# pRb Inactivation in Senescent Cells Leads to an E2F-Dependent Apoptosis Requiring p73

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## Abstract

**Senescent cells in which pRb is inactivated undergo apoptosis on attempted reinitiation of DNA synthesis. To further explore the cell death resulting from loss of pRb function in senescent cells, we employed a temperature-sensitive pRb mutant protein (tspRb). We found that tspRb inactivation results in rapid E2F reactivation and subsequent S-phase reentry associated with the up-regulation of E2F target gene expression and cyclin E-dependent kinase activity. Total inhibition of cyclin-dependent kinase 2 activity results in a cell cycle arrest on pRb loss and a nearly complete suppression of apoptosis. Furthermore, blocking of E2F activity with a dominant-negative DP1 inhibits S-phase reentry and cell death following tspRb inactivation. Finally, inhibition of p73 activity abolishes apoptosis but not S-phase entry on pRb inactivation, suggesting that activation of E2F in senescent cells can result in the use of p73 as a cell death effector. Interestingly, senescent cells rescued from apoptosis maintain their altered shape and express senescence-associated β-galactosidase despite loss of pRb function. Thus, maintenance of the terminal cell cycle arrest of senescent cells requires continuous pRb-mediated inactivation of E2F activity, the reappearance of which in these irrevocably altered cells triggers a cell death program instead of an inappropriate resumption of cell cycling.**

## Introduction

The retinoblastoma gene (*RB1*) is frequently found to be mutated in many human cancers. This is thought to be due to the ability of its protein product, pRb, to inhibit cellular proliferation by negatively regulating the $G_1$-S transition of the cell cycle (1). The antiproliferative effect of pRb is mediated partly by its ability to repress the E2F-DP1 heterodimer transcriptional activation of genes required for S-phase entry. The role of pRb in the negative regulation of cellular proliferation is highlighted by mutation of the *RB1* gene in mice. *RB1* heterozygous mice are cancer prone, and cells from the central nervous system of nullizygous embryos display an increase in free E2F, ectopic S-phase entry, and p53-dependent apoptosis (2, 3). Interestingly, the phenotypes of *RB1* heterozygous and nullizygous mice are similar to those observed on E2F overexpression—they display unscheduled cell cycle entry and, in the case of E2F1, an induction of apoptosis that has been found to be both p53 dependent and independent (4–6). Recent data indicate that E2F1-induced apoptosis, which is independent of p53, may be due to a related family member p73 (7, 8). Strongly implicating E2F as a downstream effector of pRb in cell cycle regulation, mutation of either E2F1 or E2F3 and Rb in mice suppresses the apoptosis and inappropriate S-phase entry observed in $pRb^{-/-}$ embryos (9–11). Still, the repressive effect of pRb on cell cycle progression is reversible; on proper extracellular signaling, there is relief of cyclin-dependent kinase (cdk) inhibitor (CDKI)-mediated repression of cdk activity that allows phosphorylation and inactivation of pRb. This alleviates pRb-mediated repression of E2F and allows S-phase progression (12).

pRb has also been observed to induce a nonreversible, terminal cell cycle exit termed senescence. Senescence is thought to be antitumorigenic, as it is observed in cell culture in response to prolonged proliferative signaling or after a particular number of population doublings (13–16). In both cases, by permanently removing cells from the cell cycle, the senescence process could prevent the accumulation of deleterious mutations that lead to oncogenic transformation. Senescent cells are characterized by a number of features, including up-regulation of cell cycle inhibitors, altered cell morphology, and arrest in the $G_1$ phase (17). The $G_1$ arrest has been characterized as irreversible, as senescent cells can no longer enter S phase in response to mitogenic stimulation despite the fact that the cells remain metabolically active (17). Differences in the size and nuclear/cytoplasmic balance in cells senescently arrested versus those quiescently arrested suggest that the senescence process not only blocks S-phase entry but may also change the cells such that their ability to progress in the cell cycle is irrevocably altered (18). However, expression of SV40 T-antigen has been observed to overcome senescent cell cycle arrest and induce DNA synthesis, albeit at low frequency (19). As SV40 T-antigen binds to and inactivates pRb (20, 21), this suggests that pRb may function in the irreversible senescent arrest.

More directly implicating pRb in the irreversibility of senescence, we and others have found that inactivation of reintroduced PRb in $PRb^{-/-}/p53^{-/-}$ SAOS-2 cells result in

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massive cell death 24 h subsequent to loss of pRb activity (22, 23). This cell death was associated with the initiation of DNA replication and was determined to be apoptotic, as expression of the antiapoptotic protein Bcl-2 prevented it (22). Furthermore, using an inducible system, reintroduction of the CDKI $p16^{INK4a}$ into $p16^{INK4a-/-}$ U2OS cells, effectively reconstituting the pRb pathway, led to senescence (24). Similar to the results with pRb inactivation, turning off $p16^{INK4a}$ expression in these senescent cells also resulted in some S-phase reentry and cell death (24). To begin to understand the antioncogenic nature of the senescence process, we have chosen to study the involvement of pRb in the terminality of the senescent phenotype. Thus, we have further investigated how senescent cells die on loss of pRb function using a temperature-sensitive pRb mutant protein (tspRb) to serially activate and inactivate pRb. We have found that on loss of pRb function in senescent cells, there is a reactivation of E2F, causing S-phase reentry and activation of an E2F apoptotic program requiring p73. We propose that this proliferative signaling in irreversibly altered senescent cells induces them to undergo apoptosis rather than inappropriately return them to the cell cycle. Because these cells lack p53, these results demonstrate that this process can be p53 independent and importantly suggest a cancer therapy through the sequential introduction and inactivation of Rb in tumor cells that do not require indefinite gene expression or wild-type p53 status.

## Results

### *tspRb Inactivation in Senescent Cells Leads to DNA Synthesis and Apoptosis*

We have observed previously that pRb inactivation in senescent cells results in the apoptosis of almost half of these cells within 24 h (22). To more fully understand the mechanism by which these cells undergo apoptosis, we characterized the changes senescent cells undergo when pRb function is eliminated using the tspRb XX668 (25, 26). The use of a tspRb allowed us to more rapidly control pRb activity, as the long half-life of pRb makes inducible systems less sensitive to pRb inactivation. tspRb is active at 32.5°C, inducing a $G_1$ growth arrest and senescence and inhibiting colony formation, but is only very weakly functional at 37°C (22). The ability of tspRb to function at 32.5°C as wild-type pRb does is at least partly due to its ability to stably associate with the nucleus and repress E2F transcriptional activation (22).

To study the response of cells to acute loss of tspRb function, SAOS-2 cells were cotransfected with tspRb and pBabepuro expression plasmids at 37°C, shifted to 32.5°C 48 h posttransfection, and put under puromycin selection to identify transfected cells (Fig. 1A). After 10 days at 32.5°C, senescent cells were shifted back to 37°C, and assays were performed over a 3-day period (Fig. 1A). Previous work indicated that senescent cells were actively synthesizing DNA 24 h after loss of pRb activity (22). To determine when these cells first began to reenter S phase, senescent cells were monitored for incorporation of bromodeoxyuridine (BrdUrd) over a period of 72 h after being shifted to 37°C. Cells incubated at 32.5°C for 10 days ($T = 0$) were essentially BrdUrd negative (Fig. 1B), while a significant number of cells transfected with tspRb and incubated at 37°C for 10 days were BrdUrd positive (data not shown). After the shift of these senescent cells to 37°C and subsequent loss of pRb function, they began to incorporate BrdUrd as early as 7 h later (Fig. 1, B and C). From a peak at 12 h, there was a decrease in BrdUrd-positive cells at 24 h, suggesting that the cells that had reentered the cell cycle earliest had succumbed to apoptosis (Fig. 1B). However, the number of BrdUrd-positive cells again increased by 72 h at 37°C, indicating that the remaining cells continued to enter the cell cycle (Fig. 1, B and C). We attribute this nonhomogeneous reentry of cells into S phase to the asynchronous inactivation of tspRb in different senescent cells.

To further identify the effect of loss of tspRb on apoptosis, we detected the cleavage of poly-ADP-ribose polymerase (PARP), which has been suggested to be important for the termination of DNA repair during apoptosis and extensively used as a marker of apoptosis. PARP breakdown was detectable when senescent cells expressing transfected tspRb were shifted to 37°C for 24–72 h. The original 112-kd protein was degraded to yield an 85-kd fragment during the apoptotic process (Fig. 1D). These results indicated that the activity of pRb was important for survival of senescent cells. Inactivation of pRb in senescent cells resulted in S-phase reentry and subsequent initiation of an apoptotic process.

Finally, senescent cells in which pRb had been inactivated by temperature shift were stained for a marker of senescence, senescence-associated β-galactosidase (SA-β-gal). Typically about 75% of Rb-transfected cells with an enlarged, flattened senescent morphology stain positively for SA-β-gal after 10 days (23, 27). However, there was a sharp decrease in the percentage of remaining cells that were SA-β-gal positive after 72 h at 37°C, suggesting that the senescent, flat cells were selectively dying (Fig. 1E).

### *tspRb Inactivation in Senescent Cells Leads to a Rapid Reappearance of E2F Activity and Activation of Cell Cycle Components*

We next wanted to determine if the activity of E2F was altered by loss of pRb function, possibly contributing to this S-phase reentry. To do this, we used a pRb-repressible reporter construct containing E2F-responsive elements driving green florescence protein (GFP) gene expression (28, 29). By immunofluorescence, we observed that cells with active tspRb at 32.5°C for 10 days were negative for E2F activity (Fig. 2A). However, on tspRb inactivation, senescent cells quickly exhibited GFP positivity, indicating reactivation of E2F as early as 4 h after incubation at 37°C. GFP-positive cells remained apparent throughout the time course, and many GFP-positive cells could clearly be seen to be undergoing apoptosis by 24 h at 37°C (Fig. 2A and data not shown). The timing of E2F reactivation at 4 h after tspRb inactivation suggested that the initiation of DNA synthesis we observed at 9 h may be due to E2F activity. In addition to E2F-GFP reporter gene activation, E2F *in vivo* target gene expression was also increased. Immunoblot analysis of cells at 0, 4, 8, 12, 24, 48, and 72 h postshift to 37°C after 10 days at 32.5°C indicated that cyclin E, cyclin A, and cdk2 levels, which were low in senescent cells, had increased (Fig. 2B). In contrast, the level of

cdk4 showed little change. In addition, levels of the CDKI $p27^{KIP1}$, whose up-regulation had been previously linked to pRb expression and was shown to be required for pRb-mediated cell cycle arrest and senescence in these cells (27), decreased in a time-dependent manner after incubation at 37°C. To test whether the increased expression level of cyclin E and cdk2 was accompanied by increased kinase activity, cell lysates harvested at indicated time points were immunoprecipitated with anti-cyclin E, and cyclin E-associated kinase activity was determined. The activity of cyclin E-associated kinase in senescent cells was essentially undetectable (Fig. 2C). In contrast, this activity was clearly up-regulated by 8 h after incubation at 37°C. Altogether, these results suggest that restoration of cyclin E/cdk2 activity follows E2F reactivation in tspRb-expressing cells, and this activity may be required for S-phase onset and subsequent apoptosis.

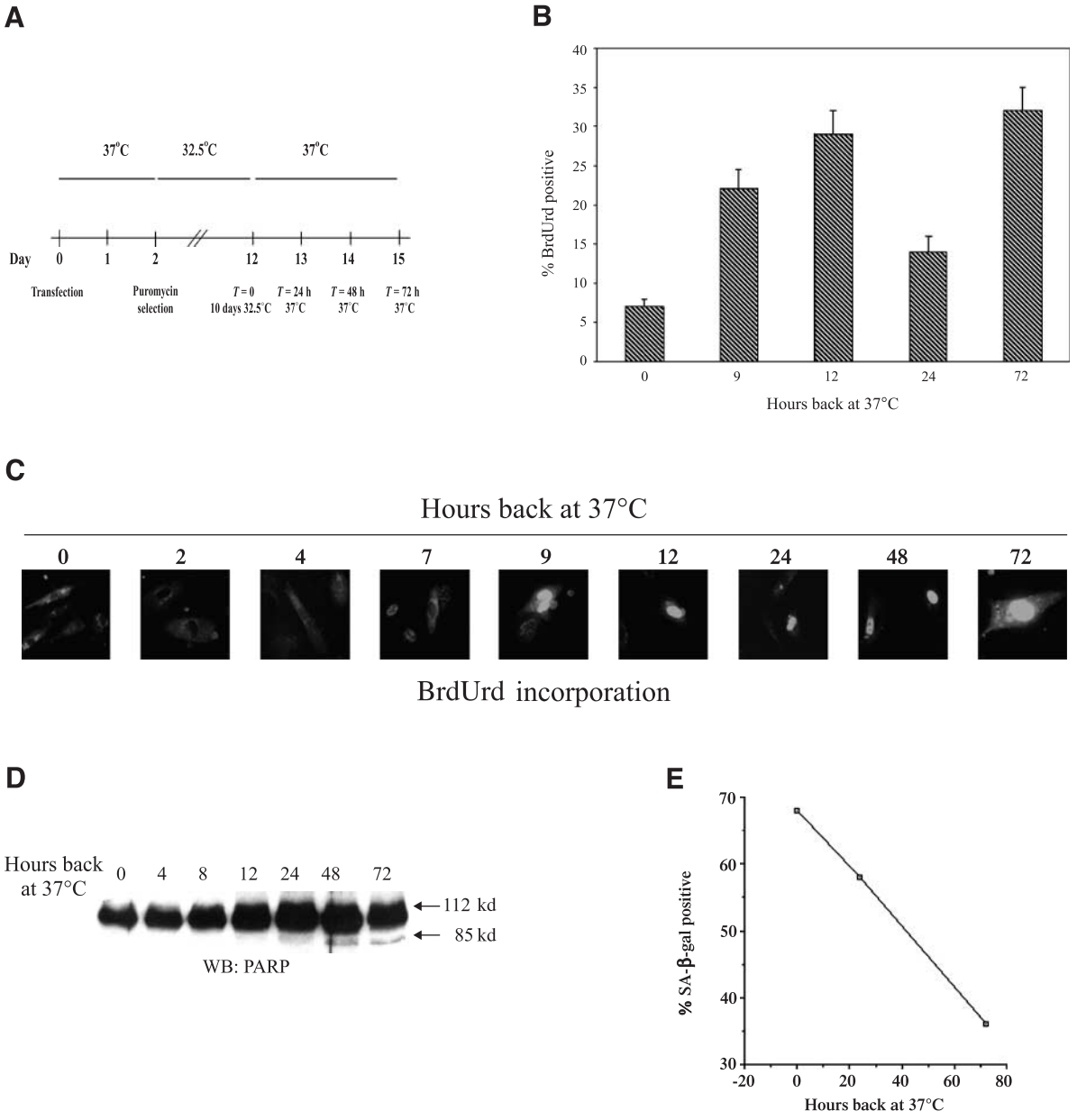



**FIGURE 1.** tspRb inactivation leads to S-phase entry and apoptosis in senescent cells. **A.** Experimental system. SAOS-2 cells are transfected with tspRb and a puromycin resistance plasmid (pBabepuro) at 37°C and shifted 24–48 h later to 32.5°C, the permissive temperature for tspRb activity. After 10 days at 32.5°C, cells are shifted back to the nonpermissive temperature of 37°C, inactivating tspRb, and assayed for various phenotypes. **B.** Cells transfected with tspRb were assayed for BrdUrd incorporation at 0, 12, 18, 24, and 72 h after cells were shifted back to 37°C. At least 100 cells were counted. **C.** At the indicated time points, cells transfected with tspRb were stained for BrdUrd incorporation. **D.** Immunoblot of tspRb-transfected cells at the indicated time points with anti-PARP monoclonal antibody. **E.** Number of tspRb-transfected cells staining positively for SA-β-gal activity at 0, 24, and 72 h after tspRb inactivation. Results indicate a representative experiment of one performed several times.

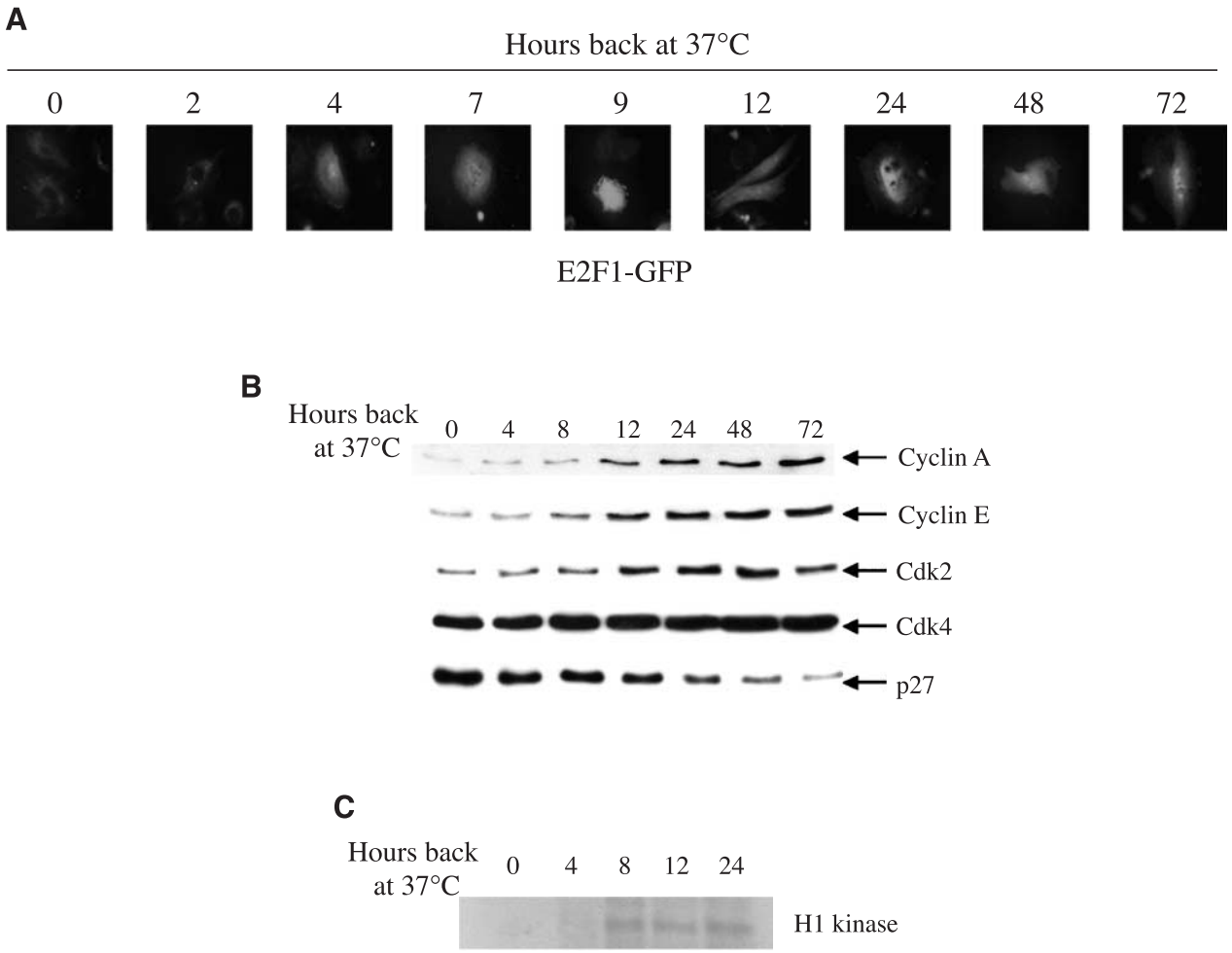



**FIGURE 2.** tspRb inactivation leads to the reactivation of E2F and cell cycle components in senescent cells. **A.** At the indicated time points, cells cotransfected with tspRb and an E2F1-GFP reporter were analyzed by fluorescence microscopy for GFP-positive cells. **B.** Immunoblot of tspRb-transfected cells at the indicated time points with cyclin A, cyclin E, cdk2, cdk4, and p27 antibodies. **C.** Cyclin E immunoprecipitations from lysates of tspRb transfectants were subjected to an *in vitro* kinase assay at the indicated time points.

*Inhibition of cdk2 Activity Prevents Senescent Cell Death Caused by Loss of pRb Function*

As E2F overexpression and resultant unscheduled S-phase entry seem linked to cellular apoptosis, we wanted to determine if the reestablishment of cell cycle arrest would protect senescent cells from apoptosis on tspRb inactivation. Inhibition of cdk2 activity via $p21^{CIP1}$, $p27^{KIP1}$, or a dominant-negative cdk2 (dncdk2) has been observed to induce a $G_1$ cell cycle arrest independent of pRb (30–33). We investigated whether these cell cycle inhibitors could block tspRb inactivation-induced cell death. Previous studies found that $p21^{CIP1}$ expression level is low in SAOS cells, while increased $p27^{KIP1}$ has been linked to pRb-mediated cell cycle arrest and senescence in these cells (27). Thus, SAOS cells cotransfected with tspRb + $p27^{KIP1}$ or dncdk2 were incubated at 32.5°C for 10 days. Cells were then monitored for the amount of cell death after shift to 37°C. $p27^{KIP1}$ and dncdk2 decreased the amount of cell death seen in tspRb-transfected cells by at most 20% within the first 24-h period at 37°C (Fig. 3A). Cell death increased with time in $p27^{KIP1}$ or dncdk2-expressing cells but remained lower than that observed in cells expressing tspRb alone. These results indicated that coexpression of the CDKIs only partially protected senescent cells from tspRb inactivation-mediated cell death.

We thought it possible that variable levels of $p27^{KIP1}$ or dncdk2 in transfected cells might leave some cells more capable of restricting cdk2 activity than others. To obtain a more homogeneous block of cdk2 activity in the cells, we used the kinase inhibitor roscovitine. Roscovitine induces a $G_1$ arrest in SAOS-2 cells and inhibits BrdUrd incorporation in both the absence and the presence of pRb (data not shown). To determine the effectiveness of roscovitine in inhibiting cdk2 activity, tspRb transfectants incubated for 10 days at 32.5°C were treated for 18 h with DMSO or roscovitine and shifted to 37°C for 24 h. Cells were harvested at those time points, and the resultant lysates were immunoprecipitated for cyclin E and assayed for cyclin E-associated kinase activity. While DMSO-treated cells in which tspRb was inactivated by temperature

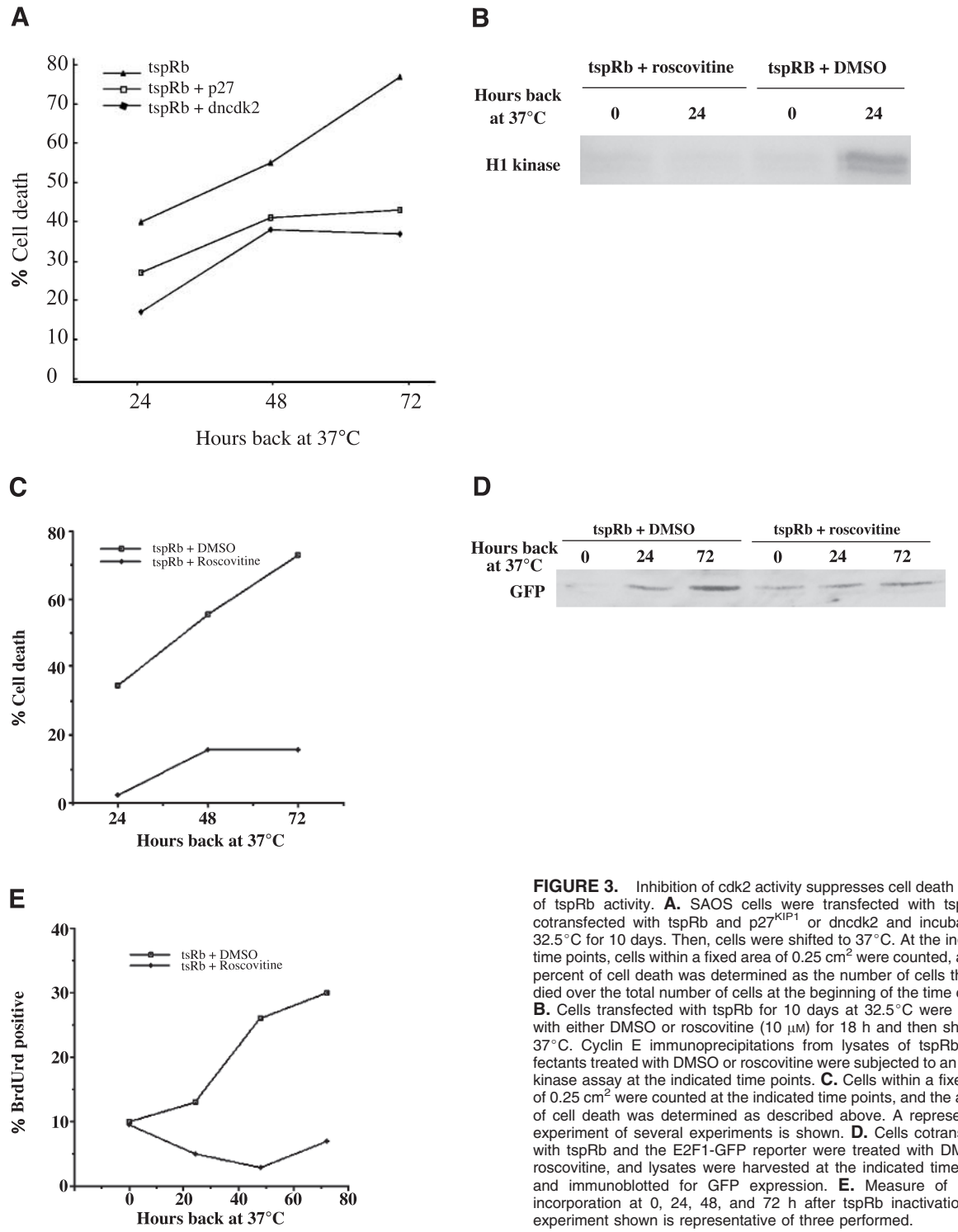



**FIGURE 3.** Inhibition of cdk2 activity suppresses cell death on loss of tspRb activity. **A.** SAOS cells were transfected with tspRb or cotransfected with tspRb and $p27^{KIP1}$ or dncdk2 and incubated at 32.5°C for 10 days. Then, cells were shifted to 37°C. At the indicated time points, cells within a fixed area of 0.25 $cm^2$ were counted, and the percent of cell death was determined as the number of cells that had died over the total number of cells at the beginning of the time course. **B.** Cells transfected with tspRb for 10 days at 32.5°C were treated with either DMSO or roscovitine (10 μM) for 18 h and then shifted to 37°C. Cyclin E immunoprecipitations from lysates of tspRb transfectants treated with DMSO or roscovitine were subjected to an *in vitro* kinase assay at the indicated time points. **C.** Cells within a fixed area of 0.25 $cm^2$ were counted at the indicated time points, and the amount of cell death was determined as described above. A representative experiment of several experiments is shown. **D.** Cells cotransfected with tspRb and the E2F1-GFP reporter were treated with DMSO or roscovitine, and lysates were harvested at the indicated time points and immunoblotted for GFP expression. **E.** Measure of BrdUrd incorporation at 0, 24, 48, and 72 h after tspRb inactivation. The experiment shown is representative of three performed.

shift to 37°C displayed increased cyclin E kinase activity, those treated with roscovitine did not (Fig. 3B). Inactivation of tspRb in roscovitine-treated senescent cells led to increased cell survival compared with DMSO-treated cells, with only about 10% of the roscovitine-treated cells dying overall (Fig. 3C). Furthermore, a GFP immunoblot of lysates from cells cotransfected with the E2F-GFP reporter construct demonstrated that after tspRb inactivation at 37°C, roscovitine was able to prevent some but not all E2F activity (Fig. 3D), suggesting that roscovitine inhibition of cdk2 activity may be acting primarily downstream of E2F reactivation. Similarly, roscovitine treatment significantly

inhibited BrdUrd incorporation on tspRb inactivation, again suggesting that roscovitine was acting to block cell cycle progression (Fig. 3E). Overall, these results demonstrate that inhibition of cell cycle progression, specifically through the inhibition of cdk2 activity, protects cells from death on loss of pRb function and implies that S-phase entry after tspRb inactivation in senescent cells leads to apoptosis.

### *Progression Through S Phase Triggers tspRb Inactivation-Mediated Cell Death*

Reentry into S phase appeared to be tightly linked to the death observed on loss of pRb activity in senescent cells. Still, it was unclear if it was the loss of a $G_1$ arrest, the process of DNA replication, or an abortive attempt of morphologically altered senescent cells to go through mitosis that resulted in cell death. To distinguish among these possibilities, we used drugs that blocked cells in various stages of the cell cycle to determine if traversal through a particular cell cycle phase was fatal to these senescent cells. After 10 days at 32.5°C, cells were treated for 18 h at 32.5°C with DMSO, hydroxyurea (HU), which arrests cells in early S phase after cdk2 activation, or nocodazole, which blocks cells in the $G_2$-M phase of the cell cycle. Treatment of senescent cells with these drugs for 18 h to several days did not induce cell death (data not shown). Interestingly, HU, by blocking cells in early S phase, largely protected senescent cells from death for 48 h after loss of tspRb function at 37°C (Fig. 4A). In fact, cells treated with HU did not undergo significant apoptosis until 72 h after tspRb inactivation (Fig. 4A). In contrast, nocodazole-treated senescent cells were afforded no protection from tspRb inactivation-mediated cell death; they underwent apoptosis at the same rate as cells treated with DMSO (Fig. 4A).

We then looked at the ability of senescent cells treated with HU and nocodazole to incorporate BrdUrd on shift to 37°C. Cells treated with DMSO became BrdUrd positive at the expected rate on loss of tspRb activity, and cells treated with nocodazole behaved in the same manner (Fig. 4B). However, HU for the most part inhibited BrdUrd incorporation for 48 h (Fig. 4B), although some cells took on a partially BrdUrd-positive phenotype like that seen in tspRb-senescent cells after 7 h at 37°C (Fig. 1C). Still, by 72 h after tspRb inactivation, some HU-treated cells were fully BrdUrd positive, correlating with the increase in death at that time (Fig. 4B). Overall, the results with roscovitine, HU, and nocodazole treatment suggested that senescent cells without functional pRb underwent apoptosis before the $G_2$-M phase but after both $G_1$ and early S phase of the cell cycle, indicating that it was progression into S phase that was fatal to these cells.

### *Repression of E2F Activity Abolishes tspRb Inactivation-Induced Cell Death*

It has been shown previously that deletion of Rb in mice leads to E2F dysregulation and apoptosis (34–36) and that E2F1–3 overexpression also results in increased S-phase entry and apoptosis (34–38). Having demonstrated that the death of cells on loss of pRb function was apoptotic (22) and due to S-phase reentry after E2F activation (Figs. 2 and 4), we wanted to determine the role of E2F in the cell cycle progression and cell death we had observed. To do this, we cotransfected tspRb with dominant-negative DP1 (dnDP1)—one of the heterodimeric partners of E2F family members that are primarily responsible for the DNA binding ability of E2F (39). dnDP1, mutated in its ability to bind DNA, thus inhibiting E2F-DNA binding, induces a $G_1$ growth arrest and suppresses E2F activity in SAOS-2 cells (39; data not shown). Cells cotransfected with tspRb and dnDP1 became flat and senescent after 10 days at 32.5°C. They were then shifted to 37°C to

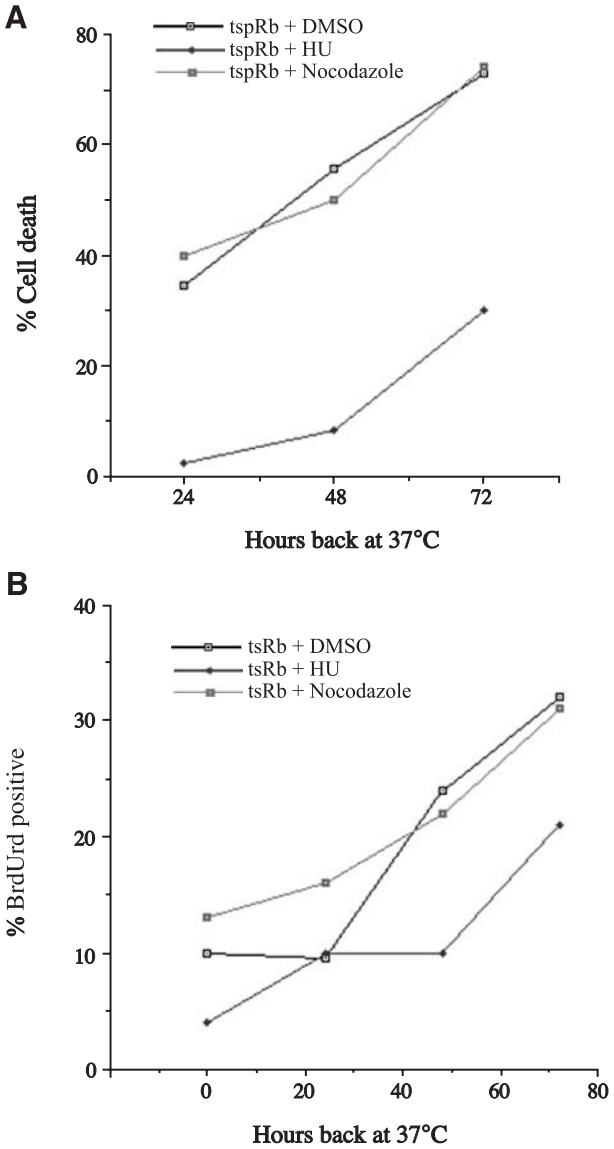



**FIGURE 4.** S-phase progression triggers cell death due to tspRb inactivation. Cells were transfected with tspRb. After 10 days at 32.5°C, cells were treated for 18 h with DMSO, HU (10 μM), or nocodazole (100 ng/ml) and shifted to 37°C. **A.** Amount of cell death was measured as the number of cells counted within a fixed area of 0.25 $cm^2$ at the indicated times over the total number of cells at the beginning of the time course ($T$ = 0). The relative cell death based on several experiments is presented. **B.** BrdUrd incorporation at 0, 24, 48, and 72 h after tspRb inactivation of transfectants treated with DMSO, HU, or nocodazole. A representative experiment of three experiments is shown.

inactivate tspRb after which a cell survival assay was performed. We found that up to 72 h (and several days) after the shift of senescent cells to 37°C, cells cotransfected with dnDP1 displayed an almost complete absence of cell death compared with tspRb-transfected cells alone (Fig. 5A), suggesting that E2F activity was responsible for the cell death on tspRb inactivation in senescent cells.

We then looked at the ability of dnDP1 to inhibit S-phase reentry to determine if cell cycle progression was in fact a result of E2F activity. Cells cotransfected with tspRb and dnDP1 that were incubated at 37°C for 10 days displayed low levels of BrdUrd incorporation indicative of $G_1$ arrest (data not shown). Interestingly, we observed that dnDP1 cotransfected with both wild-type pRb (data not shown) and tspRb and incubated for 10 days at 32.5°C perturbed tspRb $G_1$ arrest somewhat, increasing BrdUrd-positive cells over that of pRb alone (Fig. 5B). This phenomenon may be due to some dnDP1 titration of pRb-E2F complexes from E2F target promoters, resulting in derepression of S-phase-related genes. However, despite the increase in BrdUrd-positive cells, these tspRb-dnDP1 cells still became flat and senescent at the same rate as cells expressing tspRb only. When the senescent cells were shifted to 37°C to inactivate tspRb, cells cotransfected with dnDP1 maintained virtually the same amount of BrdUrd-positive cells, while those transfected with tspRb alone quickly displayed increased numbers of BrdUrd-positive cells (Fig. 5B). This repression of additional cell cycle progression by dnDP1 in tspRb-inactivated senescent cells was associated with inhibition of cyclin E-associated kinase activity. Although cells transfected with tspRb alone had increased cyclin E kinase activity on loss of tspRb function, those cotransfected with dnDP1 maintained almost complete repression of cyclin E kinase activity (Fig. 5C), consistent with the previous finding that activity of cdk2 kinase complexes is required to trigger death in these cells (Fig. 3).

To determine if the ability of dnDP1 to suppress cell death and additional S-phase entry on tspRb inactivation was indeed linked to its ability to inhibit E2F activity, cells were also cotransfected with an E2F-GFP reporter construct. Immunoblot of lysates for GFP expression demonstrated that cells expressing tspRb and dnDP1 displayed only low GFP levels 24 h after shift to 37°C (Fig. 5D). In contrast, the expression of GFP in cells cotransfected with tspRb and E2F-GFP alone was clearly increased as early as 8 h after incubation at 37°C. Through immunofluorescence, we observed that a small population of cells expressing tspRb and dnDP1 did become GFP positive (data not shown), but these were likely the few cells that went on to die after 72 h at 37°C.

Interestingly, we found that the cells in which tspRb had been inactivated but were protected from apoptosis by dnDP1 coexpression maintained the enlarged, flattened morphology of pRb-senescent cells. To determine if these cells were in fact still senescent, they were stained for SA-β-gal. Unlike cells transfected with tspRb alone, which rapidly lost the SA-β-gal-positive population of cells on shift to 37°C, surviving cells coexpressing dnDP1 remained SA-β-gal positive despite loss of pRb function (Fig. 5E). As dnDP1 does not itself induce senescence in these cells, the maintenance of the senescent phenotype could be because the cells still expressed high levels of $p27^{KIP1}$ (data not shown), which has been shown to induce senescence on its own (27, 40). However, because $p27^{KIP1}$ cannot induce the morphology change that pRb does in these cells (27), the retention of the flat cell phenotype in arrested cells at the temperature nonpermissive for tspRb function suggested that this morphology change becomes pRb independent at some point following its induction.

### *Inhibition of p73 Blocks tspRb Inactivation-Induced Cell Death Downstream of E2F Reactivation*

While inappropriate S-phase reentry due to cdk2 reactivation in tspRb-senescent cells appeared to be responsible for the apoptosis of these cells, the mechanism by which apoptosis occurred remained unclear. The apoptosis induced by E2F1 overexpression has been found to be partly the result of activation of a p53-dependent apoptotic pathway (4, 6, 41, 42). This does not explain E2F-dependent apoptosis in this system, as SAOS-2 cells do not express p53 because of gene deletion. However, recent data indicate that p73, a p53 family member that can also induce apoptosis, is able to mediate E2F1-triggered apoptosis (7, 8, 43). Thus, we asked if the restored E2F activity in senescent cells was activating a p73-dependent cell death program.

Cells were cotransfected with tspRb and a dominant-negative p73 (p73DD) or an inactive mutant of p73DD (p73LP; 7). After cells were left for 10 days at 32.5°C, they formed phenotypically normal senescent cells and underwent the process of puromycin selection in a wild-type manner, indicating that p73DD does not interfere with senescence induction by pRb. After inactivation of tspRb in these cotransfectants at 37°C, they were assayed for cell survival. While cells cotransfected with p73LP behaved similarly to cells transfected with tspRb alone, those cotransfected with p73DD were almost completely protected from cell death (Fig. 6A). This indicated that a p73-dependent apoptotic program was being induced during tspRb inactivation-mediated cell death. Cells cotransfected with tspRb and p73DD or p73LP were then assayed for BrdUrd incorporation to determine if the p73 mutants affected the ability of the cells to reinitiate DNA synthesis on loss of tspRb function. Neither p73DD nor p73LP inhibited senescent cells in which tspRb had been inactivated from becoming BrdUrd positive, indicating that S-phase reentry was independent of p73 function (Fig. 6B). Previous data (Fig. 5) had linked reinitiation of DNA synthesis to E2F activity. To ascertain if inhibition of p73-mediated apoptosis was occurring downstream of E2F activation, cells were also cotransfected with an E2F-GFP reporter construct and monitored for E2F reactivation. Through immunofluorescence, we observed that cells transfected with tspRb or cotransfected with tspRb and p73LP or p73DD all displayed GFP-positive cells with about the same kinetics on tspRb inactivation at 37°C (Fig. 6C and data not shown).

As p73 has been demonstrated to be an E2F target gene in its induction of apoptosis (7, 8), we investigated whether p73 levels changed during tspRb inactivation-mediated cell death. A Northern blot for the p73 message showed little p73 transcript in tspRb-transfected cells incubated for 10 days at 37°C or

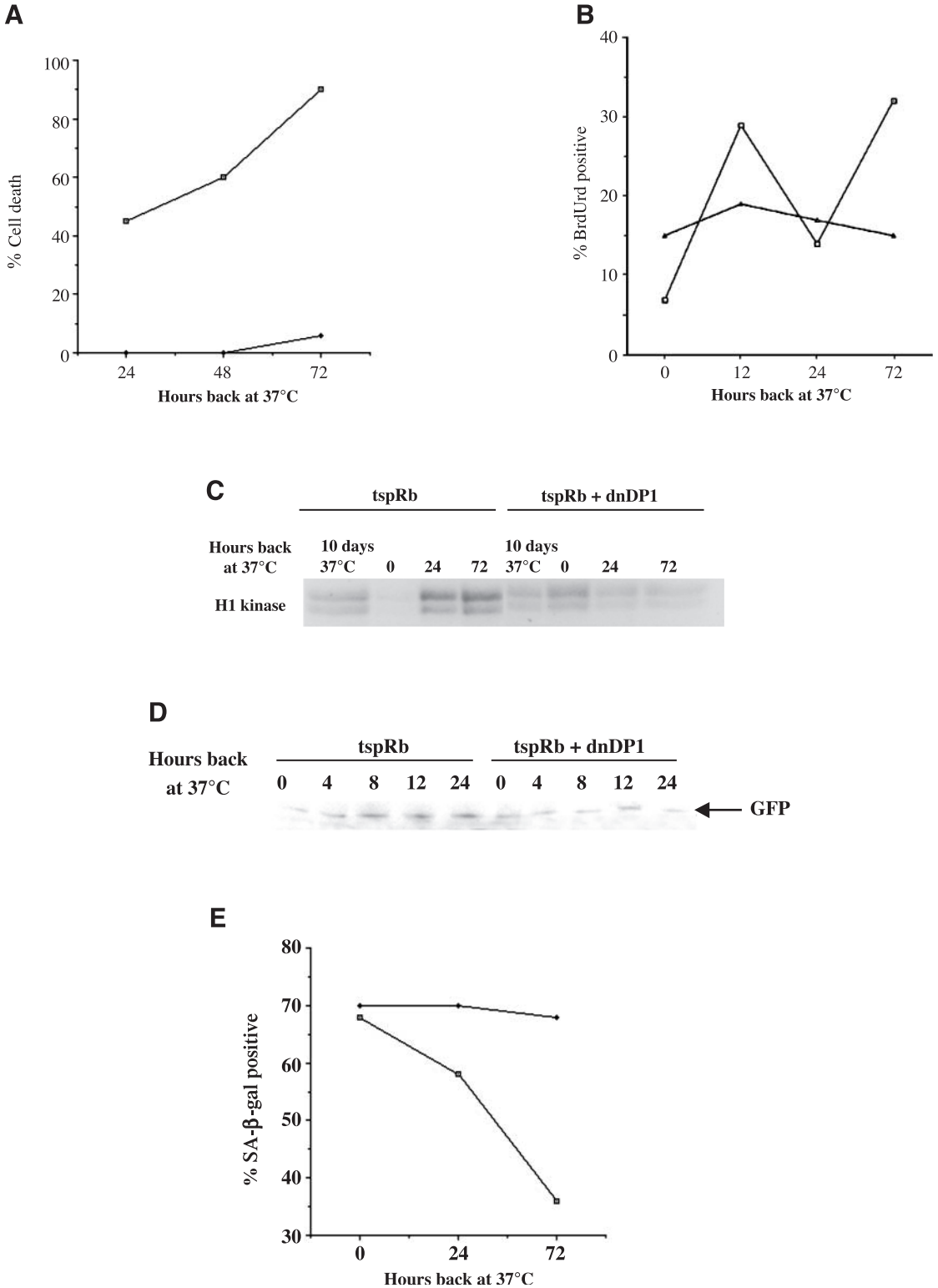
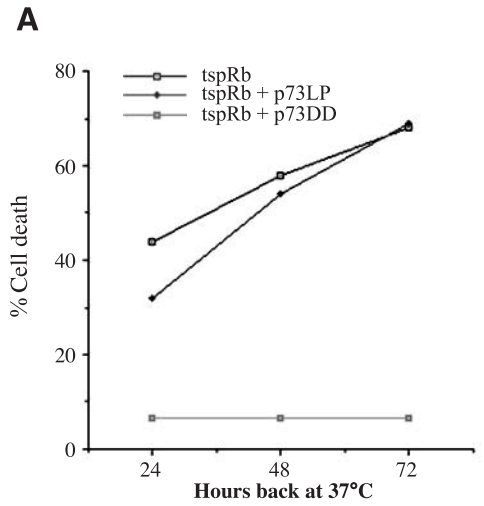
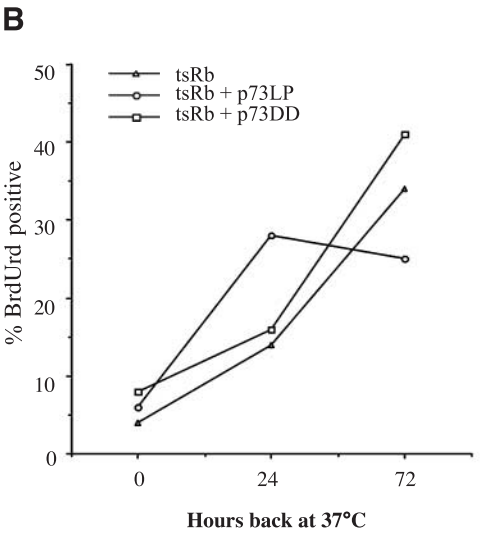
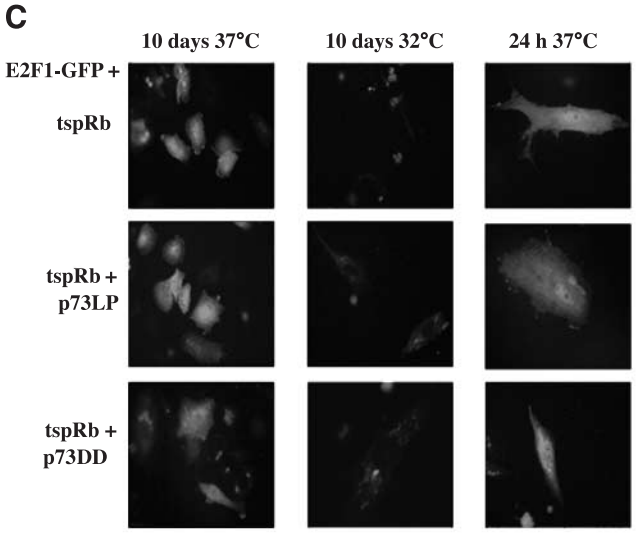
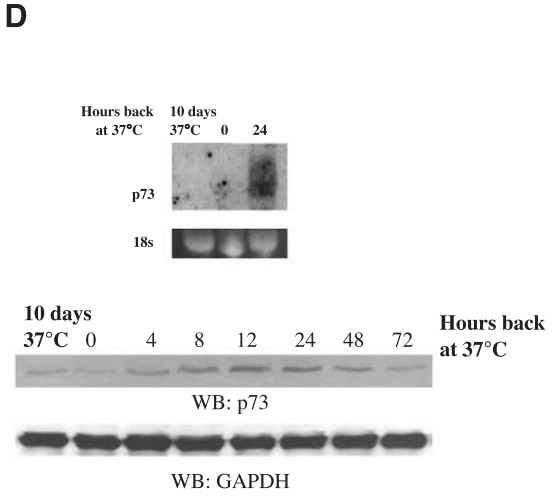


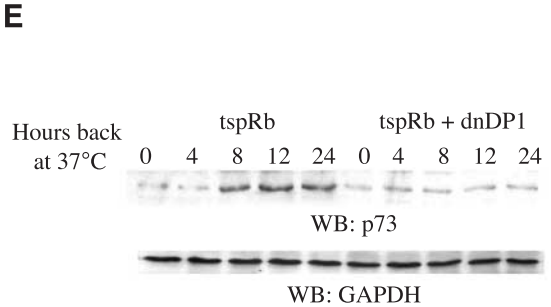

**FIGURE 5.** Repression of E2F activity protects cells from apoptosis due to loss of tspRb activity. Cells were transfected with tspRb or cotransfected with tspRb and dnDP1, incubated at the permissive temperature of 32.5°C for 10 days, shifted back to the nonpermissive temperature of 37°C, and assayed. **A.** Cells within a fixed area of 0.25 $cm^2$ were counted at the indicated time points, and the amount of cell death was determined. **B.** Incorporation of BrdUrd in transfected cells at 0, 12, 24, and 72 h after tspRb inactivation. Results are one experiment representative of several experiments. **C.** Transfected cell lysates were immunoprecipitated for cyclin E at the indicated time points and subjected to an *in vitro* kinase assay. **D.** Cells transfected with tspRb or tspRb + dnDP1 were cotransfected with an E2F1-GFP reporter, and lysates were harvested and immunoblotted for GFP at the indicated time points. **E.** Transfectants were stained for SA-β-gal activity at 0, 24, and 72 h after tspRb inactivation, and the number of flat SA-β-gal-positive cells was counted. –□–, tspRb; –◆–, tspRb + dnDP1.

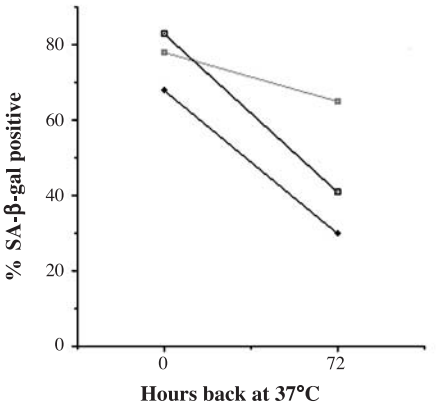


10 days at 32°C (Fig. 6D). However, there was an induction of p73 mRNA 24 h after loss of tspRb function concomitant with the reactivation of E2F (Fig. 6, C and D). p73 protein levels (α form) also increased in a manner consistent with E2F activation, peaking at 24 h after tspRb inactivation in senescent cells, indicating that increased p73 might contribute to cell death (Fig. 6D). Further, we found that on tspRb inactivation, p73 induction was decreased in those cells in which E2F activity was inhibited by cotransfection of dnDP1 (Fig. 6E). Altogether, this indicated that E2F reactivation in senescent cells caused apoptosis by both triggering S-phase progression and increasing p73 levels.

Finally, we wanted to ascertain if the tspRb cells coexpressing p73DD, and thus protected from apoptosis, maintained the senescent morphology. Indeed, like tspRb cells cotransfected with dnDP1, those cotransfected with p73DD remained SA-β-gal positive (Fig. 6F). Further, we observed that the cells maintained the senescent morphological phenotype for several days after tspRb inactivation and did not divide, as there was no increase in cell number or an appearance of mitotic cells (data not shown). Thus, p73DD protection of cells from apoptosis following tspRb inactivation again demonstrated that the pRb-senescent morphological phenotype is irreversible and becomes independent of continued pRb expression.

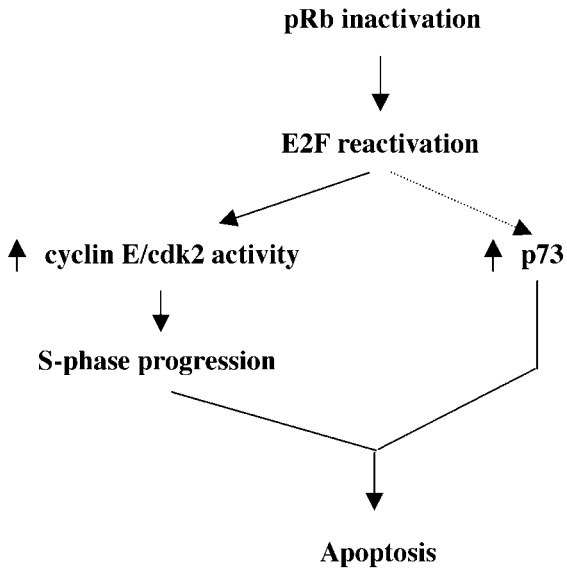



**FIGURE 7.** Induction of apoptosis in senescent cells. Inactivation of pRb in senescent cells triggers a cascade of events that culminate in cellular apoptosis. Loss of pRb function results in activation of a formerly inhibited E2F activity. This in turn activates the cdk2 kinase and triggers progression of cells from $G_1$ to S phase. The inactivation of pRb in senescent cells also causes an induction of p73 levels due to its transcriptional up-regulation by E2F. Altogether, S-phase progression and p73 cooperate to cause the death of senescent cells.

## Discussion

Loss of pRb function in senescent cells leads to apoptosis. We have found that the reason for this senescent cell death is an almost immediate reappearance of E2F activity. This reactivation of E2F alleviates repression of cyclin E-cdk2 activity, triggers S-phase progression, and activates a p73 apoptotic pathway (Fig. 7). Both S-phase progression and p73 induction contribute to and are required for the death of senescent cells in the system used here.

Interestingly, senescent cells protected by roscovitine, dnDP1, or dnp73 maintain the morphology of pRb-senescent cells and express SA-β-gal despite tspRb inactivation, demonstrating that the morphological alteration in senescent cells is irreversible. We have not yet determined with this system when the pRb-induced cell cycle arrest becomes irreversible, but work by others indicates that it is between 4 and 6 days after reestablishment of the pRb pathway (23, 24). Thus, while pRb seems to be absolutely required for the morphology change to occur, once this shape change has taken place, it cannot be reversed; the cells will not revert back to their original phenotype. Further, although pRb loss in senescent cells allows an initial S-phase entry followed by cell death, suppression of apoptosis reveals an ability of these cells to reestablish cell cycle arrest despite the lack of pRb. This may be related to a non-cell-autonomous $G_2$ arrest recently described by Lipinski *et al.* (44). This suggests that there may be a selective advantage to the morphology change associated with senescent cells, although we and others have found that it is not required for expression of other markers of senescence (27, 45). In fact, our preliminary studies indicate that at later time points after loss of pRb function, cells protected from apoptosis by p73DD exhibit high levels of $p27^{KIP1}$ expression, suggesting that $p27^{KIP1}$ expression may be the mechanism by which cells regain a cell cycle arrest. Because cell matrix signaling has recently been linked to $p27^{KIP1}$ up-regulation, it is possible that some aspect of the senescent morphology itself could lead to the maintenance of $p27^{KIP1}$ expression and reinitiation of cell cycle arrest.

We also found that it is specifically E2F activity in senescent cells that results in their death on loss of pRb function. dnDP1 completely represses this death by inhibiting E2F function. With an ability to block the activity of all E2F family members, dnDP1 is a potent inhibitor of tspRb inactivation-induced

**FIGURE 6.** Inhibition of p73 function blocks cell death triggered by the loss of tspRb activity. Cells were transfected with tspRb or cotransfected with tspRb and p73DD or an inactivating point mutant of p73LP, left at 32.5°C for 10 days, and shifted back to 37°C at the indicated time points. **A.** Cells within a fixed area of 0.25 $cm^2$ were counted at the indicated time points, and the amount of cell death was determined as described above. The experiment shown is representative of at least three experiments. **B.** Time course of S-phase reentry as detected by BrdUrd incorporation at 0, 24, and 72 h after tspRb inactivation. An experiment representative of at least three experiments is shown. **C.** Transfectants cotransfected with the E2F1-GFP reporter were observed by immunofluorescence for GFP expression. **D.** Northern blot (*top panel*) of p73 was performed. Immunoblot of tspRb-transfected cells at the indicated time points was analyzed with p73 and GAPDH antibodies (*bottom panel*). **E.** Lysates of cells used in Fig. 5D and expressing tspRb and E2F-GFP with or without dnDP1 were subjected to SDS-PAGE and immunoblotted for p73 at the indicated time points. **F.** A SA-β-gal assay of cells was performed, and the number of SA-β-gal-positive cells was determined. –□–, tspRb; –◆–, tspRb + p73LP; –■–, tspRb + p73DD.

apoptosis because it can disable those E2F proteins important for cell cycle progression and responsible for apoptosis induction. Interestingly, a large amount of E2F activity seems not to be required to trigger death in these cells. By immunoblot, we found that E2F-GFP reporter activity in tspRb-inactivated senescent cells was no higher and possibly less than that in normal cycling cells (data not shown). Thus, the same E2F activity that is tolerated in proliferating SAOS-2 cells causes senescent cells to die, suggesting that E2F activity in these cells is inappropriate. Why senescent cells are sensitized to E2F-dependent apoptosis is unclear. However, it is likely that discordant senescent and proliferative signaling results in senescent cell death. After terminally withdrawing from the cell cycle, these cells are irrevocably changed (17, 18), and E2F reactivation causes the cells to enact an apoptotic program. Thus, regardless of other probable targets of pRb in the senescence process, repression of E2F activity and inhibition of cell cycle progression are critical for the maintenance of this state.

The inhibition of senescent cell death on tspRb inactivation by blocking p73 function demonstrates that E2F not only induces S-phase reentry but also ultimately triggers a specific apoptotic program. In fact, the dramatic increase in p73 mRNA and protein levels on tspRb inactivation in senescent cells, and the inhibition of p73 induction by dnDP1, indicates that E2F likely transcriptionally activates p73, resulting in apoptosis. In addition, whereas S-phase progression seems to occur independent of p73 function, it alone is not sufficient to induce the apoptosis of these senescent cells. Similarly, roscovitine protects these cells from apoptosis by its ability to directly inhibit cdk2 activity and S-phase progression, although it is unable to completely block E2F activity or p73 induction (data not shown). Altogether, this suggests that senescent cell death is due to a collaboration between inappropriate S-phase reentry and induction of a p73 apoptotic program triggered by E2F (Fig. 7).

Clearly, E2F activity has to be carefully balanced to prevent induction of an apoptotic program; thus, it is logical that tumor cells would eliminate p53 in an effort to achieve unchecked proliferation (46). In addition, by mutating pRb, cells eliminate the threat of senescence, a state a tumorigenic cell would also want to avoid due to its irreversibility. Our work demonstrates that if cells are allowed to enter the senescent state, even oncogenic, proliferation-stimulating mutations might simply result in the death of these cells. In fact, if senescence is truly a tumor-suppressive mechanism, senescent cells may be difficult to detect *in vivo*, as proliferative genetic alterations may have induced their apoptosis. In the tspRb-senescent cells studied here, the activation of E2F, even at subphysiological levels, results in cell death. This suggests a mechanism to kill both p53 wild-type and mutant cancer cells by the transient serial activation and inactivation of the oft-mutated pRb pathway, eliminating the necessity for the continuous expression of an introduced growth-suppressing gene.

## Materials and Methods

### *Cell Culture, Plasmids, and Transfections*

The human osteosarcoma cell line SAOS-2 subclone 2.4 was used for these studies (25). The cells were maintained in DMEM (Invitrogen, Rockville, MD) supplemented with 15% heat-inactivated fetal bovine serum and penicillin streptomycin in a 5% $CO_2$ incubator at 37°C. The tspRb XX668 expression plasmid was constructed in the pSVE vector and has been described (25, 26, 47, 48). The E2F1-GFP plasmid was constructed using the E2F1 promoter from pGL2-AN (28) inserted in the promoterless GFP expression vector pEGFP-1 (Clonetech Laboratories, Inc., Palo Alto, CA). pCMVHA-$DP1^{\Delta 103-126}$ was a gift of Ed Harlow (39). pcDNA3-T7-p73LP(L371P) and pcDNA3-T7-p73DD were very generous gifts from William Kaelin and have been described (7). For puromycin selection, the vector pBabepuro was used (49).

SAOS-2 cells were transfected at 37°C at 80% confluency with tspRb or tspRb and the indicated plasmids on 10-cm plates using 2× 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid-buffered saline and calcium phosphate (25, 50) or Effectene (Qiagen, Inc., Valencia, CA). DNA precipitates were removed as described previously (51). Six-well plates with SAOS-2 cells at 80% confluency were transfected using the Fugene 6 reagent (Roche, Indianapolis, IN). Transfectants were selected with puromycin either 24 or 48 h posttransfection and shifted to 32.5°C. After 10 days at 32.5°C, cells were shifted back to 37°C and assayed for various phenotypes.

### *Immunoblotting*

Cells were lysed in 100 μl of ELB (50 mM HEPES, pH 7.2, 250 mM NaCl, 2 mM EDTA, 0.1% NP40, 1 mM DTT) plus protease and phosphatase inhibitors (1 mg/ml aprotinin, 1 μg/ml leupeptin, 100 μg phenylmethylsulfonyl fluoride, 4 mM sodium orthovanadate, 2 mM sodium $PP_i$). The concentration of proteins in cell lysates was determined by (Bio-Rad, Hercules, CA) protein assay. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. Antibodies used for immunoblotting include anti-$p27^{KIP1}$ K25020 (Transduction Laboratories, Lexington, KY), anti-GFP (Clonetech Laboratories), and anti-p73 Ab-2 (Oncogene, Boston, MA). Anti-cyclin A H-432, anti-cyclin E HE12, anti-cdk2 M2, and anti-PARP F2 were all from Santa Cruz Biotechnology (Santa Cruz, CA).

### *Kinase Assay*

To detect cyclin E kinase activity, cyclin E was immunoprecipitated with the agarose-conjugated antibody HE111 AC (Santa Cruz Biotechnology) for 1 h with CL-4B beads. Immunocomplexes were subjected to an *in vitro* kinase assay essentially as described previously with slight modifications (51). The kinase reaction was stopped with sample buffer and subjected to SDS-PAGE. The gel was stained with Coomassie blue to ensure an equal amount of histone H1, destained, dried, and exposed to autoradiography.

### *Assessment of Apoptosis*

Apoptosis was measured by immunoblot with anti-PARP F2 antibody (Santa Cruz Biotechnology). A cell survival assay was performed to detect the number of live cells. The number of cells in a fixed area of 0.25 $cm^2$ was counted at the indicated time points, and the amount of cell death was determined as the number of cells that had apoptosed over the total number of cells at the beginning of the time course ($T = 0$).

### *Analysis of DNA Synthesis*

To analyze the number of cells actively synthesizing DNA in S phase, SAOS-2 cells on 6-cm plates at 80% confluency were cotransfected with 0.5 μg of pBabepuro and 5 μg of the indicated plasmid. Twenty-four hours after transfection, cells were split to coverslips in a 24-well plate, put in selective medium 24 h after being split, and, as previously described, measured for their ability to incorporate BrdUrd at the indicated time points (27). At least 100 cells were counted using a Leica microscope (Bannockburn, IL).

### *Immunofluorescence*

SAOS-2 cells at 80% confluency were transfected with the indicated plasmids using the Fugene 6 reagent (Roche). Cells were split to coverslips 24 h after transfection and at the indicated times fixed 5 min in methanol followed by 2 min in acetone at −20°C. Coverslips were then washed three times in PBS + 0.1% BSA, incubated in primary antibody for 1 h at 37°C, washed again in PBS + 0.1% BSA, and incubated in secondary antibody for 30 min at 37°C. All visualization and photography was performed on a Leica microscope with Sony digital imaging (San Jose, CA).

### *SA-β-gal Staining Assay*

For the SA-β-gal assay, SAOS-2 cells were cotransfected with the indicated plasmids and pBabepuro plasmid. The cells were maintained in selective medium containing puromycin at 0.5 μg/ml for 10 days. The SA-β-gal assay was performed as described previously (52, 35) at the indicated time points.

### *Northern Blotting*

Total RNA was isolated from cells transfected with tspRb using Trizol (Invitrogen). Ten micrograms of RNA were resolved by electrophoresis, transferred to Hybond-N membrane, and probed with DNA probe derived from p73 gene, which was labeled with [$^{32}\alpha$] dCTP using the High Prime DNA labeling kit (Roche).

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