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Induction of DNA synthesis and apoptosis by regulated inactivation of a temperature-sensitive retinoblastoma protein

Frank Tiemann and Philip W.Hinds[1]

Department of Pathology, Harvard Medical School, Boston, MA 02115, USA

[1]Corresponding author
e-mail: phinds@warren.med.harvard.edu

The retinoblastoma protein, pRb, controls entry into the S phase of the cell cycle and acts as a tumor suppressor in many tissues. Re-introduction of pRb into tumor cells lacking this protein results in growth arrest, due in part to transcriptional repression of genes required for S phase. Several studies suggest that pRb may also be involved in terminal cell cycle exit as a result of the instigation of senescence or differentiation programs. To understand better these multiple growth-inhibitory properties of pRb, a temperature-sensitive mutant of pRb has been produced. This tspRb induces G_1 arrest and morphological changes efficiently at the permissive temperature of 32.5°C, but is weakly functional at 37°C. Consistent with this, tspRb is compromised in nuclear association and E2F regulation at the non-permissive temperature, but regains these properties at 32.5°C. Serial activation and inactivation of tspRb in SAOS-2 cells does not allow proliferation, but rather leads to apoptotic cell death. Transient activation of pRb may kill tumor cells by establishing a conflict between persistent proliferation-inhibitory signals and renewed deregulation of pRb targets such as E2F, and may thus be a more potent means of eliminating these cells than through simple re-introduction of the tumor suppressor gene.
Keywords: apoptosis/E2F/growth suppression/pRb/temperature-sensitive mutant

Introduction

The retinoblastoma susceptibility protein, pRb, functions in a cell cycle control pathway that regulates passage of cells through G_1 and into S phase (Weinberg, 1995). pRb exerts its function in part by controlling a family of heterodimeric transcription factors, collectively referred to as E2F, that can transactivate genes important for the G_1 to S phase transition. Hypophosphorylated pRb binds to E2F, resulting in a transcriptional repressor complex that constrains expression of E2F target genes. Phosphorylation of pRb in mid to late G_1 releases E2F from the complex, which now can be transcriptionally active (Weinberg, 1995; Sherr, 1996). Targeted deletion of the E2F-1 gene suggests that E2F-1 is necessary for the proliferation of some cells, but actually acts as a tumor suppressor in other cell types. This dual role of E2F-1 may result from an ability of E2F-1–pRb complexes to repress genes involved in apoptosis as well as genes whose products are needed for DNA synthesis (Qin *et al.*, 1994; Field *et al.*, 1996; Yamasaki *et al.*, 1996; Hsieh *et al.*, 1997; Phillips *et al.*, 1997).

Phosphorylation of pRb and concomitant relief of E2F repression is catalyzed by cyclin D-dependent kinases (cdk4 and 6) in response to extracellular signals. The activity of these kinases is in turn inhibited by proteins of the INK4 family (Sherr, 1996). Disruption of this so-called 'pRb pathway' is a frequent event during the pathogenesis of a variety of human tumors (Tiemann *et al.*, 1997), highlighting the importance of the integrity of this pathway in the prevention of tumor formation, as well as pRb's central role in it.

The function of pRb is lost in all retinoblastomas, where it can lead to hereditary cancer, but is also involved in a variety of other tumors, including small cell lung carcinomas, breast carcinomas, osteosarcomas, bladder carcinomas, prostate carcinomas and cervical carcinomas (Dyson *et al.*, 1989; Horowitz *et al.*, 1990; zur Hausen, 1991). Several different types of inactivating mutations in the *RB1* gene have been described, almost all of which specifically alter or eliminate the 'pocket' domain of pRb. The pocket domain was first functionally characterized as the minimal region of pRb that is necessary for binding to the viral oncoproteins E1A, E7 and large T antigen. Thus, both oncogenic mutations and viral oncoprotein binding target the same subdomain of pRb, strongly suggesting a critical role for this domain in tumor suppression (Tiemann *et al.*, 1997). Indeed, the pocket domain is sufficient for the repression of gene expression, but an extra C-terminal stretch of the pRb protein is necessary for E2F binding and growth suppression (Qin *et al.*, 1992; Hiebert, 1993). pRb shares the pocket domain with two other related proteins, p107 and p130, which also bind and regulate E2F activity, but which have not yet been shown to be mutated in cancer (Ewen *et al.*, 1992; Cobrinik *et al.*, 1993; Hannon *et al.*, 1993; Li *et al.*, 1993; Mayol *et al.*, 1993).

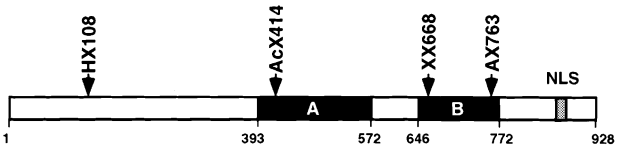
Although the pRb/E2F paradigm leads to a simple model for growth regulation through the inactivation of E2F-responsive genes, the identity of genes actually regulated by pRb is far from fully elucidated. The presence of p107 and p130 together with pRb in all cells complicates any simple model of pocket protein–E2F function, since these molecules may regulate subsets of E2F site-containing genes differentially (Hurford *et al.*, 1997). Further, pRb interacts with many proteins besides E2F, several of which can themselves directly influence gene expression (Taya, 1997). Among these are differentiation-specific transcription factors that are activated by pRb, rather than repressed, emphasizing a potential role for pRb in the process of terminal differentiation. Additionally, there is evidence that pRb may inhibit apoptosis (Haas-Kogan *et al.*, 1995; Berry *et al.*, 1996; Fan *et al.*, 1996; Macleod

et al., 1996; Shan *et al.*, 1996; Hsieh *et al.*, 1997) and somehow regulate replicative senescence, a phenomenon antithetical to tumor cell formation (Smith and Pereira-Smith, 1996).

In vivo evidence for a role for pRb in terminal cell cycle exit comes from mouse models in which both *RB1* alleles have been replaced with defective alleles by means of homologous recombination techniques. Mice homozygous for defective *RB1* die *in utero* at about the fourteenth day of gestation from abnormalities in the blood and the liver accompanied by extensive cell death in the central nervous system (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). Although pRb is also absent in all other tissues of the mouse embryo, most tissues develop grossly normally. Nevertheless, loss of pRb may lead to more subtle alterations in differentiated cell properties that have unknown consequences because of the inability to study them in dead embryos. For example, although muscle development appears normal in *RB1* nullizygotes, myotubes produced from such embryos fail to enter a truly 'terminal' state, since their nuclei can be induced to enter S phase, unlike those from genetically normal mice (Schneider *et al.*, 1994; Novitch *et al.*, 1996).

Multiple effects of pRb are also evident in cell culture model systems. For example, cells of the human osteosarcoma cell line SAOS-2 undergo a G_1 growth arrest upon pRb expression that is accompanied by a distinctive morphological change (Huang *et al.*, 1988; Templeton *et al.*, 1991; Hinds *et al.*, 1992). This phenotype is characterized by spreading and a senescence-like morphology (Templeton *et al.*, 1991; Hinds *et al.*, 1992; Qin *et al.*, 1992). This morphological alteration is not seen with dominant-negative E2F proteins or p107 overexpression, despite the probable mechanistic similarities in their ability to block progression into S phase (Zhu *et al.*, 1993; Qin *et al.*, 1995). Therefore, pRb may influence the expression of genes controlling cell morphology in a manner distinct from simple repression of E2F-dependent promoters.

In an attempt to explore the biochemical basis of pRb's functions and to produce a system to identify potential gene targets of pRb, we have generated a temperature-sensitive (ts) pRb. Characterization of this mutant supports a multifunctional role for pRb in suppression of cell proliferation, identifying both reversible and irreversible elements of growth arrest engendered by pRb. We suggest that pRb expression in this system not only prevents E2F function, but produces (possibly E2F-independent) cellular changes akin to differentiation or senescence that cannot be overcome by simple loss of pRb function, leading instead to apoptosis when the cells are once again rendered pRb-null through temperature shift. Transient restoration and removal of the differentiation and proliferation controls exerted by some tumor suppressor proteins may thus be a more potent method to eliminate tumor cells than that afforded by constitutive expression.



Protein	Mutation	Growth suppression at 37 °C	Growth suppression at 32.5 °C
HX108	RV**PLER**DL (108)	-	-
AcX414	S**IPRG**ILK (414)	-	-
XX668	ER**RSSG**LL (668)	-	+
AX763	Q**PLE**RL (763)	-	-
wtpRb	-	+	+
Δ22	deletion of exon 22	-	-

Fig. 1. Structure of *in vitro* mutagenized pRb proteins and their ability for growth suppression at 37 and 32.5°C. *In vitro* mutants were constructed by oligonucleotide linker insertion into restriction sites at certain codons of the human *RB1* cDNA. All mutants have been tested to express mutated forms of the pRb protein. The schematic diagram shows the human pRb protein and the positions where the *Xho*I linker was inserted in-frame to generate the mutants HX108, AcX414, XX668 and AX763. The solid black boxes A and B represent the two regions of the retinoblastoma protein essential for binding to E1A and SV40 large T antigen (Hu *et al.*, 1990), together known as the pocket domain of pRb. The shaded gray box represents the nuclear localization signal (NLS) of pRb (amino acids 860–876). To determine the ability of the generated *in vitro* mutants to suppress growth at 37 or 32.5°C, SAOS-2 cells were transfected with pBabepuro and HX108, AcX414, XX668, AX763, wtpRb or Δ22, and transfected cells were selected at 37 or 32.5°C. Suppression of SAOS-2 cell growth was assessed by the appearance of large, apparently non-dividing cells 7–10 days after transfection. (+) Large number of such cells. (–) No or very few enlarged cells were observed.

Results

Identification of a pRb protein temperature-sensitive for the induction of growth arrest

Regulated ectopic expression of genes involved in proliferation control is useful for studying their immediate effects upon synthesis and loss. For example, both inducible and temperature-sensitive p53 genes have been useful in identifying functional and transcriptional targets of this important tumor suppressor (Michalovitz *et al.*, 1990; Yonish-Rouach *et al.*, 1991; Barak *et al.*, 1993; El-Deiry *et al.*, 1993; Okamoto and Beach, 1994; Wu and Levine, 1994; Chen *et al.*, 1996). Because pRb's long half-life makes it an unattractive candidate for inducible systems, we attempted to identify a temperature-sensitive version of the protein that could be regulated more rapidly. Therefore, a series of *in vitro* mutagenized pRb cDNAs was produced (Mittnacht *et al.*, 1991; Hinds *et al.*, 1992) and tested for growth suppression in SAOS-2 cells at 37 and 32.5°C. As shown in Figure 1, the four linker-insertion mutants and the tumor-derived negative mutant Δ22 (Horowitz *et al.*, 1990) were found to be inactive in growth suppression at 37°C, but one mutant was found to induce growth suppression at 32.5 but not at 37°C. This mutant, XX668, carries a 12 bp *Xho*I linker inserted in-frame into an *Xmn*I site in between codons 668 and 669. This results in the insertion of four new amino acids, RSSG, changing the sequence of this mutant at codon 668 to -er[RSSG]ll- (see Figure 1).

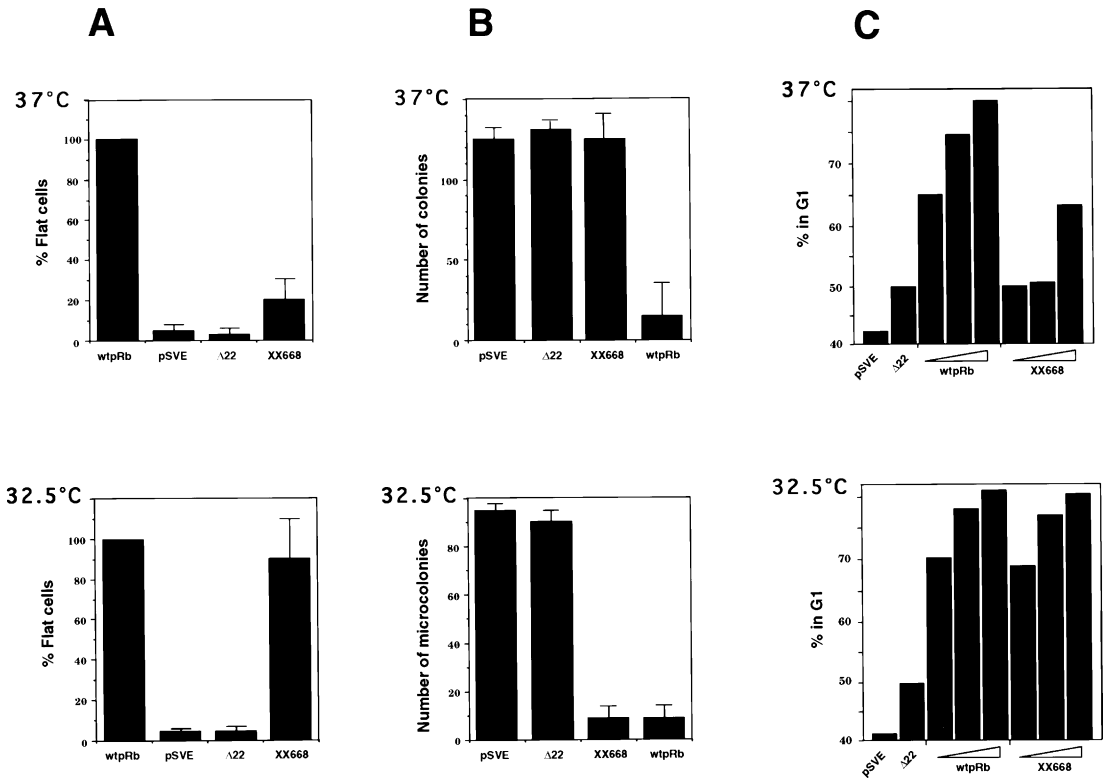


Fig. 2. Temperature-dependent growth suppression by the pRb mutant XX668. For flat cell assays (**A**) and colony formation assays (**B**), cells were transiently transfected with 1.5 μg of pBabepuro and 20 μg of the indicated pRb expression plasmid. Following transfection, cells were plated at 5×10^5 per 10 cm dish, subjected to drug selection at either 37 or 32.5°C and stained 7–10 days (A) or 14 days (B) later. Flat cells (A) or colonies (B) were counted as described in Materials and methods. The graphs (A and B) represent the mean of at least three independent experiments. For the flat cell assays (A), the percentage of flat cells relative to wtpRb (100%) is given in the graph. For cell cycle analysis (**C**), cells were co-transfected with 2 μg of pCMVCD20 and 20 μg of pSVE or Δ22, or 5, 10 and 20 μg of wtpRb or XX668. The total amount of plasmid DNA was kept constant in all samples (20 μg) by adding pSVE plasmid DNA, where needed. Cells were incubated at 37 or 32.5°C and harvested 48 h post-transfection, stained for CD20 and DNA content, and analzyed by flow cytometry. The percentage of CD20-positive cells in G_1 was plotted for each sample. The data presented are representative of multiple experiments.

One of the hallmarks of pRb-induced growth arrest in SAOS-2 cells is a distinctive morphological change which has been referred to as the flat cell phenotype (Hinds *et al.*, 1992). This phenotype is characterized by spreading and a senescence-like morphology (Templeton *et al.*, 1991; Hinds *et al.*, 1992; Qin *et al.*, 1992). Quantitation of flat cell induction, shown in Figure 2A, revealed that XX668 at 37°C produces on average 20% of the number of flat cells produced by wild-type pRb (wtpRb), but is essentially equivalent to wtpRb in flat cell production at 32.5°C. As a further measure of the ability of XX668 to suppress proliferation, colony formation assays were performed. Figure 2B demonstrates that XX668 suppressed outgrowth of SAOS-2 cell colonies with wild-type efficiency at 32.5°C, but was unable to do so at 37°C. To demonstrate conclusively that XX668 behaves as a temperature-sensitive allele not only in the production of flat cells but also in the induction of G_1 arrest in SAOS-2 cells, we used a modified flow cytometry technique that was reported previously for pRb and p107 (Zhu *et al.*, 1993). XX668 or control vectors were co-transfected into SAOS-2 cells with a plasmid expressing the cell surface marker CD20. Two days after transfection, cells that contained transfected DNA were identified by staining with an anti-CD20 monoclonal antibody, and the DNA content of the transfected cells was determined by propidium iodide staining. As shown in Figure 2C, transfection of XX668 was unable to alter the G_1 fraction of SAOS-2 cells efficiently at 37°C, whereas even low amounts of the wtpRb cDNA led to a significant increase in the G_1 population. A slight increase in the G_1 population was seen when XX668 was transfected in a very high concentration, consistent with its partial ability to induce flat cell formation. In contrast, at 32.5°C, XX668 and wtpRb both blocked SAOS-2 cells very efficiently in the G_1 phase of the cell cycle. Thus XX668 is defective in flat cell formation, colony reduction and induction of G_1 arrest when expressed at 37°C, but is indistinguishable from wtpRb at 32.5°C. In contrast, the tumor-derived mutant Δ22 was found to be non-functional in these assays at either temperature (see Figure 2A–C), consistent with previous reports classifying Δ22 as a 'loss-of-function' mutant (Horowitz *et al.*, 1990; Templeton *et al.*, 1991).

To exclude the possibility that the observed differences of XX668 in growth suppression and flat cell formation at 32.5 and 37°C may be attributable to differences in transfection efficiencies or expression levels rather than to a functional temperature-sensitivity, we performed indirect immunofluorescence and immunoblotting. In a typical experiment, regardless of the temperature or of whether XX668 or wtpRb cDNA was used, ~30% of the transfected SAOS-2 cells became pRb positive (data not shown). In

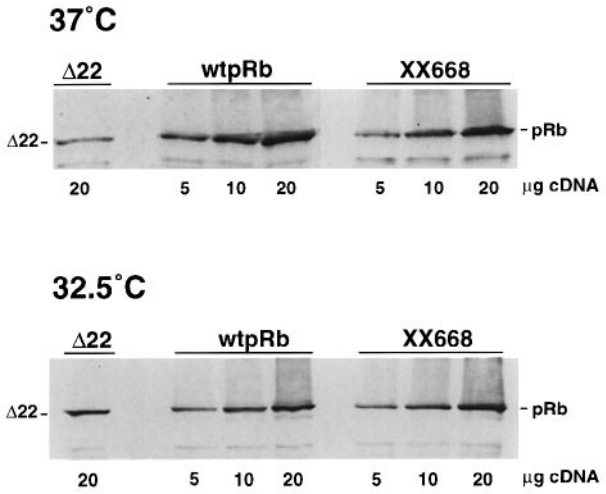


Fig. 3. Expression of wild-type and mutant pRb cDNAs in SAOS-2 cells. SAOS-2 cells, transfected as described in Figure 2B with the indicated amounts of Δ22, wtpRb or XX668 and incubated for 48 h post-transfection at 37 or 32.5°C, were lysed and aliquots were immunoblotted with an anti-pRb monoclonal antibody. The position of wtpRb and XX668 proteins (pRb) and the position of the slightly faster migrating Δ22 protein are indicated.

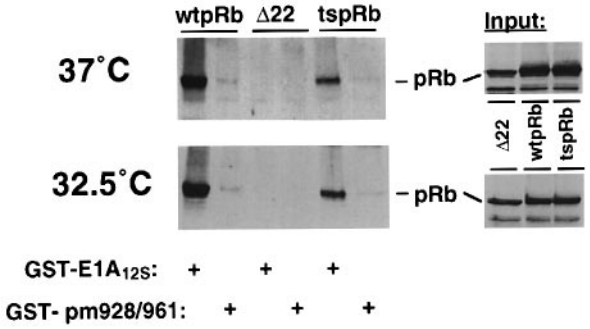


Fig. 4. *In vitro* binding of pRb proteins to E1A. SAOS-2 cells were transfected with 20 μg of Δ22, wtpRb or tspRb (XX668) and maintained for 48 h post-transfection at 37 or 32.5°C. Aliquots of the different cell lysates were incubated with glutathione–Sepharose loaded with GST–$E1A_{12S}$ or GST–pm928/961. Bound proteins were separated by SDS–PAGE and immunoblotted for pRb. To control the input of the pRb proteins, aliquots of the same lysates were immunoblotted directly for pRb (Input).

addition, protein levels expressed from increasing amounts of XX668 or wtpRb cDNA were essentially equal at both temperatures (Figure 3).

These data suggest that XX668 encodes a mutant pRb protein that is essentially wild-type for suppression of proliferation and flat cell formation at 32.5°C, but is only very weakly functional at 37°C, despite its persistence in the cell. XX668 therefore can be considered as a temperature-sensitive allele of *RB1*.

Biochemical properties of tspRb

We explored the biochemical properties of tspRb in an effort to understand the defect that inactivates the protein at the non-permissive temperature. Using an *in vitro* binding assay, we first determined if tspRb's ability to associate with the pocket-binding viral oncoprotein E1A could correlate with its temperature-dependent growth-suppressing function. Extracts from SAOS-2 cells transfected with either XX668, wtpRb or Δ22, and cultured at 37 or 32.5°C, were mixed with E1A that was made as a GST fusion protein in *Escherichia coli* and incubated at 4°C. The fraction of pRb that was bound by the GST–E1A protein was visualized by immunoblot with a pRb-specific monoclonal antibody (Figure 4). Equal amounts of input pRb protein were assured by performing direct immunoblots with aliquots of the cell extracts used in the *in vitro* binding experiments (Figure 4, Input). Regardless of the temperature at which transfected cells had been cultured, only ~10% of tspRb was associated with the E1A oncoprotein when compared with wtpRb. Similar results were observed when *in vitro* binding was performed at 25 or 37°C (data not shown). Thus, the four amino acid insertion in the B-pocket of tspRb severely compromises the ability of the mutant protein to associate with E1A at both the permissive and the non-permissive temperatures. Further, based on the assay employed, there is no correlation between tspRb's ability to suppress cell growth and its ability to bind to the E1A oncoprotein.

In contrast to E1A binding, nuclear association of pRb is well correlated to its functional state (Mittnacht and Weinberg, 1991; Mittnacht *et al.*, 1991; Templeton *et al.*, 1991). Functionally active pRb stably associates with nuclear structures, whereas pRb that has been inactivated by hyperphosphorylation or mutation fails to do so (Mittnacht and Weinberg, 1991). This biochemical property of pRb can be measured by treatment of the transfected cells with a detergent-containing buffer of low salt concentration. Under these conditions, functional pRb is retained in the nucleus, but inactive pRb is extracted (Mittnacht and Weinberg, 1991; Mittnacht *et al.*, 1994). To examine this property of tspRb, SAOS-2 cells were transfected with XX668, wtpRb or control vectors, cultured for 2 days at 32.5 or 37°C and finally lysed in a hypotonic buffer containing 0.1% Triton X-100. Lysates then were fractionated by low-speed centrifugation into a low-salt-soluble supernatant and an insoluble nuclear pellet fraction. Distribution of the pRb proteins to the fractions was determined by immunoblotting and is shown in Figure 5. As expected, wtpRb was found predominantly in the nuclear pellet fraction at both temperatures, whereas the majority of the tumor-derived mutant Δ22 was extracted into the soluble supernatant (low-salt extract). In contrast, tspRb was stably associated with the nucleus and therefore was detectable mainly in the nuclear pellet fraction at 32.5 but not at 37°C, where it was extracted efficiently into the low-salt fraction. Thus, tspRb encoded by XX668 is temperature-sensitive for nuclear tethering and, consistent with previous studies of tumor-derived mutants (Mittnacht and Weinberg, 1991; Mittnacht *et al.*, 1991; Kratzke *et al.*, 1994), this activity correlates well with its growth-suppressing function.

Viral oncoprotein binding to pRb is thought to mimic and to compete with the association of cellular proteins that can bind to the pocket domain of pRb. The best understood cellular targets of pRb are the members of the E2F family of transcription factors. Since physical association between E2F and pRb is postulated to result in transcriptional repression and growth arrest, we wished to determine if the defects in E1A binding, growth suppression and nuclear tethering displayed by tspRb were associated with an inability to suppress transcription and/

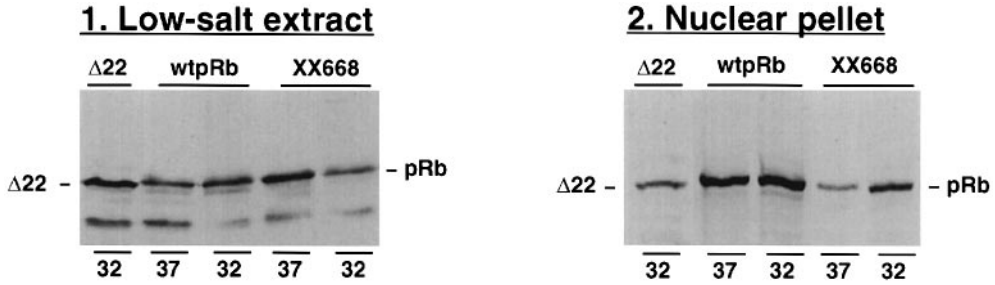


Fig. 5. Nuclear association of pRb. SAOS-2 cells were transfected with 20 μg of the indicated pRb expression construct and incubated at either 37 or 32.5°C. At 48 h after the removal of DNA precipitates, transfected cells were subjected to subcellular fractionation. Aliquots of the low-salt extract fraction and the nuclear pellet fraction were analyzed for pRb by immunoblotting with an anti-pRb-specific monoclonal antibody. The position of wtpRb and XX668 proteins (pRb) and the position of the slightly faster migrating Δ22 protein are indicated.

or bind to E2F. To determine the functional consequence of tspRb expression on E2F-dependent transcription, we employed a reporter consisting of the luciferase gene driven by the E2F-1 promoter. The E2F-1 promoter fragment used in this experiment has been shown to be subject to repression by pRb (Sellers *et al.*, 1995). This reporter construct was co-transfected with tspRb cDNA, wtpRb or control plasmids into SAOS-2 cells, and the cells were incubated at either 37 or 32.5°C. As shown in Figure 6A, increasing amounts of tspRb were much less effective in repressing the E2F-1 promoter at 37°C, but gained this ability with wild-type efficiency at 32.5°C. This result clearly demonstrates a temperature-dependent activity of tspRb in suppression of E2F-dependent transcription.

To determine if the inability of tspRb to repress transcription through E2F is due to an inability of this mutant protein to interact with E2F, we obtained an antibody against E2F-4 (kind gift of J.Lees, MIT, Cambridge, MA) and used this to immunoprecipitate E2F-4 from SAOS-2 cells transfected with the various pRb expression constructs. Because E2F-4 is the most abundant family member in SAOS-2 cells, we anticipated that pRb–E2F-4 complexes would be detectable without further overexpression of E2F-4 (J.Lees, personal communication). Figure 6B presents the results of an anti-pRb immunoblot of the anti-E2F-4 immunoprecipitate. Whereas Δ22 is incapable of associating with endogenous E2F-4, a clear signal is obtained when wtpRb is produced in the transfectants. Transfection with XX668 yields a tspRb protein that is clearly capable of interacting with E2F-4 at 37°C, but at a reduced level. This binding does not increase when the protein is produced at 32.5°C. These data suggest that tspRb binds E2Fs more weakly than wtpRb, and this binding is not temperature-sensitive. Thus it is possible that sufficient pRb–E2F complex is present to repress E2F-dependent transcription, but repression only occurs at 32.5°C, when tspRb gains transcriptional repressor ability through an as yet undetermined mechanism.

Inactivation of tspRb leads to DNA synthesis and apoptotic cell death

The data obtained so far clearly demonstrate that tspRb in SAOS-2 cells can induce cell cycle arrest, a senescence-like morphology and E2F repression in a temperature-dependent manner. We next wished to determine if tspRb produced at the permissive temperature could be inactivated by a shift to the non-permissive temperature, and ultimately wished to study the reversibility of the pRb phenotype. To determine if active tspRb can be inactivated after transient transfection of SAOS-2 cells, tspRb-induced, growth-arrested flat cells produced by a 1 week incubation at the permissive temperature were shifted back to the non-permissive temperature and scored for re-entry into S phase by measuring the ability to incorporate bromodeoxyuridine (BrdU) (Figure 7). Flat cells produced by wtpRb were found to be incapable of incorporating BrdU at either temperature, consistent with their arrest in G_1 (or G_0). Similarly, tspRb-produced flat cells at 32.5°C could not incorporate this nucleotide. Strikingly, when the latter were shifted to 37°C, ~30% of the cells were found to incorporate detectable amounts of BrdU as early as 24 h after the shift. In contrast, growth-arrested cells produced by wtpRb expression at 32.5°C did not regain an ability to incorporate BrdU upon temperature upshift. Parental SAOS-2 cells permanently cultivated at 32.5 and 37°C and XX668-transfected SAOS-2 cells permanently cultivated at the non-permissive temperature were used as a positive control for BrdU incorporation (Figure 7). Thus, the block to S phase entry caused by tspRb appears to be rapidly reversible by temperature shift.

The apparent reversibiliy of the S phase block induced by tspRb allowed us to ask if the transiently growth-arrested SAOS-2 cells could re-enter the cell cycle permanently and/or reverse the senescence-like morphology. Incorporation of BrdU into the tspRb-transfected, temperature-shifted SAOS-2 cells occurred while the cells still possessed the flat cell morphology. To determine if inactivation of tspRb would allow the cells to regain their normal shape and proliferation capacity, flat cells were produced by transient transfection of SAOS-2 cells with XX668 and wtpRb followed by drug selection for 7–10 days at 32.5°C. Flat cells were then removed from the drug and either further incubated at 32.5°C or shifted to 37°C. Photographs of cells within a fixed area of 0.25 cm^2 were taken and the cells were counted on seven consecutive days (see Table I). While the number of wild-type transfected cells at both temperatures as well as tspRb-expressing cells at 32.5°C remained unchanged, tspRb-expressing flat cells at 37°C quickly disappeared from the plate, with loss of ~50% of the cells occurring within 24 h and loss of all cells occurring within 7 days after temperature shift. Periodic visual inspection of the remaining flat cells suggested that nuclear division commonly precedes the death of these cells, perhaps consistent with their ability to re-enter S phase prior to death

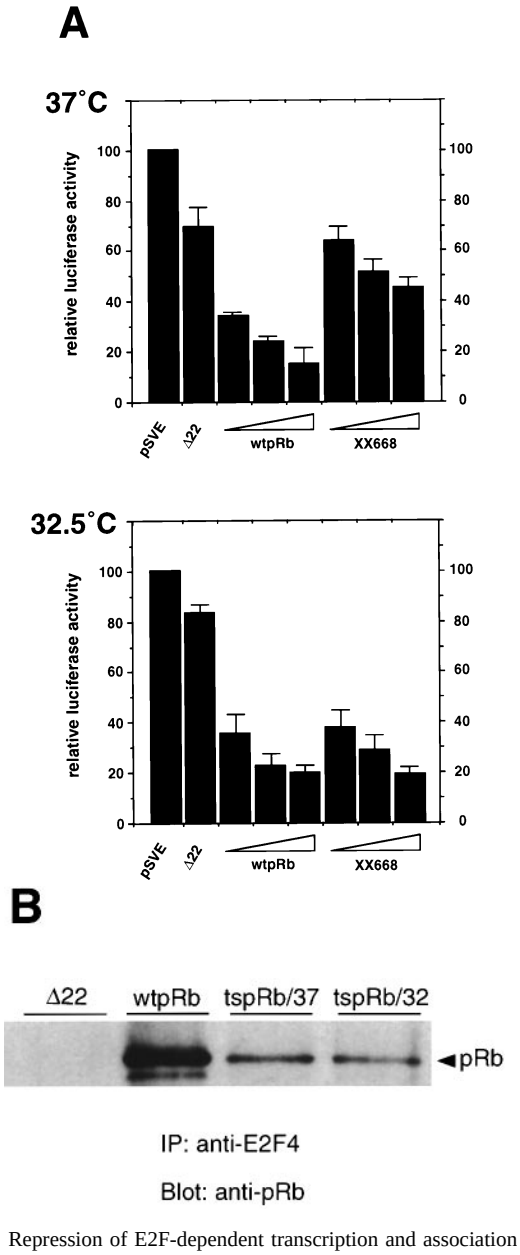


Fig. 6. Repression of E2F-dependent transcription and association of pRb proteins with E2F-4. (**A**) SAOS-2 cells were co-transfected with 5 μg of pGL2-AN, 2 μg of pCMV-βgal and 20 μg of pSVE or Δ22, or 5, 10 and 20 μg of wtpRb or XX668. The total amount of plasmid DNA was kept constant in all samples (20 μg) by adding pSVE plasmid DNA, where needed. Following transfection, cells were incubated for 48 h at 37 or 32.5°C, cell extracts were prepared and β-galactosidase and luciferase activities were assayed. Luciferase values were normalized for β-galactosidase activity and plotted relative to the activity observed for the reporter in the presence of pSVE. Data shown are representative of three experiments. (**B**) SAOS-2 cells were transfected with the indicated pRb-expressing constructs, incubated at either 37 or 32.5°C for 48 h and then subjected to lysis. Soluble proteins were immunoprecipitated with an anti-E2F-4 monoclonal antibody and the immunoprecipitates were subjected to immunoblot with anti-pRb antibodies.

(data not shown). Thus, flat cells produced by transient transfection with tspRb at the permissive temperature appear to display a reversible block to S phase entry, but are terminally blocked from proliferation, since they die after shift to the non-permissive temperature.

Because pRb loss has been reported to induce apoptosis (Lee *et al.*, 1994; Maandag *et al.*, 1994; Morgenbesser *et al.*, 1994; Macleod *et al.*, 1996; Zacksenhaus *et al.*, 1996), we wished to determine if the cell death we observed was apoptotic in nature. Therefore, we first attempted to block cell death by introducing Bcl-2 or adenovirus E1B 19K protein along with pRb. Interestingly, co-transfection of these anti-apoptotic genes along with tspRb prevented cell death upon temperature upshift (see Table I), suggesting that the observed cell death is apoptotic.

To quantitate the percentage of apoptotic cells within the total flat cell population, we determined the DNA content of the affected cells by flow cytometry analysis following propidium iodide staining (Figure 8). The percentage of apoptotic cells in the wtpRb-induced flat cell population was very low, usually <10%, regardless of the incubation temperature. Similary, flat cells produced with tspRb at 32.5°C did not contain very many apoptotic cells. In contrast, when the latter were shifted to 37°C, a significant number of cells with a sub-G_1 DNA content was observed 24 h after the shift, representing some 40% of the total cell population. At 48 h post-shift, the percentage of apoptotic cells was a little lower, but still significant at 30–40% of the total cell population. Co-introduction of either Bcl-2 or E1B 19K led to a 3- to 5-fold decrease in apoptotic cells. Furthermore, as a consequence of the additional expression of either apoptosis inhibitor, small, proliferating SAOS-2 cells, positive for the expression of tspRb, were generated (Figure 9).

Thus, cellular changes caused by prolonged pRb expression appear to preclude proliferation (but not cell cycle progression) upon removal of pRb as a result of programed cell death. Inhibition of this death by known protein inhibitors of apoptosis allows the outgrowth of colonies of pRb-positive, morphologically 'normal' SAOS-2 cells, suggesting that the flat cell phenotype can be reversed once the apoptotic signals are overcome.

Discussion

We have produced a genetically engineered pRb mutant, XX668, carrying an insertion of four amino acids at codon 668 in the B-pocket, that can exert very diverse effects on transiently transfected SAOS-2 cells, depending on the temperature at which the culture is maintained. At 37°C, despite its persistence in the cell, XX668, unlike wtpRb, is defective in flat cell formation, colony reduction and induction of G_1 arrest. In this respect, it behaves like tumor mutants described here and in earlier work (Templeton *et al.*, 1991; Hinds *et al.*, 1992). However, unlike these tumor mutants, XX668 was found to be indistinguishable from wtpRb for suppression of proliferation when the cells were kept at 32.5°C. Furthermore, at this temperature, it can induce formation of morphologically altered 'flat' cells very efficiently. Thus, XX668 encodes a mutant pRb protein that is essentially wild-type at the low temperature, but is only very weakly functional at 37°C. These findings strongly suggest that XX668 is a temperature-sensitive mutant of pRb.

Not surprisingly, the insertion in the B-pocket of tspRb severely compromised its ability to interact with E1A. tspRb interacts only weakly with E1A at 37°C, and this binding cannot be improved upon changing the

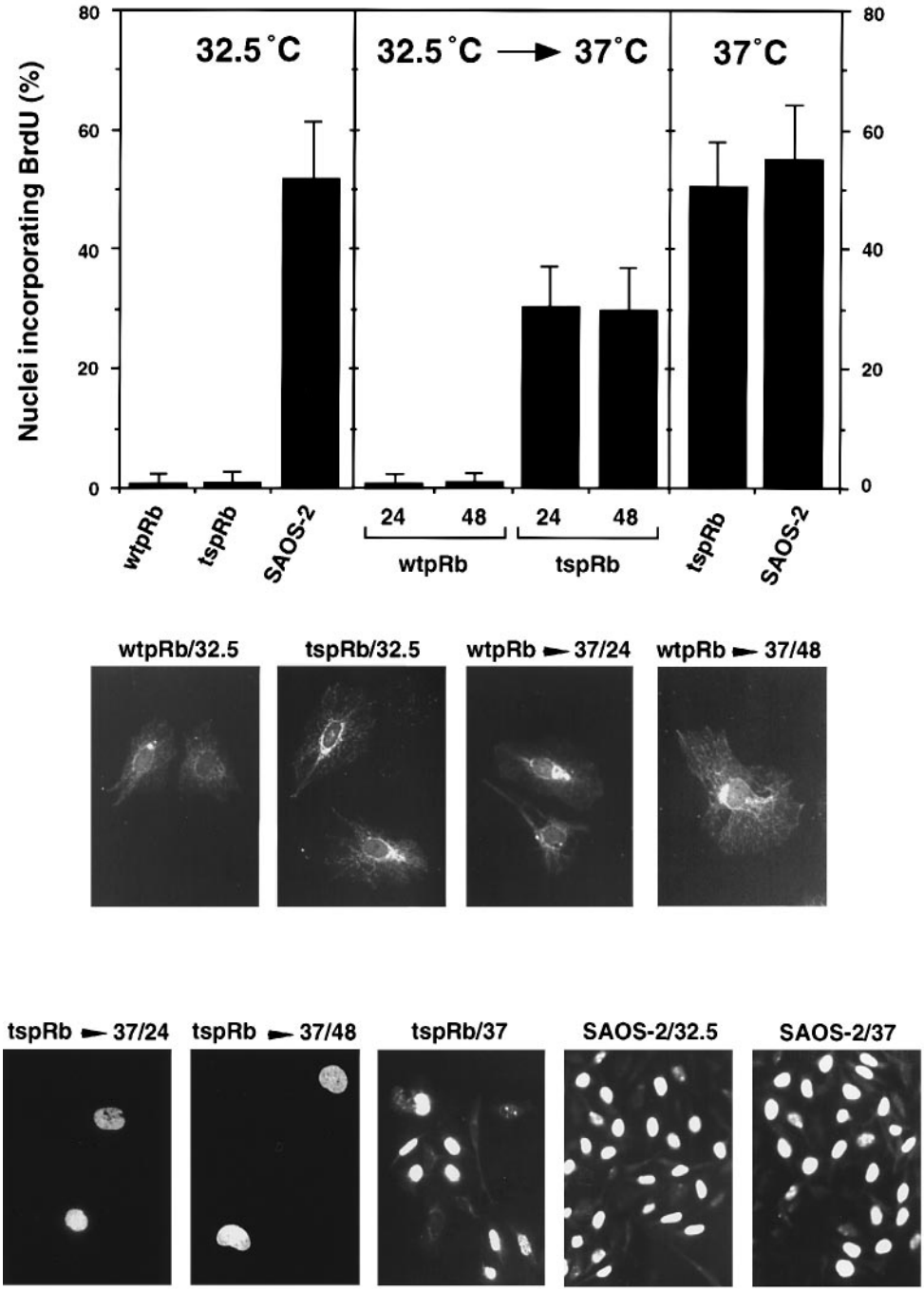


Fig. 7. Re-entry of tspRb-induced growth-arrested flat cells into S phase upon temperature shift to the non-permissive temperature. SAOS-2 cells were co-transfected with 1.5 μg of pBabepuro and 20 μg of wtpRb or tspRb (XX668). Following transfection, cells were subjected to drug selection at 32.5°C. In addition, tspRb-transfected cells were selected at 37°C. After 7–10 days, when only flat cells appeared on the plates, cells were removed from the drug and either incubated further at their former temperature or shifted from 32.5 to 37°C for 24 or 48 h. Shifted and indicated control cells were scored for re-entry into S phase by measuring their ability to incorporate BrdU. The percentage of BrdU-incorporating cells for each sample is shown in the graph. The results shown represent three independent experiments and the error bars indicate the standard deviations. Representative pictures of all samples stained for BrdU were taken at the same magnification.

temperature at which the protein is produced. Therefore, binding to E1A seems not to correlate with pRb's ability to suppress proliferation. This is consistent with previous studies in which pRb mutants with mutations within the B-pocket were reported that retained the ability to suppress cell proliferation but failed to associate with E1A (Mittnacht *et al.*, 1991; Kratzke *et al.*, 1994). A caveat to these E1A-binding experiments is that although tspRb proteins were produced in cells incubated at either the permissive or non-permissive temperature, *in vitro* binding was performed at 4°C, raising the possibility that the temperature-sensitive nature of the interaction might be altered by exposure of tspRb to the low temperature. Several observations argue against this possibility. First, tspRb produced in cells incubated at either temperature displayed equivalent *in vitro* binding to E1A (and equivalent E2F-4 co-immunoprecipitation) rather than showing an increase at the functionally permissive temperature.

Table I. Inactivation of pRb after flat cell induction results in cell death

Flat cell phenotype induced at 32.5°C with[a]	No. of cells (%) after further incubation at 32.5°C[b]		No. of cells (%) after temperature shift to 37°C[b]			
	Day 0	Day 7	Day 0	Day 1	Day 2	Day 7
Wild-type pRb	100	100	100	100	100	100
tspRb	100	100	100	55	45	0–1
tspRb + Bcl-2	100	100	100	100	100	150
tspRb + E1B 19K	100	100	100	100	100	140

[a]SAOS-2 cells were co-transfected with 1.5 μg of pBabepuro and 20 μg of wtpRb or tspRb (XX668) and 5 μg of Bcl-2 or E1B 19K, where indicated. Following transfection, cells were subjected to drug selection at 32.5°C. After 7–10 days, when only flat cells appeared on the plate, cells were removed from the drug and either incubated further at 32.5°C or shifted from 32.5 to 37°C for up to 7 days.
[b]Average cell numbers based on four experiments were calculated (see Materials and methods) and are presented relative to the cell numbers on day 0 of either temperature which were set as 100%.

This argues that the starting pools of conformationally wtpRb were equivalent, even if an effect of 4°C incubation might lead to partial restoration of pRb binding function. Second, incubation of GST–E1A with pRb-containing lysates at 25 or 37°C failed to increase the difference between wild-type and tspRb (data not shown), demonstrating that the temperature to which tspRb is subjected *in vitro* does not necessarily influence its E1A-binding ability. Finally, the XX668 mutant is also severely compromised in its ability to associate with the nucleus at 37°C, a loss of function common to inactive, tumor-derived mutants (Mittnacht and Weinberg, 1991; Mittnacht *et al.*, 1991; Templeton *et al.*, 1991; Kratzke *et al.*, 1994) but, in contrast to E1A binding, tspRb produced at 32.5°C regains the ability to associate with the nucleus, suggesting a temperature-dependent structural alteration of the pocket region required for nuclear tethering. Because this property of tspRb is also assayed at 4°C, it is clear that some physical differences between tspRb produced at permissive and non-permissive temperatures persist *in vitro*.

It is believed that transcriptional repression of genes containing E2F sites mediated by complexes of pRb and E2F contributes significantly to pRb's role as a tumor suppressor. Consistent with this, we find that tspRb is much less effective than wtpRb in repressing the E2F-1 promoter at the non-permissive temperature, but gains this ability with wild-type efficiency at 32.5°C. This result clearly suggests a temperature-dependent activity of tspRb in suppression of E2F-dependent transcription, correlating with its nuclear tethering and proliferation-suppressing activity. Interestingly, our data suggest that the inability of tspRb to suppress transcription at the non-permissive temperature is not due simply to a defect in association with E2F, since we have observed equivalent levels of pRb–E2F complexes at both the permissive and non-permissive temperatures. The XX668 mutation may thus be somewhat similar to a recently identified, low penetrance allele of *RB1*, called 661W, that is defective for E1A and E2F association, yet retains the ability to arrest cells (Kratzke *et al.*, 1994). However, as with E1A, E2F binding in the experiments described here was assayed after incubation of cell lysates at 4°C, and was restricted to analysis of an association with E2F-4. Although we cannot rule out entirely temperature-dependent affinity differences *in vivo*, the clear functional changes observed with tspRb are not accompanied by obvious changes in E1A or E2F association *in vitro*. Thus, it remains possible that temperature-dependent interactions with one or more E2F family members within the cell may be found. Alternatively, tspRb may be defective for transcriptional repression but not E2F association.

We suspect that the temperature dependence of tspRb's ability to repress E2F-dependent transcription may be due to a defect in its ability to interact with the transcriptional machinery adjacent to the E2F-binding sites. It has been proposed that the pRb pocket, after being tethered to a specific promoter through E2F, could either bind surrounding transcription factors, preventing their interaction with the basal transcription machinery (TFIID-like factors; the factor PU.1) (Chow and Dean, 1996), or bind one or more unknown proteins 'X' that have an intrinsic repressor activity (Sellers *et al.*, 1995). Both possibilities would result in the repression of transcription of the affected gene. TspRb may be unable to form such contacts at the non-permissive temperature, but could regain this ability at the permissive temperature. The suppression of proliferation mediated by tspRb at the permissive temperature may thus result from one or more pRb-containing multiprotein complexes that act to repress the transcription of growth-promoting genes. Clearly, our knowledge of pRb's interaction with E2F only scratches the surface of pRb's true molecular role in proliferation control. The tspRb protein promises to be a powerful tool to unravel the molecular mechanism of pRb-mediated transcriptional repression and should also be a very useful tool for identifying genes that are turned on or off soon after temperature shift. Such genes would be good candidates for direct control by pRb. As a first step in the identification of such genes, we recently have established cell lines stably expressing tspRb that undergo growth arrest at the permissive temperature (F.Tiemann and P.W.Hinds, unpublished observations).

The utility of tspRb as a reagent to study pRb's molecular functions in tumor suppression is enhanced by its apparent ability to be inactivated efficiently by temperature upshift. Cell cycle re-entry of tspRb-arrested SAOS-2 cells occurred well within 24 h as determined by BrdU incorporation, suggesting pRb's control of E2F and other factors is rather rapidly reversible. Most interestingly, although these cells displayed a reversible block to S phase entry, they were terminally blocked from proliferation, since they died by apoptosis after shift to the non-permissive temperature. An intriguing property of pRb-expressing SAOS-2 cells is the appearance of the so-

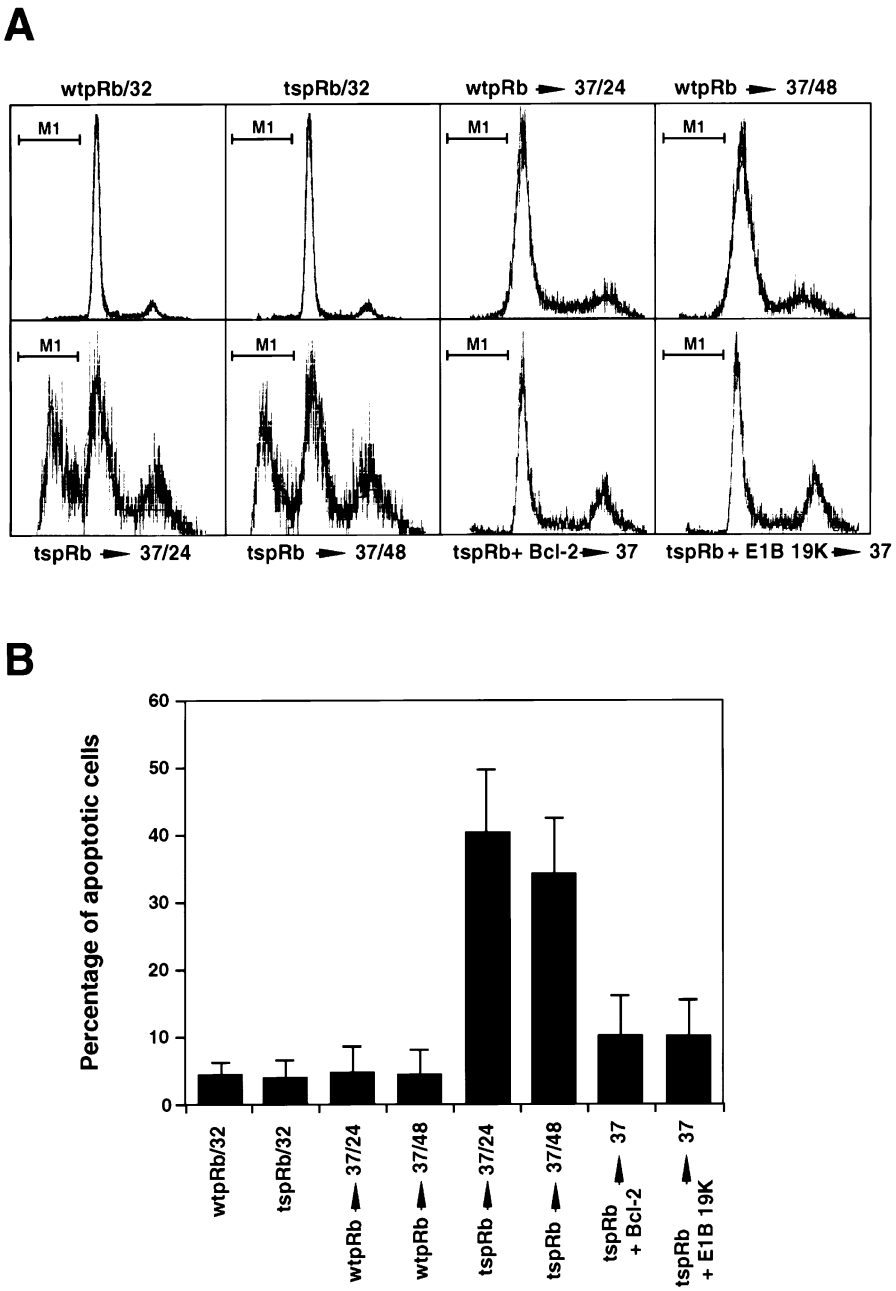


Fig. 8. Inactivation of tspRb after induction of the flat cell phenotype leads to apoptosis. SAOS-2 cells were co-transfected with 1.5 μg of pBabepuro and 20 μg of wtpRb or tspRb (XX668) and 5 μg of Bcl-2 or E1B 19K, where indicated. Following transfection, cells were subjected to drug selection at 32.5°C. After 7–10 days, when only flat cells appeared on the plate, cells were removed from the drug and either incubated further at 32.5°C or shifted from 32.5 to 37°C. WtpRb- and tspRb-transfected cells that were shifted from 32.5 to 37°C were harvested for flow cytometry analysis 24 and 48 h after temperature shift. All other cells were harvested for flow cytometry analysis 48 h after the former were shifted. Total flat cell populations were analyzed. Apoptosis was measured by the accumulation of cells with a sub-G_1 DNA content in an area indicated as M1. A representative FACS analysis is shown (**A**). The graph (**B**) represents the percentage of cells with sub-G_1 content (apoptotic cells) with mean values derived from three independent experiments.

called flat cell phenotype (Templeton *et al.*, 1991; Hinds *et al.*, 1992; Zhu *et al.*, 1993; Qin *et al.*, 1995). It was suggested that these flat cells resemble the senescent phenotype of primary fibroblasts seen after extended time in culture (Templeton *et al.*, 1991), and that this biological activity of pRb may be a manifestation of a less reversible type of exit from the cell cycle leading to senescence or differentiation (Qin *et al.*, 1995; Weinberg, 1995). Prolonged expression of pRb may produce a 'terminal' pseudo-differentiated or senescent state characterized by the flat cell phenotype that is distinct from pRb–E2F-type cell cycle arrest and which is poorly reversible upon loss of pRb. Indeed, some property of the flat cells themselves, such as cytoskeletal structure or cell shape, may contribute directly to the relative irreversibility of the arrested state. Thus, cellular changes caused by the prolonged expression of functional tspRb appear to preclude rather than restore proliferation (but not cell cycle progression) upon removal of a functional pRb as a result of programed cell death. Interruption of this apoptotic response could be achieved

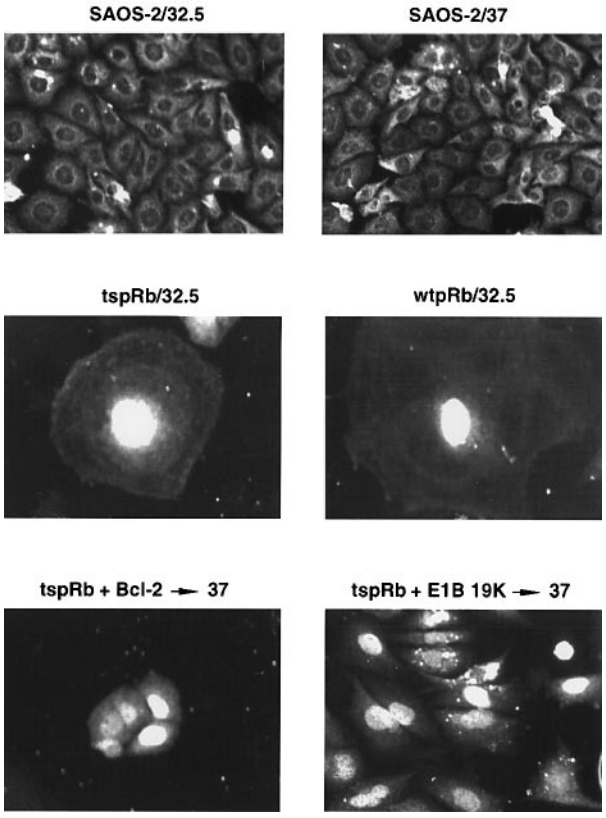


Fig. 9. Inhibition of cell death by apoptosis inhibitors allows the outgrowth of pRb-positive SAOS-2 cells with a normal cell morphology. SAOS-2 cells transfected with tspRb and the apoptosis inhibitors Bcl-2 or E1B 19K, respectively, or with tspRb or wtpRb that were generated at day 7 in the experiment described in Table I, were immunocytochemically stained for pRb and compared with SAOS-2 cells that were cultivated at 37 or 32.5°C. Representive photographs were taken at the same magnification.

by co-transfection of Bcl-2 or E1B 19K, which led to the outgrowth of small, proliferating SAOS-2 cells, positive for the expression of tspRb. As observed by the photographs taken, the outgrowth of colonies was rather slow, with the first appearance of colonies positive for pRb staining at day 7 after temperature shift to the non-permissive temperature. This can be explained in part by the fact that the block to apoptosis was not complete (see Figure 8). Additionally, we cannot rule out a requirement for additional events to restore proliferative capacity to cells that have escaped apoptosis through expression of Bcl-2 or E1B 19K.

Given these results, it is of note that SAOS-2 cells express high levels of the CDK inhibitor gene encoding p16, which is overexpressed in senescent cells, and which is one of the candidate causal senescence genes (Smith and Pereira-Smith, 1996). Since loss of pRb in SAOS-2 cells may allow bypass of the putative p16 function in senescence (Sherr, 1996), re-introduction of a hypophosporylated form of pRb in pRb-negative tumor cells could promote a terminal cell cycle exit by switching on elements of a senescence program. The functional inactivation of pRb once the senescent phenotype is established may lead to apoptosis as a result of two conflicting signals: (i) a growth-promoting signal triggered by a deregulated E2F and (ii) a growth-inhibitory signal maintained by the senescence machinery. In support of this are studies that suggest that several proteins that play a role in regulating cell cycle progression also have an apoptotic potential and that the apoptotic signal induced by such proteins is a direct consequence of conflicting growth control functions. Of particular interest are recent studies that suggest a p53-independent apoptotic activity of E2F-1 that is dependent on the deregulation of a functional pRb–E2F-1 repressor complex. Thus, derepression of genes containing E2F sites may lead directly to apoptosis if the cell's interpretation of its environment is otherwise at odds with a decision to proliferate (Hsieh *et al.*, 1997; Phillips *et al.*, 1997).

In summary, we propose that the introduction of a functional pRb protein into SAOS-2 cells acts as a negative epigenetic signal that is followed by the start of a senescence program (or alternatively a differentiation program) that in turn causes the observed S phase block accompanied by the shape change of the cells. Once the growth-inhibitory signal is established, removal of functional pRb restores S phase entry, but this may conflict with irreversible negative signals resulting from other downstream effects of pRb with the consequent death of the affected cells. Achieved here with a temperature-sensitive pRb, such a transitory reactivation of the pRb pathway has broader conceptual implications for cancer therapy, since techniques designed to restore the pRb pathway only temporarily in tumor cells may prove more efficient at cell killing than permanent expression of a given cell cycle regulator.

Materials and methods

Cell culture, plasmids and transfections

The human osteosarcoma cell line SAOS-2, subclone 2.4 (Hinds *et al.*, 1992) was used for all studies. Cells were maintained in Dulbecco's modified Eagle's medium (Gibco/BRL) supplemented with 15% heat-inactivated fetal bovine serum in a 3% CO_2 incubator at 37°C.

All pRb expression plasmids were constructed in pSVE (Templeton *et al.*, 1991). The human pRb expression vector phRbc-SVE (here referred to as wtpRb) has been described previously (Templeton *et al.*, 1991; Hinds *et al.*, 1992) and was used in all transfection experiments to express wild-type pRb. Several mutant cDNAs were constructed in this vector by inserting an *Xho*I linker in-frame into different restriction sites at certain codons (Mittnacht *et al.*, 1991; Hinds *et al.*, 1992). HX108 has a 12 bp linker inserted at the *Hinc*II site at codon 108, resulting in the insertion of four amino acids (P-L-E-R) at this position. AcX414 has an insertion of four amino acids (I-P-R-G) at codon 414, resulting from a 10 bp linker inserted into an *Acc*I site. The mutant XX668 carries the 12 bp linker inserted at the *Xmn*I restriction site at codon 668, with four new amino acids (R-S-S-G) at this position. AX763 was constructed by inserting a 12 bp linker into an *Alw*NI site at codon 763, and has three new amino acids (P-L-E) at this position.

The expression vector pSVΔ22 encoding a pRb protein deleted in exon 22 (here referred to as Δ22) was used as a negative control for pRb expression and function. The Δ22 cDNA was derived from the NCI-H592 small cell lung carcinoma (Horowitz *et al.*, 1990) and subcloned into pSVE (Templeton *et al.*, 1991).

The vector pBabepuro (Morgenstern and Land, 1990) was used to mediate resistance to puromycin.

SAOS-2 cells ($1–2\times10^6$) were transfected with the indicated plasmids on 10 cm dishes for 18 h by using the 2× Bes-buffered saline (2× BBS)/calcium phosphate method of Chen and Okayama (1987), with modifications as described previously (Hinds *et al.*, 1992).

Flat cell assay and colony formation assay

For flat cell assays and colony formation assays, cells were transiently transfected with 1.5 μg of pBabepuro and 20 μg of the indicated pRb expression plasmid. Following transfection, cells were plated at 5×10^5 per 10 cm dish. Puromycin (Sigma) was added at 0.5 μg/ml 24 h after plating and cells were selected at either 37 or 32.5°C. After 7–10 days

of selection, cells were stained with crystal violet and flat cells were quantitated as described by Hinds *et al.* (1992). For the quantitation of colonies, cells were stained with crystal violet after 14 days of selection, when macroscopic colonies became detectable. Since no macroscopic colonies were detectable at this time on plates maintained at 32.5°C, microcolonies were quantitated as described for the flat cells.

Flow cytometry analysis

Flow cytometry analysis for the determination of cell cycle profiles of pRb-transfected cells was performed as described previously for pRb and p107 (Zhu *et al.*, 1993). Briefly, 2 μg of the expression plasmid for the B cell surface marker CD20 (pCMVCD20) (van den Heuvel and Harlow, 1993) was co-transfected with the indicated amounts of pRb expression constructs into SAOS-2 cells. At 48 h after the removal of DNA precipitates, cells were rinsed off the plates with phosphate-buffered saline (PBS) containing 0.1% EDTA, pelleted, and stained with 20 μl of a fluorescein isothiocyanate (FITC)-conjugated anti-CD20 monoclonal antibody (PharMingen). Subsequently, cells were fixed with 90% ethanol on ice for several hours. Before flow cytometry analysis, the cells were treated with 200 μg/ml RNase A for 15 min at 37°C and stained with a solution containing 20 μg/ml of propidium iodide. Flow cytometry analysis was performed on a Becton-Dickinson FACScan. The intensity of propidium iodide staining was analyzed with the CellFIT Cell Cycle Analysis software to determine the DNA content and hence the cell cycle profiles on cells that were positive for FITC staining. We observed small variations in the G_1, S and G_2/M populations between samples that were transfected independently with the same plasmids. However, the differences between controls and testing samples observed in each particular experiment were significantly consistent in all separate experiments.

Immunoblotting, immunoprecipitations and in vitro binding assay

The expression of transfected cDNAs was monitored by immunoblotting. Aliquots of SAOS-2 cells, transfected with the indicated amounts of pRb expression constructs for cell cycle analysis, reporter assays or *in vitro* binding assays, were lysed in ELB (50 mM HEPES, pH 7.2; 250 mM NaCl; 2 mM EDTA; 0.1% NP-40) 48 h after the removal of DNA precipitates as described (Latham *et al.*, 1996). Protein concentrations in the cell lysates were determined by the Bio-Rad protein assay. Proteins were separated by SDS–PAGE and transferred to nitrocellulose by standard procedures. pRb proteins were monitored by blotting with the monoclonal antibodies 245 (PharMingen) or Ab-5 (Oncogene Science). Detection was performed, after incubation with peroxidase-conjugated donkey anti-mouse IgG (Jackson Immunoresearch), by enhanced chemiluminescence (Amersham), as described previously (Latham *et al.*, 1996).

For E2F-4 immunoprecipitations, lysates containing 200 μg of protein were subjected to immunoprecipitation with 100 μl of hybridoma supernatant from the monoclonal antibody 2-4/E9 (kind gift from J.Lees), as described (Latham *et al.*, 1996).

Plasmids encoding the GST–E1A$_{12S}$ and E1A$_{12S}$ pm928/961 fusion proteins (Kraus *et al.*, 1994) were used to produce the proteins for the GST pull down experiments. GST fusion proteins were induced, purified and recovered on glutathione–Sepharose beads as described earlier (Kaelin *et al.*, 1991). The beads were rocked with aliquots of cell lysates for 2 h at 4°C and then washed five times with ELB. The beads were then boiled in sample buffer and bound proteins were resolved by SDS–PAGE. pRb proteins were visualized by immunoblotting as described above. With each aliquot of cell lysate, ~150 μg of total protein was analyzed.

Nuclear extraction

SAOS-2 cells were transfected with 20 μg of the indicated pRb expression construct and incubated at either 37 or 32.5°C. At 48 h after the removal of DNA precipitates, transfected cells were subjected to subcellular fractionation as described previously (Mittnacht and Weinberg, 1991). Briefly, cells were lysed in a hypotonic buffer (10 mM HEPES-KOH, pH 7.9; 10 mM KCl; 1.5 mM $MgCl_2$; 0.5 mM dithiothreitol) containing 0.1% Triton X-100, and fractionated by low-speed centrifugation into a low-salt-soluble supernatant and an insoluble nuclear pellet. The insoluble nuclear pellet fraction then was extracted further with ELB. pRb in aliquots of both extracts was detected by immunoblotting as described above. To control for pRb expression, a small aliquot of each sample was lysed directly in ELB and monitored for pRb expression by immunoblotting.

E2F transcription assay

Luciferase assays using the luciferase reporter plasmid pGL2-AN, containing the E2F-1 promoter upstream of the luciferase cDNA (Neuman *et al.*, 1994), were performed according to standard protocols (Ausubel *et al.*, 1990). Cells were co-transfected with 5 μg of pGL2-AN, 2 μg of pCMV-βgal and the indicated amount of different pRb expression plasmids, and incubated at 37 or 32.5°C. At 48 h after the removal of DNA precipitates, β-galactosidase and luciferase activities were assayed.

Analyses of DNA synthesis and cell death, and immunocytochemical staining for pRb

SAOS-2 cells were transfected with 1.5 μg of pBabepuro, 20 μg of the indicated pRb expression plasmid and 5 μg of either the Bcl-2 expression vector SFFV-Bcl-2 (kindly provided by Dr S.Korsmeyer) or the E1B 19K expression vector pCMV-E1B 19K (Han *et al.*, 1996), when indicated. Following transfection, cells were plated and selected at 32.5°C as described for the flat cell assays. After 7–10 days, when only flat cells appeared on the plates, cells were removed from the drug and either incubated further at 32.5°C or shifted to 37°C. Treated cells as well as control cell populations were then scored for (i) DNA synthesis by measuring their ability to incorporate BrdU, (ii) cell death by cell number comparison and flow cytometry analysis of propidium iodide-labeled cells and (iii) pRb expression by immunocytochemical staining for pRb.

(i) BrdU incorporation was detected exactly as described earlier (Latham *et al.*, 1996). At least 100 nuclei per sample were counted.

(ii) To compare cell numbers, photographs of cells within a fixed area of 0.25 cm^2 were taken on seven consecutive days and the cells in this fixed area were counted. Relative cell numbers based on several experiments are presented. Flow cytometry analysis was carried out and total flat cell populations were gated and analyzed as described (Phillips *et al.*, 1997).

(iii) The immunocytochemical staining for pRb was performed as described (Latham *et al.*, 1996).

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References

Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (1990) *Current Protocols in Molecular Biology.* Green Publishing Associates/Wiley-Interscience, New York.

Barak,Y., Juven,T., Haffner,R. and Oren,M. (1993) mdm-2 expression is induced by wild-type p53 activity. *EMBO J.*, **12**, 461–468.

Berry,D.E., Lu,Y., Schmidt,B., Fallon,P.G., O'Connell,C., Hu,S.-X., Xu,H.-J. and Blank,G. (1996) Retinoblastoma protein inhibits IFN-gamma induced apoptosis. *Oncogene*, **12**, 1809–1819.

Chen,C. and Okayama,H. (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.*, **7**, 2745–2752.

Chen,X., Ko,L.J., Jayaraman,L. and Prives,C. (1996) p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells. *Genes Dev.*, **10**, 2438–2451.

Chow,K.N. and Dean,D.C. (1996) Domains A and B in the Rb pocket interact to form a transcriptional repressor motif. *Mol. Cell. Biol.*, **16**, 4862–4868.

Clarke,A.R., Maandag,E.R., van Roon,M., van der Lugt,N.M., van der Valk,M., Hooper,M.L., Berns,A. and te Riele,H. (1992) Requirement for a functional Rb-1 gene in murine development. *Nature*, **359**, 328–330.

Cobrinik,D., Whyte,P., Peeper,D.S., Jacks,T. and Weinberg,R.A. (1993) Cell cycle-specific association of E2F with the p130 E1A-binding protein. *Genes Dev.*, **7**, 2392–2404.

Dyson,N., Howley,P.M., Munger,K. and Harlow,E. (1989) The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science*, **243**, 934–937.

El-Deiry,W.S. *et al.* (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell*, **75**, 817–825.

Ewen,M.E., Faha,B., Harlow,E. and Livingston,D.M. (1992) Interaction of p107 with cyclin A independent of complex formation with viral oncoproteins. *Science*, **255**, 85–87.

Fan,G.-S., Ma,X.-M., Kren,B. and Steer,C.J. (1996) The retinoblastoma gene product inhibits TGF-β1 induced apoptosis in primary rat hepatocytes and human HuH-7 hepatoma cells. *Oncogene*, **12**, 1909–1919.

Field,S.J., Tsai,F.Y., Kuo,F., Zubiaga,A.M., Kaelin,W.G.,Jr, Livingston,D.M., Orkin,S.H. and Greenberg,M.E. (1996) E2F-1 functions in mice to promote apoptosis and suppress proliferation. *Cell*, **85**, 549–561.

Haas-Kogan,D.A., Kogan,S.C., Levi,D., Dazin,P., T'Ang,A., Fung,Y.-K.T. and Israel,M.A. (1995) Inhibition of apoptosis by the retinoblastoma gene product. *EMBO J.*, **14**, 461–472.

Han,J., Sabbatini,P., Perez,D., Rao,L., Modha,D. and White,E. (1996) The E1B 19K protein blocks apoptosis by interacting with and inhibiting the p53-inducible and death-promoting Bax protein. *Genes Dev.*, **10**, 461–477.

Hannon,G.J., Demetrick,D. and Beach,D. (1993) Isolation of the Rb-related p130 through its interaction with CDK2 and cyclins. *Genes Dev.*, **7**, 2378–2391.

Hiebert,S.W. (1993) Regions of the retinoblastoma gene product required for its interaction with the E2F transcription factor are necessary for E2 promoter repression and pRb-mediated growth suppression. *Mol. Cell. Biol.*, **13**, 3384–3391.

Hinds,P.W., Mittnacht,S., Dulic,V., Arnold,A., Reed,S.I. and Weinberg,R.A. (1992) Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell*, **70**, 993–1006.

Horowitz,J.M., Park,S.H., Bogenmann,E., Cheng,J.C., Yandell,D.W., Kaye,F.J., Minna,J.D., Dryja,T.P. and Weinberg,R.A. (1990) Frequent inactivation of the retinoblastoma anti-oncogene is restricted to a subset of human tumor cells. *Proc. Natl Acad. Sci. USA*, **87**, 2775–2779.

Hsieh,J.-K., Fredersdorf,S., Kouzarides,T., Martin,K. and Lu,X. (1997) E2F1-induced apoptosis requires DNA binding but not transactivation and is inhibited by the retinoblastoma protein through direct interaction. *Genes Dev.*, **11**, 1840–1852.

Huang,H.J., Yee,J.K., Shew,J.Y., Chen,P.L., Bookstein,R., Friedmann,T., Lee,E.Y. and Lee,W.H. (1988) Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells. *Science*, **242**, 1563–1566.

Hurford,R.K., Cobrinik,D., Lee,M.-H. and Dyson,N. (1997) pRb and P107/p130 are required for the regulated expression of different sets of E2F responsive genes. *Genes Dev.*, **11**, 1447–1463.

Jacks,T., Fazeli,A., Schmitt,E.M., Bronson,R.T., Goodell,M.A. and Weinberg,R.A. (1992) Effects of an Rb mutation in the mouse. *Nature*, **359**, 295–300.

Kaelin,W.G., Pallas,D.C., DeCaprio,J.A., Kaye,F.J. and Livingston,D.M. (1991) Identification of cellular proteins that can interact specifically with the T/E1A-binding region of the retinoblastoma gene product. *Cell*, **64**, 521–532.

Kratzke,R.A., Otterson,G.A., Hogg,A., Coxon,A.B., Geradts,J., Cowell,J.K. and Kaye,F.J. (1994) Partial inactivation of the RB product in a family with incomplete penetrance of familial retinoblastoma and benign retinal tumors. *Oncogene*, **9**, 1321–1326.

Kraus,V.B., Inostroza,J.A., Yeung,K., Reinberg,D. and Nevins,J.R. (1994) Interaction of the Dr1 inhibitory factor with the TATA binding protein is disrupted by adenovirus E1A. *Proc. Natl Acad. Sci. USA*, **91**, 6279–6282.

Latham,K.M., Eastman,S.W., Wong,A. and Hinds,P.W. (1996) Inhibition of p53-mediated growth arrest by overexpression of cyclin-dependent kinases. *Mol. Cell. Biol.*, **16**, 4445–4455.

Lee,E.Y., Chang,C.Y., Hu,N., Wang,Y.C., Lai,C.C., Herrup,K., Lee,W.H. and Bradley,A. (1992) Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. *Nature*, **359**, 288–294.

Lee,E.Y., Hu,N., Yuan,S.S., Cox,L.A., Bradley,A., Lee,W.H. and Herrup,K. (1994) Dual roles of the retinoblastoma protein in cell cycle regulation and neuron differentiation. *Genes Dev.*, **8**, 2008–2021.

Li,L.J., Naeve,G.S. and Lee,A.S. (1993) Temporal regulation of cyclin A–p107 and p33cdk2 complexes binding to a human thymidine kinase promoter element important for G_1–S phase transcriptional regulation. *Proc. Natl Acad. Sci. USA*, **90**, 3554–3558.

Maandag,E.C., van der Valk,M., Vlaar,M., Feltkamp,C., O'Brien,J., van Roon,M., van der Lugt,N., Berns,A. and te Riele,H. (1994) Developmental rescue of an embryonic-lethal mutation in the retinoblastoma gene in chimeric mice. *EMBO J.*, **13**, 4260–4268.

Macleod,K.F., Hu,Y. and Jacks,T. (1996) Loss of Rb activates both p53-dependent and independent cell death pathways in the developing mouse nervous system. *EMBO J.*, **15**, 6178–6188.

Mayol,X., Grana,X., Baldi,A., Sang,N., Hu,G. and Giordano,H. (1993) Cloning of a new member of the retinoblastoma gene family (pRb2) which binds to the E1A transforming domain. *Oncogene*, **8**, 2561–2566.

Michalovitz,D., Halevy,O. and Oren,M. (1990) Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. *Cell*, **62**, 671–680.

Mittnacht,S. and Weinberg,R.A. (1991) G_1/S phosphorylation of the retinoblastoma protein is associated with an altered affinity for the nuclear compartment. *Cell*, **65**, 381–393.

Mittnacht,S., Hinds,P.W., Dowdy,S.F. and Weinberg,R.A. (1991) Modulation of retinoblastoma protein activity during the cell cycle. *Cold Spring Harbor Symp. Quant. Biol.*, **56**, 197–209.

Mittnacht,S., Lees,J.A., Desai,D., Harlow,E., Morgan,D.O. and Weinberg,R.A. (1994) Distinct sub-populations of the retinoblastoma protein show a distinct pattern of phosphorylation. *EMBO J.*, 13, 118–127.

Morgenbesser,S.D., Williams,B.O., Jacks,T. and DePinho,R.A. (1994) p53-dependent apoptosis produced by Rb-deficiency in the developing mouse lens. *Nature*, **371**, 72–74.

Morgenstern,J.P. and Land,H. (1990) Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.*, **18**, 3587–3596.

Neuman,E., Flemington,E.K., Sellers,W.R. and Kaelin,W.G. (1994) Transcription of the E2F-1 gene is rendered cell cycle dependent by E2F DNA-binding sites within its promoter. *Mol. Cell. Biol.*, **14**, 6607–6615.

Novitch,B.G., Mulligan,G.J., Jacks,T. and Lassar,A.B. (1996) Skeletal muscle cells lacking the retinoblastoma protein display defects in muscle gene expression and accumulate in S and G_2 phases of the cell cycle. *J. Cell Biol.*, **135**, 441–456.

Okamoto,K. and Beach,D. (1994) Cyclin G is a transcriptional target of the p53 tumor suppressor protein. *EMBO J.*, **13**, 4816–4822.

Phillips,A.C., Bates,S., Ryan,K.M., Helin,K. and Vousden,K.H. (1997) Induction of DNA synthesis and apoptosis are separable functions of E2F-1. *Genes Dev.*, **11**, 1853–1863.

Qin,X.Q., Chittenden,T., Livingston,D.M. and Kaelin,W.G.,Jr (1992) Identification of a growth suppression domain within the retinoblastoma gene product. *Genes Dev.*, **6**, 953–964.

Qin,X.Q., Livingston,D.M., Kaelin,W.G. and Adams,P.D. (1994) Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. *Proc. Natl Acad. Sci. USA*, **91**, 10918–10922.

Qin,X.Q., Livingston,D.M., Ewen,M., Sellers,W.R., Arany,Z. and Kaelin,W.G.,Jr (1995) The transcription factor E2F-1 is a downstream target of RB action. *Mol. Cell. Biol.*, **15**, 742–755.

Schneider,J.W., Gu,W., Zhu,L., Mahdavi,V. and Nadal-Ginard,B. (1994) Reversal of terminal differentiation mediated by p107 in Rb–/– muscle cells. *Science*, **264**, 1467–1471.

Sellers,W.R., Rodgers,J.W. and Kaelin,W.G. (1995) A potent transrepression domain in the retinoblastoma protein induces a cell cycle arrest when bound to E2F sites. *Proc. Natl Acad. Sci. USA*, **92**, 11544–11548.

Shan,B., Durfee,T. and Lee,W.H. (1996) Disruption of RB/E2F-1 interaction by single point mutations in E2F-1 enhances S-phase entry and apoptosis. *Proc. Natl Acad. Sci. USA*, **93**, 679–684.

Sherr,C. (1996) Cancer cell cycles. *Science*, **274**, 1672–1677.

Smith,J.R. and Pereira-Smith,O.M. (1996) Replicative senescence: implications for *in vivo* aging and tumor suppression. *Science*, **273**, 63–67.

Taya,Y. (1997) From RB kinases and RB-binding proteins—new points of view. *Trends Biochem. Sci.*, **22**, 14–17.

Templeton,D.J., Park,S.H., Lanier,L. and Weinberg,R.A. (1991) Nonfunctional mutants of the retinoblastoma protein are characterized by defects in phosphorylation, viral oncoprotein association, and nuclear tethering. *Proc. Natl Acad. Sci. USA*, **88**, 3033–3037.

Tiemann,F., Musunuru,K. and Hinds,P.W. (1997) The retinoblastoma tumour suppressor protein and cancer. In Swallow,D.M. and Edwards,Y.H. (eds), *Protein Dysfunction and Human Genetic Disease*. BIOS Scientific Publishers, Oxford, UK, 163–185.

van den Heuvel,S. and Harlow,E. (1993) Distinct roles for cyclin-dependent kinases in cell cycle control. *Science*, **262**, 2050–2054.

Weinberg,R.A. (1995) The retinoblastoma protein and cell cycle control. *Cell*, **81**, 323–330.

Wu,X. and Levine,A.J. (1994) p53 and E2F-1 cooperate to mediate apoptosis. *Proc. Natl Acad. Sci. USA*, **91**, 3602–3606.
Yamasaki,L., Jacks,T., Bronson,R., Goillot,E., Harlow,E. and Dyson,N.J. (1996) Tumor induction and tissue atrophy in mice lacking E2F-1. *Cell*, **85**, 537–548.
Yonish-Rouach,E., Resnitzky,D., Lotem,J., Sachs,L., Kimchi,A. and Oren,M. (1991) Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. *Nature*, **352**, 345–347.
Zacksenhaus,E., Jiang,Z., Chung,D., Marth,J.D., Phillips,R.A. and Gallie,B.L. (1996) pRb controls proliferation, differentiation, and death of skeletal muscle cells and other lineages during embryogenesis. *Genes Dev.*, **10**, 3051–3064.
Zhu,L., van den Heuvel,S., Helin,K., Fattaey,A., Ewen,M., Livingston,D., Dyson,N. and Harlow,E. (1993) Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. *Genes Dev.*, **7**, 1111–1125.
zur Hausen,H. (1991) Human papillomaviruses in the pathogenesis of anogenital cancer. *Virology*, **184**, 9–13.

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