data, it was shown that E1A promotes entry into S phase by overcoming p130/E2F4-mediated repression of E2F target genes.³¹ In sum, these results indicate that E1A protects the ploidy of cells despite continuous upregulation of E2F-responsive S-phase genes.

It was shown that Rb acts as a downstream target for ATR that is important for the inhibition of DNA rereplication, particularly upon conditions of DNA damage.^{32–34} We found that expression of E1A causes increase in protein levels of checkpoint kinases Chk1 and Chk2, which also play a pivotal role in regulating cell cycle arrest upon DNA damage or stalled replication (Figure 7c). Levels of phosphorylation-activated Chk1-S345, H2AX-S139, and Rb-S612 (markers of a persistent DNA damage response) were also constitutively elevated in E1A-expressing cells (Figure 7d and Supplementary Figure 7D). BrdU labeling of etoposide-treated MEFs revealed a sharply reduced S phase in E1A-transduced p53^{-/-} cells compared to control-infected cells (Figure 7e), thus indicating that the S-phase DNA damage checkpoint is more responsive in E1A-expressing cells.

E1A associates with a large number of cellular proteins that are critical for normal cell cycle progression (see Supplementary Figure 5C). To identify the underlying mechanism by which E1A blocks proliferation of polyploid cells, we performed additional mutational analysis. Remarkably, all N-terminal E1A mutants unable to bind p300/CBP or p400/TRRAP were nearly as effective as WT E1A in blocking the proliferation of tetraploid p53^{-/-} MEFs (Supplementary Figure 8). By contrast, E1A mutants lacking the CR1 or CR2 domains were impaired in this capacity (Supplementary Figure 8). As outlined above, the CR2 mutant does not induce Rb stabilization. In contrast, the defect of the CR1 mutant is its inability to compete with Rb-bound E2Fs, forming instead triple complexes with Rb and E2Fs,¹¹ and this contributes to genomic instability, as shown above.

Discussion

Adenoviral E1A is believed to exert its influence on host cells by globally disrupting Rb–E2F interactions via the CR2 and CR1 domains.^{4–6,35} Our study challenges this simple paradigm by demonstrating previously unappreciated complexity. We show that E1A-expressing primary and transformed cells are characterized by the persistence of Rb–E2F1 complexes. We find that the augmented function of Rb–E2F1 plays a significant role in E1A-induced phenotype. As proteolysis is impaired in E1A-expressing cells, Rb and E2F1 proteins are increased, leading to an increase in functional Rb–E2F1 complexes, which are required for the genomic stabilization found in cells expressing E1A.

The opposing properties of E1A to activate p53, but at the same time to promote cellular immortalization and oncogenic transformation have long been difficult to reconcile. However, recent studies showed that concomitant inactivation of the Rb family proteins or acute interference with E2F-mediated transcriptional regulation also immortalizes cells despite the presence of functional p53.^{14,15,36,37} Given that E1A effectively disrupts Rb–E2F2-4 and p107/p130–E2F4 complexes, we reasoned that E1A-induced immortalization depends primarily upon the disruption of these repressive complexes of Rb, p107, and p130. In contrast, inactivation of Rb–E2F1 is not a prerequisite for cellular immortalization, at least in the context of E1A expression. Instead, Rb controls progression through S phase and may be required for regulation of DNA replication, particularly in response to DNA damage.^{32,34}

Studies to date on E1A have focused on its role as a transforming oncogene in rodent cells. A popular model for how Rb family members regulate G1/S-phase-specific gene expression predicts a complex pattern of protein–protein and protein–DNA interactions that change as cells progress through the cell cycle.^{18,33} In addition, the Rb and E2F family members are involved in stable gene repression, indicating that Rb–E2F complexes are able to mediate different types of regulation under different circumstances.¹⁹ The situation may be even more complex in that a cooperative regulation of E2F-responsive genes by several Rb family proteins may be required.³⁸ A unifying theme emerging from these observations is that Rb exerts its cellular functions by controlling activities that involve nucleosome phasing. Because chromatin structure has a well-documented significance in both gene expression and DNA replication, one can envision that Rb–E2F1 will function as regulators of transcription or regulators of replication depending upon the availability of cofactors that can influence their DNA-binding specificity. These chromatin changes can be implemented by recruiting histone deacetylases, histone methyltransferases, DNA methyltransferases, SWI/SNF complex members, and polycomb proteins.^{18,19}

Although these data point out a potential mechanism by which cell cycle control and epigenetic changes may be linked with the genome stabilization, further studies are required to characterize the components of Rb- and E2F1-based chromatin-remodeling complexes in E1A-expressing cells. More importantly, would such genome-surveillance complexes be seen at any time in the normal cell cycle, or in DNA-damaged cells? Finally, is it possible to specifically induce the formation of such complexes in the absence of E1A expression? Because E1A reverses the transformed phenotype of many human cancer cells,⁶ answering these questions could make it feasible to maximize both the potency and extent of Rb's engagement within the tumor cells and to optimize the therapeutic benefit of its activation afterwards.

Materials and Methods

Cells and tissue culture. HEK293 and IMR90 cells were maintained in DMEM supplemented with 10% FBS and antibiotics. Where indicated, cells were treated with 0.5 mM L-mimosine, 0.5 μg/ml aphidicolin, 0.5 mM hydroxyurea, 2 μM VM26, 1 μM etoposide, or 0.12 μg/ml nocodazole (all from Sigma). WT, p53^{-/-}, and p53^{-/-}E2F1^{-/-} MEFs were derived from day 13.5 embryos (all on the 129S1/SvImJ genetic background). Rb^{-/-} MEFs were a gift from G Leone (Ohio State University, Columbus, OH, USA). Rb R654W MEFs were a gift from DW

Goodrich (Roswell Park Cancer Institute, Buffalo, NY, USA). Rb Δ L/ Δ L MEFs were a gift from F Dick (University of Western Ontario, London, ON, Canada).

Retroviral vectors. We used replication-defective retroviral vectors encoding E1A12S, human E2F1, E2F4, or DB-E2F1 and DB-E2F4 mutants as described previously.¹² Additional retroviral vectors encoding E1A and mutants Δ 2-11, Δ N2-36, Δ 26-35, Δ CR1, Δ CR2, and pm47/124 were a gift from S Lowe (Cold Spring Harbor Laboratory); retroviral plasmid encoding shRNA against Rb was a gift from J DeGregori (University of Colorado at Denver and Health Sciences Center).

Expression analysis. For immunoprecipitation, cells were resuspended in buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton, 1 mM EDTA, protease inhibitor cocktail (Roche), and lysed by freezing and thawing. The lysates were precleared by the addition of protein G/A-Sepharose (Amersham) and centrifugation. Here, 1–2 mg of total cell lysate (1 ml) was incubated with antibodies (1 μ g) and 40 μ l of 50% protein G/A-Sepharose. Immunoprecipitates were washed four times in the lysis buffer, solubilized by boiling in Laemmli buffer, and subjected to SDS-polyacrylamide gel electrophoresis. Chromatin binding by Rb and E2F1 was examined as described.³⁹

Cytogenetic analyses. For immunofluorescence, cells were plated on cover slips, fixed in 4% paraformaldehyde and stained with antibodies specific for Rb (554136; Pharmingen), E2F1 (KH-95; Santa Cruz), γ -tubulin (GTU-88; Sigma), followed by TRITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories), and counterstaining with Hoechst 33342. For cell cycle analysis, trypsinized cells were fixed in 70% ethanol, stained with propidium iodide (Sigma), and analyzed using FACS Calibur (Becton-Dickinson) and ModFit LT software (Verity). Spectral karyotyping was performed at the Roswell Park Cancer Institute SKY/FISH facility.

Acknowledgements. This study was supported by the Long Island Cancer Center (OP), New York State Department of Health Research Science Board, and M Carol Baldwin Breast Cancer Research Award (UMM). We have no conflicting financial interests.

Note added in proof. While this paper was in preparation, Seifried *et al.*⁴⁰ showed that Rb–E2F1 complexes are resistant to E1A-mediated disruption.

1. Van der Eb AJ, Mulder C, Graham FL, Houweling A. Transformation with specific fragments of adenovirus DNAs. I. Isolation of specific fragments with transforming activity of adenovirus 2 and 5 DNA. *Gene* 1977; **2**: 115–132.
2. Ruley HE. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. *Nature* 1983; **304**: 602–606.
3. Matlashewski G, Schneider J, Banks L, Jones N, Murray A, Crawford L. Human papillomavirus type 16 DNA cooperates with activated ras in transforming primary cells. *EMBO J* 1987; **6**: 1741–1746.
4. Whyte P, Williamson NM, Harlow E. Cellular targets for transformation by the adenovirus E1A proteins. *Cell* 1989; **56**: 67–75.
5. Nevins JR. E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. *Science* 1992; **258**: 424–429.
6. Frisch SM, Mymryk JS. Adenovirus-5 E1A: paradox and paradigm. *Nat Rev Mol Cell Biol* 2002; **3**: 441–452.
7. Bandara LR, La Thangue NB. Adenovirus E1a prevents the retinoblastoma gene product from complexing with a cellular transcription factor. *Nature* 1991; **351**: 494–497.
8. Bagchi S, Weinmann R, Raychaudhuri P. The retinoblastoma protein copurifies with E2F-1, an E1A-regulated inhibitor of the transcription factor E2F. *Cell* 1991; **65**: 1063–1072.
9. Chellappan SP, Hiebert S, Mudryj M, Horowitz JM, Nevins JR. The E2F transcription factor is a cellular target for the RB protein. *Cell* 1991; **65**: 1053–1061.
10. Cao L, Faha B, Dembski M, Tsai LH, Harlow E, Dyson N. Independent binding of the retinoblastoma protein and p107 to the transcription factor E2F. *Nature* 1992; **355**: 176–179.
11. Fattaey AR, Harlow E, Helin K. Independent regions of adenovirus E1A are required for binding to and dissociation of E2F–protein complexes. *Mol Cell Biol* 1993; **13**: 7267–7277.
12. Petrenko O, Moll UM. Macrophage migration inhibitory factor MIF interferes with the Rb–E2F pathway. *Mol Cell* 2005; **17**: 225–236.

13. Mitnacht S, Weinberg RA. G1/S phosphorylation of the retinoblastoma protein is associated with an altered affinity for the nuclear compartment. *Cell* 1991; **65**: 381–393.
14. Dannenberg JH, van Rossum A, Schuijff L, te Riele H. Ablation of the retinoblastoma gene family deregulates G(1) control causing immortalization and increased cell turnover under growth-restricting conditions. *Genes Dev* 2000; **14**: 3051–3064.
15. Sage J, Mulligan GJ, Attardi LD, Miller A, Chen S, Williams B *et al*. Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. *Genes Dev* 2000; **14**: 3037–3050.
16. de Stanchina E, McCurrach ME, Zindy F, Shieh SY, Ferbeyre G, Samuelson AV *et al*. E1A signaling to p53 involves the p19(ARF) tumor suppressor. *Genes Dev* 1998; **12**: 2434–2442.
17. Cross SM, Sanchez CA, Morgan CA, Schimke MK, Ramel S, Idzerda RL *et al*. A p53-dependent mouse spindle checkpoint. *Science* 1995; **267**: 1353–1356.
18. Harbour JW, Dean DC. The Rb/E2F pathway: expanding roles and emerging paradigms. *Genes Dev* 2000; **14**: 2393–2409.
19. Frolov MV, Dyson NJ. Molecular mechanisms of E2F-dependent activation and pRB-mediated repression. *J Cell Sci* 2004; **117**: 2173–2181.
20. Isaac CE, Francis SM, Martens AL, Julian LM, Seifried LA, Erdmann N *et al*. The retinoblastoma protein regulates pericentric heterochromatin. *Mol Cell Biol* 2006; **26**: 3659–3671.
21. Sun H, Chang Y, Schweers B, Dyer MA, Zhang X, Hayward SW *et al*. An E2F binding-deficient Rb1 protein partially rescues developmental defects associated with Rb1 nullizygosity. *Mol Cell Biol* 2006; **26**: 1527–1537.
22. Hofmann F, Martelli F, Livingston DM, Wang Z. The retinoblastoma gene product protects E2F-1 from degradation by the ubiquitin–proteasome pathway. *Genes Dev* 1996; **10**: 2949–2959.
23. Campanero MR, Flemington EK. Regulation of E2F through ubiquitin–proteasome-dependent degradation: stabilization by the pRB tumor suppressor protein. *Proc Natl Acad Sci USA* 1997; **94**: 2221–2226.
24. Hateboer G, Kerkhoven RM, Shvarts A, Bernards R, Beijersbergen RL. Degradation of E2F by the ubiquitin–proteasome pathway: regulation by retinoblastoma family proteins and adenovirus transforming proteins. *Genes Dev* 1996; **10**: 2960–2970.
25. Nakajima T, Morita K, Tsunoda H, Imajoh-Ohmi S, Tanaka H, Yasuda H *et al*. Stabilization of p53 by adenovirus E1A occurs through its amino-terminal region by modification of the ubiquitin–proteasome pathway. *J Biol Chem* 1998; **273**: 20036–20045.
26. Turnell AS, Grand RJ, Gorbea C, Zhang X, Wang W, Mymryk JS *et al*. Regulation of the 26S proteasome by adenovirus E1A. *EMBO J* 2000; **19**: 4759–4773.
27. Zhang X, Turnell AS, Gorbea C, Mymryk JS, Gallimore PH, Grand RJ. The targeting of the proteasomal regulatory subunit S2 by adenovirus E1A causes inhibition of proteasomal activity and increased p53 expression. *J Biol Chem* 2004; **279**: 25122–25133.
28. Samuelson AV, Lowe SW. Selective induction of p53 and chemosensitivity in RB-deficient cells by E1A mutants unable to bind the RB-related proteins. *Proc Natl Acad Sci USA* 1997; **94**: 12094–12099.
29. Kops GJ, Weaver BA, Cleveland DW. On the road to cancer: aneuploidy and the mitotic checkpoint. *Nat Rev Cancer* 2005; **5**: 773–785.
30. Kanda T, Sullivan KF, Wahl GM. Histone-GFP fusion protein enables sensitive analysis of chromosome dynamics in living mammalian cells. *Curr Biol* 1998; **8**: 377–385.
31. Ghosh MK, Harter ML. A viral mechanism for remodeling chromatin structure in G0 cells. *Mol Cell* 2003; **12**: 255–260.
32. Avni D, Yang H, Martelli F, Hofmann F, Elshamy WM, Ganesan S *et al*. Active localization of the retinoblastoma protein in chromatin and its response to S phase DNA damage. *Mol Cell* 2003; **12**: 735–746.
33. Cam H, Dynlacht BD. Emerging roles for E2F: beyond the G1/S transition and DNA replication. *Cancer Cell* 2003; **3**: 311–316.
34. Kennedy BK, Barbie DA, Classon M, Dyson N, Harlow E. Nuclear organization of DNA replication in primary mammalian cells. *Genes Dev* 2000; **14**: 2855–2868.
35. Chowdhury D, Keogh MC, Ishii H, Peterson CL, Buratowski S, Lieberman J. Gamma-H2AX dephosphorylation by protein phosphatase 2A facilitates DNA double-strand break repair. *Mol Cell* 2005; **20**: 801–809.
36. Peeper DS, Dannenberg JH, Douma S, te Riele H, Bernards R. Escape from premature senescence is not sufficient for oncogenic transformation by Ras. *Nat Cell Biol* 2001; **3**: 198–203.
37. Rowland BD, Bernards R. Re-evaluating cell-cycle regulation by E2Fs. *Cell* 2006; **127**: 871–874.
38. Wells J, Boyd KE, Fry CJ, Bartley SM, Farnham PJ. Target gene specificity of E2F and pocket protein family members in living cells. *Mol Cell Biol* 2000; **20**: 5797–5807.
39. Mendez J, Stillman B. Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplicative complexes in late mitosis. *Mol Cell Biol* 2000; **20**: 8602–8612.
40. Seifried LA, Talluri S, Cecchini M, Julian LM, Mymryk JS, Dick FA. pRB–E2F1 complexes are resistant to adenovirus E1A-mediated disruption. *J Virol* 2008 (in press).

Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)