CDK2 Transcriptional Repression Is an Essential Effector in p53-Dependent Cellular Senescence—Implications for Therapeutic Intervention

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Abstract

Cellular senescence, a form of cell-cycle arrest, is a tumor-suppressor mechanism triggered by multiple tumor-promoting insults, including oncogenic stress and DNA damage. The role of cyclin-dependent kinase 2 (CDK2) regulation has been evaluated in models of replicative senescence, but little is known regarding its role in other senescence settings. Using in vitro and in vivo models of DNA damage–and oncogene-induced cellular senescence, it was determined that activation of the tumor-suppressor protein p53 (TP53) resulted in repression of the CDK2 transcript that was dependent on intact RB. Ectopic CDK2 expression was sufficient to bypass p53-dependent senescence, and CDK2-specific inhibition, either pharmacologically (CVT313) or by use of a dominant-negative CDK2, was sufficient to induce early senescence. Pharmacologic inhibition of CDK2 in an in vivo model of pinal tumor decreased proliferation and promoted early senescence, and it also decreased tumor penetrance and prolonged time to tumor formation in animals lacking p53. In conclusion, for both oncogene- and DNA damage–induced cellular senescence, CDK2 transcript and protein are decreased in a p53- and RB-dependent manner, and this repression is necessary for cell-cycle exit during senescence.

Implications: These data show that CDK2 inhibition may be useful for cancer prevention in premalignant hyperproliferative lesions, as well as established tumors. Mol Cancer Res; 13(1); 29–40. ©2014 AACR.

Introduction

Cellular senescence is a well-documented tumor-suppressor response, which in most instances is dependent on functional TP53 (p53), and results in irreversible cell-cycle exit, so preventing further tumor progression under the influence of oncogenic or genotoxic insults (reviewed in refs. 1, 2). Replicative senescence occurs due to telomere shortening, but senescence can also be induced by oncogene activation, loss of tumor suppressors, or direct DNA damage (reviewed in ref. 3). Cell-cycle regulators are critically involved in the execution of the senescence response. In human fibroblasts, the cyclin-dependent kinase (CDK) inhibitors, CDKN2a (p16
\(^{ink4a}\)) and CDKN1A (p21
\(^{Cip1}\)), are activated in response to oncogenic signals and are essential for cell-cycle exit during cellular senescence (4–8). In senescence caused by genotoxic insults, such as irradiation or topoisomerase inhibition, p53 is activated and this results in p21
\(^{Cip1}\) induction, which is essential for cell-cycle exit and senescence (5, 9, 10). In yet other in vitro settings, other CDK inhibitors, such as CDKN2B (p15
\(^{ink4b}\)) and CDKN2C (p18
\(^{ink4c}\)), have also been shown to play roles in this process (11, 12).

In contrast with the well-documented roles of the CDK inhibitors (p16
\(^{ink4a}\), p21
\(^{Cip1}\), p15
\(^{ink4b}\), and p18
\(^{ink4c}\)) in cellular senescence, the roles of the respective CDKs are not well defined.

The role of CDK2 has been primarily studied in the setting of replicative senescence, in which several investigators have shown that activity of CDK2 decreases in senescent fibroblasts, likely through inhibition of Cyclin A–CDK2 complexes by p21
\(^{Cip1}\), whereas CDK2 protein levels remain unchanged (13–15); whereas in replicative senescence of cultured human umbilical vein endothelial cells (HUVEC), reduction of both CDK2 activity and protein levels were noted (16). Transduction of replicatively senescent cells with Cyclin E–CDK2 complexes results in cell-cycle reentry in a subset of cells, indicating that reduction of CDK2 activity is indeed essential for replicative senescence (17).

In oncogene-induced senescence, limited data are available, but suggest a physiologic role for CDK2 in opposing senescence. For instance, in RASV12-induced senescence, endogenous CDK2 activity is reported to be decreased (6). MYC-induced senescence occurs in a Cdk2-null background (and upon pharmacologic CDK2 inhibition in fibroblasts), but not in Cdk4-null or Cdk6-null backgrounds (18). We previously showed that in a mouse model of CCND1 (Cyclin D1)-driven senescence, CDK2 protein levels are also markedly decreased (19). All the above data together prompted us to investigate the role of CDK2 in oncogene-induced and DNA damage–induced senescence. In this report, we uncover a central physiologic role for p53-mediated repression of CDK2 during oncogene-induced senescence, and
highlight CDK2 as a possible target to limit premalignant lesions, as well as inhibit progression of established cancer, via induction of senescence.

Materials and Methods

Mouse studies

Irbp-Cyclin D1 transgenic mice (20) were bred with p53fl/fl mice (The Jackson Laboratory) or p53ERTAMki/kimice (21) and maintained in a mixed C57BL/6 × 129/Sv genetic background. PCR for targeted alleles was used to verify mouse genotypes as described previously (20–22). Animals were euthanized at defined time points or when obviously ill in accordance with the American University of Beirut Institutional Animal Care and Use Committee guidelines; all animal studies were approved by this committee.

For all in vivo studies, CTVT313 was administered by once daily i.p. injection of 1.25 mg/kg. For bromodeoxyuridine (BrdUrd) incorporation assays, mice were given an i.p. injection of 50 mg/kg of BrdUrd (Sigma-Aldrich), every 2 hours × 5 and sacrificed 2 hours later.

Plasmid constructs, virus production, and transduction

CMV–CDK2–HA and CMV–CDK2DN–HA plasmid constructs were obtained from Addgene [Addgene plasmids 1884 and 1885 (ref. 23); www.addgene.org], and subcloning of the constructs was performed into a lentivirus backbone expressing GFP separated by an IRES site. The RasV12-GFP plasmid was purchased from Clonetech. HEK 293T cells were used for lentivirus production (RasV12 constructs), and retrovirus production (RasV12 constructs), using calcium phosphate transfection with the appropriate respective packaging plasmids.

Cell culture

Human BJ foreskin fibroblasts (ATCC) were cultured at 37°C and 5% CO2 in a cell incubator, in DMEM containing 10% FBS, 1% glutamine, and 1% pen/strep. Mouse embryonic fibroblasts (MEF) were isolated from E13.5 embryos from wild-type (WT), p53fl/fl, p53ERTAMkikiw, and Rbfl/fl mice (in which exon 19 of the Rb gene is flanked by two LacI sites, allowing induction ofCre-mediated recombination; ref. 24). MEFs were cultured in DMEM containing 10% FBS, 1% nonessential amino acids, 1% sodium pyruvate, 1% glutamine, and 1% pen/strep. For MEFs derived from p53ERTAMkikiw mice, p53 was reversibly switched on and off by addition or withdrawal of 100 nmol/L 4-OH tamoxifen, as specified. For Rbfl/fl MEFs, Rb knockout was performed by using Cre-mediated recombination via lentiviral transduction. Successful recombination was assessed by PCR and Western blot analysis.

For senescence induction, cells were exposed to 20 Gy irradiation at 1 Gy/min, or treated with 10 μmol/L etoposide. Cells were treated with CVT313 at 5 μmol/L (Santa Cruz Biotechnology) for CDK2 inhibition, with NSC625987 at 1 μmol/L (Sigma-Aldrich) for CDK4 inhibition, with flavopiridol at 1 μmol/L (Sigma-Aldrich) for total CDKs inhibition or corresponding volume of DMSO vehicle; media were renewed every 3 days. For viral transductions, cells were plated in 6-well plates at a density of 200,000 cells per well. Virus was added to the cells with 8 μg/mL polybrene (hexadimethrine bromide; Sigma). Spinnoculation was performed at 32°C, 2500 rpm for 2 hours and medium was replaced after 3 hours. The following day, the procedure was repeated. For SABG (senescence-associated beta-galactosidase) staining, cells were fixed and stained overnight at 37°C, and counterstained with eosin, as in our previous studies (12, 19). For BrdUrd incorporation assays, cells were treated with BrdUrd at a concentration of 33 μmol/L for 2 hours, fixed with 50% methanol/50% acetone solution for 2 minutes, then treated with 2N HCL for 10 minutes, and neutralized by Borate buffer for 12 minutes. After blocking, slides were probed with anti-BrdUrd antibody (Santa Cruz Biotechnology). Cy3-conjugated secondary antibody (Jackson ImmunoResearch) or Alexa 488–conjugated antibody (Invitrogen) was used for detection. Stained cells were covered with aqueous mounting medium containing DAPI (Vector Laboratories) and visualized by immunofluorescence microscopy. The number of BrdUrd-positive cells was manually counted from at least five representative fields, at ×20 magnification, and normalized to total cell number (counted as DAPI-positive nuclei). Digital photomicrographs were obtained using a Zeiss 510 NLO multiphoton/confocal laser scanning microscope. Composite images were constructed using Photoshop CS6 software (Adobe Systems).

Cell accumulation assay

Cells were plated in 6-well plates at a density of 200,000 cells per well. For senescence induction, cells were exposed to 20 Gy irradiation at 1 Gy/min, treated with 10 μmol/L etoposide or transduced with RasV12-GFP. The cells were collected at 7 days (after RasV12 transduction) or 14 days (after DNA damage), fixed with methanol: acetic acid 3:1 for 5 minutes, then stained in 0.5% Crystal Violet in methanol for 15 minutes. A dissecting microscope was used to view the cells under a magnified field.

Cell explantation and ex vivo culture

Mice were euthanized according to the IACUC-approved protocol. Pineales were explanted at postnatal day 10 (P10), mechanically dispersed, and cells were plated onto 8-well permoculture slides (Nunc), and cultured in DMEM with 10% FBS, 1% glutamine, and 1% pen/strep. Explanted cells were treated with CVT313 (Santa Cruz Biotechnology) or DMSO vehicle; media were renewed every 3 days, and cells were fixed after 7 days. BrdUrd and SAGB staining and cell counting were performed as detailed above. Digital photomicrographs were analyzed using Adobe Photoshop CS4 software.

Histologic studies and immunostaining

Brain tissue was fixed in 4% paraformaldehyde for 72 hours then embedded in paraffin. Four- to 8-μm sections were cut from paraffin-embedded tissues and deparaffinized. Antigen retrieval was performed in a steamer for 40 minutes in citrate antigen retrieval buffer (pH 6.0). For immunohistochemistry, slides were incubated with anti-Ki67 antibody (BD Pharmingen), anti-phosphorylated histone 1 (Millipore), anti-CDK2, anti-CDK4 (Santa Cruz Biotechnology) or anti-phospho RB Ser612 (MBL International), followed by biotinylated secondary antibody, and detected using streptavidin conjugated to horseradish peroxidase and DAB substrate (DAKO). For immunofluorescence staining, anti-H3K9me3 (Upstate Laboratories), and anti-BrdUrd (Santa Cruz Biotechnology) antibodies were detected with Cyanine 2, Cyanine 3, or Alexa Fluor 488 secondary antibodies, and counterstained with DAPI (Vector Laboratories). The numbers of positive cells were manually counted from five representative fields, at ×40 magnification. Results were reported as normalized...
to the control animals treated with vehicle. Images were taken as detailed above.

Analysis of protein expression
Protein lysates were prepared from cultured cells or pineal tissue in Universal Lysis Buffer, as previously described (19). Electrophoresis was performed using 12% Tris-Chloride gels, and proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories), and detected using antibodies to p21\(^{Cip1}\), p15\(^{INK4b}\), p16\(^{INK4a}\), CDK4, CDK1, CDK2, DEC1, DCR2, HSC70, and GAPDH (all from Santa Cruz Biotechnology); phospho-Histone1 (Millipore), phospho-RB to the control animals treated with vehicle. Images were taken as detailed above.

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Ser612 (MBL International); total RB (BD-Pharmingen) and Tubulin (Abcam).

RNA extraction and quantitative real-time PCR
Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. DNAse-treated total RNA was used for cDNA synthesis with random hexamers using the RevertAid 1st Strand cDNA Synthesis Kit (Fermentas). Real-time PCR was performed using the iQ SYBR Green Supermix in a CFX96 system (Bio-Rad Laboratories). Products were amplified using the following primers: GAPDH, forward = AGCCAAAGGGTATCATCT; reverse = GGAGCCATCCACGTTCTC; mature Cdk2 mRNA, forward = CTGCACTTGGCTGAAATG; reverse = GATCCGGAAGAGTTGGTCAAT; preprocessed Cdk2 mRNA, forward = AGTACACCTGCTGTCCTTCT, reverse = TGAGTGAGGATCCGCTTGGT. PCR parameters consisted of denaturation at 95°C for 15 minutes followed by 40 cycles of 95°C for 15 seconds, 55°C for 1 minute. Annealing temperature was 60°C for Gapdh and mature Cdk2, and 55°C for the preprocessed Cdk2 transcript. A final extension at 72°C for 10 minutes was performed followed by a melting curve, with temperature gradually increased (0.5°C) to 95°C. Standard curves were plotted using serially diluted cDNA. The geometric mean of the housekeeping gene GAPDH was used as an internal normalization control.

Results
CDK2 protein levels are decreased during both oncogene-induced and DNA damage–induced senescence
We had previously noted that CDK2 protein levels decrease in an in vivo model of cellular senescence, wherein exogenous expression of Cyclin D1 in pineal cells leads to a pretumorigenic hyperplastic lesion limited by p53-dependent senescence (19). To verify whether downregulation of CDK2 holds true in other instances of senescence induction, we used two well-documented in vitro models of senescence—RasV12–induced senescence and DNA damage–induced senescence, using MEFs and human BJ fibroblasts. Senescent cells in both of these well-established and previously studied systems displayed cell-cycle exit (evidenced by absence of BrdUrd incorporation into DNA) and positive staining for SABG (Fig. 1A), in addition to other markers of senescence such as expression of p21Cip1, p15Ink4b, and DcR2 (Fig. 1B). Interestingly, CDK2 protein levels were specifically decreased in senescent cells whereas the levels of another S-phase kinase

![Image](https://example.com/image.png)

Figure 2. CDK2 repression during senescence occurs at an RNA level, in a p53-dependent manner. Quantitative real-time PCR analysis of mature Cdk2 mRNA transcripts in WT MEFs (A) and p53−/− MEFS (B), 5 days after RasV12 transduction, and in Intp-Cyclin D1 pineal glands at presenescence (PG-PS) and senescent (PG-S) time points (C). The Y axis, Cdk2 transcript levels normalized to Gapdh. D and E, real-time PCR analysis of primary Cdk2 mRNA transcript in WT MEFs and p53−/− MEFS, respectively, 5 days after RasV12 transduction and Intp-Cyclin D1 pineal glands at presenescence (PG-PS) and senescent (PG-S) time points (F). The Y axis represents Cdk2 transcript levels normalized to Gapdh; *, P < 0.05. G, Western blotting for the indicated proteins in WT, p53+/−, and p53ER(TAM)+/− MEFs, treated with 4-hydroxytamoxifen (4OH-Tam) or vehicle, and treated with 10 μmol/L etoposide (Eto) or vehicle, as indicated. Cells were collected 48 hours after treatment for WT control cells (lanes 1 and 2), and at day 7 for the remaining cells (lanes 3–8). GAPDH is used as a loading control. H, Western blotting for the indicated proteins in Rb flox/flox MEFS, after Cre-mediated recombination as indicated, and transduction with RasV12 as indicated. TUBULIN is used as a loading control.
CDK1, and the G₁-S kinase CDK4, were unchanged (Fig. 1C and D). Concomitant with the decrease in CDK2 levels, we found decreased levels of its target phospho-Histone 1 (Fig. 1C), and decreased phosphorylation of the retinoblastoma protein RB1 (RB) at Ser612, a CDK2-specific phosphorylation site (Fig. 1C; ref. 25). FACS analysis showed that senescent cells were arrested in G₀-G₁, as expected (data not shown). Timed analysis showed that the decrease in CDK2 protein levels occurred at around 5 days after the insult (Fig. 1D), which correlated temporally with the timing of cell-cycle exit and preceded SABG positivity (Fig. 1E and F).

CDK2 repression during senescence occurs at an mRNA level, in a p53-dependent and RB-dependent manner

This temporal lag of 5 days between oncogenic or DNA damage–inducing insult and CDK2 downregulation (see Fig. 1D) suggested the possibility of transcriptional repression of CDK2 during senescence induction. To investigate this, we evaluated the expression levels of mature and primary Cdk2 mRNA transcripts during senescence both in vitro and in vivo. We found the levels of mature Cdk2 mRNA to be significantly decreased at senescence in vitro in p53-WT MEFs (Fig. 2A), but not in p53-null MEFs that were exposed to the same insults but failed to undergo senescence (Fig. 2B). Similarly, in the Irbp-Cyclin D1 mouse, a well-characterized in vivo model of p53-dependent Cyclin D1–induced senescence that limits development of the brain tumor pineoblastoma (12, 19), tissue from senescent pineal hyperplastic lesions driven by Cyclin D1 (PG-S) exhibited marked downregulation of mature Cdk2 mRNA compared with presenescent hyperplastic lesions (PG-PS; Fig. 2C). We next evaluated the expression of the primary (preprocessed) Cdk2 mRNA transcript and found that levels of expression were also significantly decreased during senescence in p53-WT MEFs (Fig. 2D), but not in nonsenescent p53-null MEFs (Fig. 2E). Similarly, primary mRNA transcript expression levels were decreased in senescent pineal cells in vivo (Fig. 2F).

The lack of repression of CDK2 in p53-null cells and tissues (see Fig. 2B and E) suggested that CDK2 downregulation is a downstream consequence of p53 activation. To test this, we used MEFs derived from the reversibly regulatable p53ER(TAM)KI/KI mouse, in which the endogenous p53 gene is fused with a modified form of the estrogen receptor. p53 function in cells and...
tissues from such animals is totally dependent upon provision of the ER(TAM) ligand 4-hydroxy tamoxifen (4OHT; ref. 21). When exposed to etoposide-induced DNA damage, CDK2 was repressed in p53ER(TAM)KI/KI MEFs only when the cells were treated with 4OHT (Fig. 2G, compare lane 8 with the control lanes 5–7). The decrease in CDK2 did not occur in nonsenescent p53-null MEFs at a similar time point after etoposide treatment (Fig. 2G, lanes 3 and 4), nor in p53-WT MEFs immediately following etoposide treatment but before onset of senescence (Fig. 2G, compare lanes 1 and 2). This repression was specific to CDK2, as CDK4 levels were...
unchanged (Fig. 2G). We conclude that CDK2 repression in response to oncogenic and genotoxic stress occurs at an mRNA level and downstream of p53 activation.

CDK2 has been shown to be a transcriptional repression target of the RB/E2F complex (26, 27). In addition, a well-recognized mechanism of p53-induced cell-cycle exit is via induction of $p21^{\text{CIP1}}$, which then results in inhibition of S-phase Cyclin–CDK complexes, leading to dephosphorylation and activation of RB (28, 29). To formally assess whether p53-dependent CDK2 repression occurred downstream of RB activation, we used MEFs derived from $Rb^{\text{fl/ex}}$ mice, in which exon 19 of the $Rb$ gene can be deleted by Cre-mediated recombination, essentially producing $Rb^{-/-}$ MEFs.
(24). As expected, genetic ablation of Rb resulted in increased baseline protein levels of Cdk2 (Fig. 2H, compare lanes 1 and 3). In response to oncogene activation, we found that absence of Rb prevented the previously observed downregulation of Cdk2 (Fig. 2H, compare lanes 2 and 4).

We therefore conclude from the above results that Cdk2 downregulation during oncogene-induced senescence occurs at the mRNA level, via a p53-dependent and RB-mediated mechanism. We hypothesize that the most likely mechanism is through p53-mediated p21<sup>WAF1</sup> induction (which occurs during senescence as seen in Fig. 1B), leading to RB activation and subsequent repression of Cdk2 transcription (26, 27). However, this still needs to be formally proven in future studies.

**CDK2 repression is necessary for senescence induction**

To test directly whether Cdk2 repression is required for oncogene- and DNA damage–induced senescence, we asked whether constitutive expression of Cdk2 could inhibit induction of senescence. Indeed, in both human BJ fibroblasts and in MEFs, constitutive ectopic expression of Cdk2 abrogated senescence in response to either DNA damage (irradiation), or oncogenic stress (HRas<sup>V12</sup> transduction), as evidenced by continued proliferation by day 14 and absence of SABG staining (Fig. 3A and B), along with reduction in other protein markers of senescence (Fig. 3C and D). Importantly, exogenous expression of Cdk2 resulted in increased cell accumulation, rather than simply increased entry into the cell cycle, effectively bypassing senescence, though this was more evident in Ras<sup>V12</sup>-induced cells than in irradiated cells, which seemed to have reduced ability to undergo cellular proliferation irrespective of senescent state (Fig. 3E, and see Fig. 5D and more below). Notably, constitutive Cdk2 expression after senescence was well established, could not reverse the phenotype (Fig. 3B, third panel; and Fig. 3D, lane 3). Of note, the levels of exogenously expressed Cdk2 in this system were found to be much higher than endogenous levels in unstressed cells (see Fig. 3C and D), but similar to levels seen in cells after RB disruption (see Fig. 2H).

To ascertain whether downregulation of Cdk2 activity is sufficient to induce senescence, we inhibited Cdk2 activity in two ways—by expression of a dominant-negative Cdk2 construct (Cdk2-DN; ref. 23) and using the specific Cdk2 inhibitor CVT313 (30). Both promoted acceleration of senescence (by day 5 instead of days 7–10) in response to either HRas<sup>V12</sup> expression (Fig. 4A and B) or DNA damage (Fig. 4C and D). This effect was unique to Cdk2, because the specific Cdk4 inhibitor NSC625987 caused only cell-cycle exit but no SABG positivity or other features of senescence (Fig. 4E and data not shown), whereas the pan-Cdk inhibitor flavopiridol showed a similar phenotype of senescence to Cdk2 inhibition (Fig. 4E).

We next assessed the effect of Cdk2 inhibition on “normal” cells in the absence of any oncogenic or DNA damage. Interestingly, Cdk2 inhibition by either CVT313 or Cdk2-DN in unstressed fibroblasts resulted only in cell-cycle exit without other signature features of senescence, such as SABG positivity (Fig. 4F and G). From these studies, we conclude that Cdk2 repression is necessary for the induction of senescence, but is not sufficient in the absence of a genotoxic or oncogenic insult, which indicates that other effectors of the senescence program are needed to establish the complete phenotype.

Interestingly, inhibition of Cdk2 in p53-null MEFs, by either CVT313 or Cdk2-DN, failed to induce senescence even when combined with oncogenic or genotoxic stress (Fig. 5A and B, and additional data not shown). In addition, HRas<sup>V12</sup>-transduced MEFs in which the Rb gene is nonfunctional also failed to undergo senescence after Cdk2 inhibition (Fig. 5C). Thus, in addition to Cdk2 repression, other components of a p53- and RB-activation response appear to be necessary for execution of the full senescent phenotype in MEFs.

Interestingly, despite inability to cause features of senescence, longer-term analysis showed that, at 14 days, Cdk2 inhibition in HRas<sup>V12</sup>-transduced p53<sup>−/−</sup> MEFs resulted in decreased cell accumulation, whereas cell accumulation in p53<sup>−/−</sup> MEFs after irradiation was already reduced and did not further decrease with Cdk2 inhibition (Fig. 5D). Cells exposed to etoposide treatment had the same response to Cdk2 inhibition as cells exposed to irradiation (data not shown). Finally, treatment of explanted pineal tumor cells from Irbp–Cyclin D1, p53<sup>−/−</sup> mice with CVT313 did result in features of senescence, evidenced by decrease in BrdUrd incorporation into DNA, and positive staining for SABG (Fig. 5E), though the decrease in BrdUrd incorporation occurred to a lesser extent than in pretumorigenic Irbp–Cyclin D1, p53<sup>−/−</sup> pineal cells (Fig. 5F). These findings suggest that Cdk2 inhibition can effectively decrease cell accumulation in the absence of p53, irrespective of senescence phenotype, and that the ability of Cdk2 inhibition to induce senescence in the absence of p53 varies depending on the specific cellular context.

**Pharmacologic inhibition of Cdk2 in vivo promotes cellular senescence**

To evaluate the efficacy of Cdk2 inhibition as a therapeutic strategy in pretumorigenic and tumor tissue, we used the Irbp–Cyclin D1 mouse model of p53-dependent Cyclin D1–induced senescence (12, 19). Treatment of young mice at postnatal day...
(P)10 with CVT313 resulted in effective CDK2 inhibition in Irbp–Cyclin D1 mice of both WT and p53-null background, as shown by decreased phosphorylation of the CDK2 target Histone 1 in harvested pineal tissue (Fig. 6A). Although the pineal cells of control, untreated mice show no senescence phenotype until around 7 weeks of age (postnatal day (P)49; refs. 12, 19), inhibition of CDK2 for as few as 10 days (P10–P20) resulted in a dramatically advanced onset of senescence, evident as loss of Ki67 positivity and development of senescence-associated heterochromatin foci (SAHF) that start to become visible as early as 6 days after treatment with the CDK2 inhibitor (P16), and become readily apparent by 10 days after treatment (P20; Fig. 6B).

We next evaluated the effect of CDK2 inhibition in p53-deficient Irbp–Cyclin D1 mice. In such animals, proliferation in the transgenic pineal gland continues unabated without senescence induction due to absence of p53 and invasive pineal tumors develop uniformly by 3 months of age (12). Treatment of these Irbp–Cyclin D1, p53−/− mice with CVT313 between P10 and P20 resulted in mixed phenotypes, some mice showing enhanced pineal cell senescence, whereas others appeared unaffected by CVT313 (Fig. 6C, left: (1) and (2) represent different mice, in which (1) shows persistent Ki67 staining and absence of H3K9me3 foci whereas (2) shows the opposite). Low-to-negative staining for CDK2 and its targets, phosphorylated Histone 1 (pH1) and RB phosphorylated at serine 612 (pRB-Ser612), was seen differentially in pineal glands that responded to CVT313 treatment and displayed features of senescence, whereas CDK4 staining was unchanged or increased (Fig. 6C, right; and quantitation in Fig. 6D). Conversely, pineal glands in which CVT313 treatment had no detectable inhibitory effect on proliferation showed high levels of phosphorylation of the CDK2 targets pH1 and pRB-Ser612 (Fig. 6C and D). Importantly, in both cases, the cells that acquired SAHF were distinct from cells that continued to proliferate, shown by distinct staining for H3K9me3 and BrdUrd in dual stained sections (Fig. 6E). To evaluate any impact of CDK2 repression on tumor onset, we treated a cohort of Irbp–Cyclin D1, p53−/− mice (n = 8) with CVT313 for 10 days (P10 through P20, as above) and monitored their survival. Although untreated mice developed clinically apparent pineal tumor at a median age of 85 days, the treated mice exhibited significant delay in tumor onset, with a median prolongation of tumor-free survival of more than 1 month (Fig. 6F; P < 0.001).

**Discussion**

Our study now identifies p53-dependent and RB-mediated downregulation of CDK2 as an essential physiologic tumor-suppressive mechanism, triggered by both oncogene-induced and DNA damage–induced senescence programs. Much insight has been gained over the past decade into mechanisms underlying induction of cellular senescence in tumor suppression, including the central role of the p53 pathway, typically activated by DNA damage response signals, and the importance of the RB pathway, which contributes to formation of SAHF (reviewed in refs. 1, 2, 31). During the onset of senescence, the CDK2 inhibitor p21Cip1 is upregulated together with the CDK4/6 inhibitor p16INK4a, both of which contribute to cell-cycle exit, as are the CDK4/6 inhibitors p15INK4b and p16INK4a in some settings (reviewed in refs. 3, 4, 32). Notably, CDK activities are primarily controlled by regulation of expression of their regulatory Cyclin subunits and of the members of the Ink4 and Cip/Kip families of CDK inhibitor proteins (reviewed in refs. 33, 34). Unlike replicative senescence, in which important work has demonstrated a role for a decrease in CDK2 kinase activity (see more below), very limited information is available regarding the role of CDK2 in oncogene-induced senescence. Serrano and colleagues (6) have shown that during HRasV12-induced senescence, there is a decline in the levels of cyclin A and CDK2 kinase activity. CDK2 has also been previously implicated as a possible target to induce senescence, specifically in MYC-transformed cells that have circumvented the senescent response (18). Interestingly, MYC was found to be itself a target of CDK2, and, in cells cotransformed by MYC and RAS, MYC was able to repress RAS-induced senescence, in a manner dependent upon MYC phosphorylation by CDK2 (35). These studies show that CDK2 activity is critical for circumventing senescence during MYC-induced transformation. Our work now establishes an essential role for physiologic CDK2 downregulation during p53-mediated senescence induced by DNA damage or oncogenic insult.

As mentioned above, previous work has established that CDK2 kinase activity (but not CDK2 protein levels) is decreased during replicative senescence (13–15, 36). Expression of recombinant CYCLIN E–CDK2 in replicatively senescent human fibroblasts resulted in DNA synthesis in approximately 10% of cells (17). Although several studies did not show any notable changes in CDK2 protein levels despite decrease in kinase activity in replicatively senescent fibroblasts, one study using HUVECs did show a decrease in CDK2 protein levels (16), suggesting cell-type–specific regulation of CDK2 in this setting. Our study now shows that, in both oncogene-induced and DDR-induced cellular senescence, the cell-cycle exit that underpins senescence induction requires p53-dependent downregulation of CDK2. In our studies, expression of exogenous CDK2 before senescence resulted in complete bypass of senescence, evidenced by continued DNA synthesis and restoration of proliferative capacity in the face of oncogenic or DNA damage stress. However, in our system, the levels of exogenously expressed CDK2 were much higher than those seen in parental cells, and therefore we cannot exclude the possibility that some of the effects seen may be related to nonphysiologic effects due to overexpression of the protein. Notably, levels of CDK2 protein were similarly high in RB-deficient cells (likely due to loss of RB/E2F-mediated transcriptional repression), and these RB-deficient cells also do not undergo senescence. A possible role for the level of protein expression of CDK2, if any, would need to be further evaluated using more refined tools that would allow titration of CDK2 protein expression level, in both RB-deficient and RB-WT cells. Interestingly, we found that expression of CDK2 after onset of senescence failed to reverse the phenotype, suggesting that CDK2 repression is needed to execute the cell-cycle exit accompanying senescence, but is not needed for maintenance of senescence once established.

Our work further shows that the p53-dependent downregulation of CDK2 is also dependent on intact RB, suggesting that RB activation is upstream of CDK2, likely through E2F-mediated transcriptional repression, as CDK2 is a well-described target of active RB–E2F complexes (26, 27). RB is activated during senescence through inhibition of the activity of Cyclin–CDK complexes by p21Cip1 (induced by p53), as well as by INK4 proteins, both of which are induced during senescence in...
the above described models, as previously shown (see Fig. 1B and refs. 12, 31, 37).

Although the most well-studied phosphorylation target of CDK2 is the RB protein, CDK2 acts to phosphorylate multiple other proteins important in cell-cycle regulation. CDK2 becomes activated in late G1 phase of the cell cycle, upon complexing with Cyclin E. The Cyclin E–CDK2 complex then acts to phosphorylate multiple substrates, including the RB protein (pRB), Histone 1, p27Kip1, and the Cyclin E

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positivity, but did limit proliferative capacity. This suggests that,

in response to DNA damage or oncogenic stress, other down-

stream p53 pathway effectors are needed for the full phenotype

of senescence to be manifest. Nonetheless, when we inhibited

CDK2 activity in Cyclin D1-expressing pineal cells both in vitro

and in vivo, we could induce early senescence in p53-proficient

pineal cells, and a senescence phenotype in p53-deficient pineal

cells, resulting in a significant delay in tumor onset in vivo even

after cessation of the drug. All together, the above findings show

that CDK2 inhibition in oncogene-expressing p53−/− cells can

indeed decrease cell accumulation, despite no change in ability to

enter S-phase. In addition, they uncover a differential effect on

induction of senescence in different cellular contexts (MEFs vs. pineal cells) and oncogenic pathways (HRAsV12 vs. Cyclin D1 expression).

Of note, although the senescence phenotype in p53-deficient animals was quite variable, it seemed to correlate with the variability in the extent of inhibition of CDK2 activity. As such, persistent phosphorylation of the CDK2 substrates Histone 1 and RB (at Ser 612) correlated with continued proliferation and lack of formation of SAHF, suggesting that complete inhibition of CDK2 activity is needed for induction of the senescent phenotype. Although in p53-proficient mice, CDK2 activity was completely abolished by the used doses of CVT13, this was obviously not the case in p53-deficient mice, and varied among animals. Possibly, this reflects variable degrees of complementation by other CDKs—perhaps CDK1, which is known to be capable of phosphorylating CDK2 targets (39) and may be expressed at increased levels in cells lacking p53 (40).

Why CDK2 inhibition elicits features of senescence in Ibrh-Cyclin D1, p53−/− pineal cells both in vivo and in vitro when it fails to do so in HRAsV12-transduced p53−/− MEFs in vitro, remains unclear but may be due to intrinsic cell-type–specific differences, as well as oncogene-specific responses. Indeed, similar differences have been observed in other studies; for example, Campaner and colleagues (18) found that lymphomas arising in Eu-myκ mice are delayed when mice are crossed into a CDK2−/− genotype background; however, in the absence of p53, this delay is abolished, indicating that it is p53 dependent. On the other hand, when they tested a panel of MYC-expressing cell lines, they found that CDK2 inhibition facilitated MYC-induced senescence even in the absence of p53. In another study, transfection of SAOS osteosarcoma tumor cells with dnCdk2-induced senescence, even in the absence of pRB and p53 (41). These data, in addition to our work, suggest that the response of different cell types to CDK2 inhibition, and the relationship of that response to the specific oncogenic insult, needs to be further investigated so to optimize CDK2 as a target in cancer therapy.

Our study identifies p53-dependent repression of CDK2 as a key mechanism during cellular senescence, essential for cell-cycle exit. It also suggests that, clinically, CDK2 inhibition may be effective in driving pretumorigenic cells into a senescent state, thereby abrogating tumor progression by preventing further proliferation and possible accumulation of genetic aberrations that may otherwise lead to bypass of senescence. Our data also support the notion that CDK2 inhibition will be therapeutically useful in inducing senescence and preventing further proliferation even in lesions that have already lost p53; however, in such cases, and as can be deduced from findings of our in vivo studies, it will be essential to achieve complete inhibition of CDK2 activity, together with any potential redundant CDK activities, to achieve effective cell-cycle exit and inhibition of tumor progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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