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 \textit{in vitro} and \textit{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 which 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 \textit{in vivo} model of pineal 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 is useful for cancer prevention of premalignant hyper-proliferative lesions, as well as established tumors.
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 (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 (3)). Cell cycle regulators are critically involved in the execution of the senescence response. In human fibroblasts, the cyclin-dependent kinase (CDK) inhibitors CDKN2a (p16Ink4a) and CDKN1A (p21Cip1), 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 p21Cip1 induction, which is essential for cell cycle exit and senescence (5, 9, 10). In yet other in vivo settings, other CDK inhibitors, such as CDKN2B (p15Ink4b) and CDKN2C (p18Ink4c), have also been shown to play roles in this process (11, 12).

In contrast to the well-documented roles of the CDK-inhibitors (p16Ink4a, p21Cip1, p15Ink4b, p18Ink4c) 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, where several investigators have shown that activity of CDK2 decreases in senescent fibroblasts, likely through inhibition of Cyclin A/CDK2 complexes by p21Cip1, while CDK2 protein levels remain unchanged (13-15); whereas in replicative senescence of cultured human umbilical vein endothelial cells, 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 re-entry in a subset of cells, indicating that reduction of CDK2 activity is indeed essential for replicative senescence (17).

In oncogene-induced senescence, limited data is available, but suggests a physiologic role for CDK2 in opposing senescence. For instance, in RAS \(^{V12}\)-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 p53+/− mice (Jackson Laboratory, Maine) or p53ERTAM ki/ki mice (21) and maintained in a mixed C57BL/6 × 129/Sv genetic background. PCR for targeted alleles was used to verify mouse genotypes as described (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, CVT313 was administered by once daily intraperitoneal injection of 1.25 mg/kg. For BrdU incorporation assays, mice were given an intraperitoneal injection of 50 mg/Kg of BrdU (Sigma-Aldrich, St Louis, MO), 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 (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 (CDK2 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 Dulbecco's Modified Eagles Medium (DMEM) containing 10% fetal bovine serum, 1% glutamine, and 1% Pen/Strep. Mouse embryonic fibroblasts (MEFs) were isolated from E13.5 embryos from wild type, p53⁻/⁻, p53ERTAM¹/¹, and Rb²⁻/⁻ mice (where exon 19 of the Rb gene is flanked by two LoxP sites, allowing induction of Cre-mediated recombination (24)). MEFs were cultured in DMEM containing 10% fetal bovine serum, 1% Non Essential Amino Acids, 1% Sodium Pyruvate, 1% glutamine, and 1% Pen/Strep. For MEFs derived from p53ERTAM¹/¹ mice, p53 was reversibly switched on and off by addition or withdrawal of 100nM 4-OH tamoxifen, as specified. For Rb²⁻/⁻ MEFs, Rb knock out was performed by using Cre-mediated recombination via lentiviral transduction. Successful recombination was assessed by PCR and western blot. For senescence induction, cells were exposed to 20 Gy irradiation at 1 Gy/min, or treated with 10 μM Etoposide. Cells were treated with CVT313 at 5 μM (Santa Cruz Biotechnology, Santa Cruz, CA) for CDK2 inhibition, with NSC625987 at 1 μM (Sigma-Aldrich) for CDK4 inhibition, with Flavopiridol at 1 μM (Sigma-Aldrich) for total CDKs inhibition or corresponding volume of DMSO vehicle; media was 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). Spinoculation 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 staining, cells were fixed and stained overnight at 37°C, and counterstained with eosin, as in our previous studies (12, 19). For BrdU incorporation assays, cells were treated with BrdU at a concentration of 33 μM 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-BrdU antibody (Santa Cruz Biotechnology). Cy3-conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA) or Alexa 488-conjugated antibody (Invitrogen, Carlsbad, CA) was used for detection. Stained cells were covered with aqueous mounting medium containing DAPI (Vector Laboratories, Burlingame, CA), and visualized by immunofluorescence microscopy. The number of BrdU-positive cells was manually counted from at least 5 representative fields, at 20x 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, Mountain View, CA).

**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 μM 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 15min. A dissecting microscope was used to view the cells under a magnified field.

**Cell Explantation and ex-vivo culture:** Mice were euthanized according to IACUC-approved protocol. Pineal cells were explanted at postnatal day 10 (P10), mechanically
dispersed, and cells were plated onto 8-well permanox chamber slides (Nunc, Rochester, NY), 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 was renewed every 3 days, and cells were fixed after 7 days. BrdU and SABG staining and cell counting were performed as detailed above. Digital photomicrographs were analyzed using Adobe Photoshop CS4 software.

**Histological studies and Immunostaining**

Brain tissue was fixed in 4% paraformaldehyde for 72 hours then embedded in paraffin. 4-8 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, San Diego, CA) anti-phosphorylated Histone 1 (Millipore, California, CA), anti-CDK2, anti-CDK4 (Santa Cruz Biotechnology) or anti-phospho RB Ser612 (MBL International, Woburn, MA), followed by biotinylated secondary antibody; and detected using streptavidin conjugated to horseradish peroxidase and DAB substrate (DAKO, Carpinteria, CA). For immunofluorescence staining, anti-H3K9me3 (Upstate Laboratories, Syracuse, NY), and anti-BrdU (Santa Cruz Biotechnology) antibodies were detected with Cyanine 2, Cyanine 3, or Alexafluor488 secondary antibodies, and counterstained with DAPI (Vector Laboratories). The numbers of positive cells were manually counted from 5 representative fields, at 40x 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, Hercules, CA), and detected using antibodies to p21<sup>Cip1</sup>, p15<sup>ink4b</sup>, p16<sup>ink4a</sup>, CDK4, CDK1, CDK2, DEC1, DCR2, HSC70, GAPDH, (all from Santa Cruz Biotechnology); phospho-Histone1 (Millipore), phospho- RB Ser612 (MBL International, Woburn, MA); total RB (BD-Pharmingen, San Diego, CA) and Tubulin (Abcam, Cambridge, UK).

**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 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 = AGCCAAAGGTCATCATCT; reverse = GGGGCCATCCACAGTCTTCT; mature Cdk2 mRNA, forward = CTGCATCTTGGCTGAAATGG, reverse = GATCCGGAAGAGTTGGTCAAT; pre-processed Cdk2 mRNA, forward = AGTACACCTGCTGGTCAAT; reverse = TGGCTGAAATCCGCTTGTT. PCR parameters consisted of denaturation at 95°C for 15 min followed by 40 cycles of 95°C for 15 sec, and 72°C for 1 min. Annealing temperature was 60°C for Gapdh and mature
Cdk2, and 55°C for preprocessed Cdk2 transcript. A final extension at 72°C for 10 min 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 housekeeping gene GAPDH was used as an internal normalization control.

**Statistical Analysis:**

Comparisons between experimental groups were performed using Student’s t-test, and a p-value below 0.05 was considered as statistically significant. Survival curves were generated as per the method of Kaplan and Meier.
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 pre-tumorigenic hyperplastic lesion limited by p53-dependent senescence (19). To verify whether down-regulation 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 BrdU incorporation into DNA) and positive staining for senescence-associated beta-galactosidase (SABG) [Figure 1A], in addition to other markers of senescence such as expression of p21Cip1, p15Ink4b, p16Ink4a, and DcR2 [Figure 1B]. Interestingly, CDK2 protein levels were specifically decreased in senescent cells whereas the levels of another S-phase kinase CDK1, and the G1/S kinase CDK4, were unchanged [Figure 1C and 1D]. Concomitant with the decrease in CDK2 levels, we found decreased levels of its target phospho-Histone 1 [Figure 1C], and decreased phosphorylation of the retinoblastoma protein RB1 (RB) at Ser612, a CDK2-specific phosphorylation site (25) [Figure 1C]. 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 [Figure 1D], which correlated temporally with the timing of cell cycle exit and preceded SABG positivity [Figures 1E and 1F].
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 down-regulation [see Figure 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-wild type MEFs [Figure 2A], but not in p53-null MEFs that were exposed to the same insults but failed to undergo senescence [Figure 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 down-regulation of mature Cdk2 mRNA compared with pre-senescent hyperplastic lesions (PG-PS) [Figure 2C]. We next evaluated the expression of the primary (pre-processed) Cdk2 mRNA transcript and found that levels of expression were also significantly decreased during senescence in p53-wild type MEFs [Figure 2D], but not in non-senescent p53-null MEFs [Figure 2E]. Similarly, primary mRNA transcript expression levels were decreased in senescent pineal cells in vivo [Figure 2F].

The lack of repression of CDK2 in p53-null cells and tissues [see Figures 2B and 2E] suggested that CDK2 down-regulation 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) (21). When exposed to etoposide-induced DNA damage, CDK2 was repressed in \( p53ER(TAM)^{K/K} \) MEFs only when the cells were treated with 4OHT [Figure 2G, compare lane 8 to the control lanes 5, 6, and 7]. The decrease in CDK2 did not occur in non-senescent \( p53\)-null MEFs at a similar time-point after etoposide treatment [Figure 2G, lanes 3 and 4], nor in \( p53\) wild-type MEFs immediately following etoposide treatment but before onset of senescence [Figure 2G, compare lanes 1 and 2]. This repression was specific to CDK2, as CDK4 levels were unchanged [Figure 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 \( p21^{Cip1} \), 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 utilized MEFs derived from \( Rb^{\beta/\beta} \) mice, where exon 19 of the \( Rb \) gene can be deleted by Cre-mediated recombination, essentially producing \( Rb^{\beta/} \) MEFs (24). As expected, genetic ablation of \( Rb \) resulted in increased baseline protein levels of Cdk2 (Figure 2H, compare lanes 1 and 3). In response to oncogene activation, we found that absence of RB prevented the previously observed downregulation of Cdk2 (Figure 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\textsuperscript{Cip1} induction (which occurs during senescence as seen in Figure 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\textsuperscript{V12} transduction), as evidenced by continued proliferation by day 14 and absence of SABG staining [Figures 3A and 3B], along with reduction in other protein markers of senescence [Figures 3C and 3D]. 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\textsuperscript{V12}-induced cells than in irradiated cells, which seemed to have reduced ability to undergo cellular proliferation irrespective of senescent state [Figure 3E, and see Figure 5D and more below]. Notably, constitutive CDK2 expression after senescence was well established could not reverse the phenotype [Figure 3B, 3\textsuperscript{rd} panel; and Figure 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 Figures 3C and 3D], but similar to levels seen in cells after RB disruption [see Figure 2H].

To ascertain whether down-regulation 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) (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 HRasV12 expression [Figures 4A and 4B] or DNA damage [Figures 4C and 4D]. This effect was unique to CDK2, since the specific CDK4 inhibitor NSC625987 caused only cell cycle exit but no SABG positivity or other features of senescence [Figure 4E and data not shown], while the pan-CDK inhibitor flavopiridol showed a similar phenotype of senescence to CDK2 inhibition [Figure 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 [Figure 4F and 4G]. 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.
[Figures 5A and 5B, and additional data not shown]. In addition, HRas$^{V12}$-transduced MEFs in which the $Rb$ gene is nonfunctional also failed to undergo senescence after CDK2 inhibition [Figure 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$^{V12}$-transduced p53$^{-/-}$ MEFs resulted in decreased cell accumulation, while cell accumulation in p53$^{-/-}$ MEFs after irradiation was already reduced and did not further decrease with CDK2 inhibition [Figure 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$^{-/-}$* mice with CVT313 did result in features of senescence, evidenced by decrease in BrdU incorporation into DNA, and positive staining for SABG [Figure 5E], though the decrease in BrdU incorporation occurred to a lesser extent than in pre-tumorigenic *Irbp-Cyclin D1,p53$^{+/+}$* pineal cells [Figure 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 pre-tumorigenic 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 post-natal day (P)10) with CVT313 resulted in effective CDK2 inhibition in Irbp-Cyclin D1 mice of both wild-type and p53-null background, as shown by decreased phosphorylation of the CDK2 target Histone 1 in harvested pineal tissue [Figure 6A]. While the pineal cells of control, untreated mice show no senescence phenotype until around 7 weeks of age (postnatal day (P)49) (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 CDK2 inhibitor (P16), and become readily apparent by 10 days after treatment (P20) [Figure 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, while others appeared unaffected by CVT313 [Figure 6C, left panel: (1) and (2) represent different mice, where (1) shows persistent Ki67 staining and absence of H3K9me3 foci while (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 which responded to CVT313 treatment and displayed features of senescence, while CDK4 staining was unchanged or increased [Figure 6C right panel, and quantitation in
Figure 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 [Figures 6C and 6D]. Importantly, in both cases, the cells that acquired SAHF were distinct from cells that continued to proliferate, shown by distinct staining for H3K9me3 and BrdU in dual stained sections [Figure 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 ten days (P10 through P20, as above) and monitored their survival. While 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 one month [Figure 6F, p < 0.001].

**Discussion:**

Our current study now identifies p53-dependent and RB-mediated down-regulation of Cdk2 as an essential physiological tumor suppressive mechanism, triggered by both oncogene-induced and DNA-damage-induced senescence. 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 (1, 2, 31)). During the onset of senescence, the CDK2-inhibitor p21Cip1 is up-regulated together with the CDK4/6-inhibitor p16Ink4a, both of which contribute to cell cycle exit, as are the CDK4/6 inhibitors p15Ink4b and p18Ink4c.
in some settings (reviewed in (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 (33, 34)). Unlike replicative senescence, where 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 et al. have shown that during HRasV12-induced senescence, there is a decline in the levels of cyclin A and CDK2 kinase activity (6). 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 co-transformed 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 down-regulation 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). While several studies did not show any notable changes in CDK2 protein levels despite decrease in kinase activity in replicatively senescent fibroblasts, one study using human umbilical vein endothelial cells (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 down-regulation of CDK2. In our studies, 
expression of exogenous CDK2 prior to 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 non-physiologic effects due to over-expression of the protein. Notably, levels 
of CDK2 protein were similarly high in RB-deficient cells (likely due to loss of the 
feedback inhibitory loop via 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-wild type 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 p21^{Cip1} (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 Figure 1B and references (12, 31, 37)).

While 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 retinoblastoma protein (pRB), Histone 1, p27^{Kip1}, and MYC (reviewed in (34, 38)). Hyper-phosphorylation of RB by CDK2, added to that already mediated by Cyclin D-CDK4/6 complexes in early/mid-G1, results in the release and activation of E2F-mediated transcription, precipitating S-phase entry (reviewed in (34)). It is therefore likely that the mechanism by which CDK2 repression contributes to senescence is through facilitation of cell cycle exit, however, as RB activation itself is necessary to cause downregulation of the CDK2 message, we propose that a feed-forward loop is activated to further enhance the decrease in CDK2 activity (by message reduction and not only inhibition of kinase activity), augmenting the inhibition of phosphorylation of the multiple downstream targets of CDK2. The specific targets or substrates of CDK2 that are important in this context remain to be investigated.

Our *in vitro* studies showed that exogenous CDK2 inhibition led to early onset of senescence after oncogenic or DNA damage-induced stress in both MEFs and human fibroblasts, while down-regulation or inhibition of CDK2 alone elicited cell cycle exit but
not other markers of the senescent state, such as SABG positivity. In addition, CDK2 inhibition in \( p53-/\) MEFs after oncogenic or DNA damage-induced stress did not lead to cell cycle exit nor to other features of senescence such as SABG positivity, but did limit proliferative capacity. This suggests that, in response to DNA damage or oncogenic stress, other downstream 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 \textit{in vivo} and \textit{in vitro}, 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 \textit{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 versus pineal cells) and oncogenic pathways (HRas\textsuperscript{V12} versus Cyclin D1 expression).

Of note, while 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. While in p53-proficient mice, CDK2 activity was completely abolished by the used doses of CVT313, 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 Irbp-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 et al. found that lymphomas arising in Eμ-myc 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 (18). 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 current 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 as 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 pre-tumorigenic 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, in order to achieve effective cell cycle exit and inhibition of tumor progression.
**Author Contributions:** HZ, BN, and MH participated in design of the study, and carried out most of the cellular and molecular studies, and participated in drafting the manuscript. HB, SG, and FG performed some of the cellular and molecular studies, and helped in drafting the manuscript. NG provided the RB fl/fl MEFs and participated in the design and execution of the related experiments. GIE participated in the design and coordination of the study, and review of the manuscript. RS conceived the study, and oversaw its design and coordination and drafted the manuscript. All authors read and approved the final manuscript.

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Figure Legends:

Figure 1: CDK2 protein levels are decreased during both oncogene-induced and DNA damage-induced senescence. A) Representative staining for BrdU (middle), corresponding DAPI nuclear stain (left), and SABG staining (right), in wild-type MEFs 7 days after Ras-induced senescence (RIS) or backbone plasmid-transduced controls (C) (upper panel); and human BJ fibroblasts after irradiation-induced senescence (S-IR) or Etoposide-induced senescence (S-Et) (lower panel), compared to control (C) untreated cells. Quantitative assessment of percent BrdU-positive cells is shown on the right of each panel. B) Western blotting for the indicated proteins in Ras-Induced Senescence (RIS) in MEFs and Etoposide-induced senescence (S-Et) in BJ fibroblasts, as above, compared to control; GAPDH is used as a loading control. C) Western blotting for the indicated proteins in Irradiation-induced senescence (S-IR) and Etoposide-induced senescence (S-Et) in MEFs and BJ fibroblasts, compared to control. D) Western blotting for the indicated proteins in MEFs at the indicated days after Ras$^{V12}$ transduction. E) Representative staining for BrdU (middle), corresponding DAPI nuclear stain (left), and SABG staining (right) in control plasmid-transduced (C) and Ras-transduced (RIS) MEFs at the indicated days. F) Upper panel: Quantitation of percent BrdU-positive cells at the indicated time points; dark grey columns denote control GFP-transduced cells, while light tan columns denote Ras$^{V12}$-transduced cells; Asterisks indicate significant p-value < 0.05. Lower panel: quantitation of percent SABG-positive cells in Ras$^{V12}$-transduced cells at the indicated time points.
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 A) wild-type MEFs and B) p53/-/- MEFs, 5 days after Ras\textsuperscript{V12} transduction, and in C) Irbp-Cyclin D1 pineal glands at pre-senescent (PG-PS) and senescent (PG-S) time points. Y-axis represents Cdk2 transcript levels normalized to Gapdh. D) & E) Real-time PCR analysis of primary Cdk2 mRNA transcript in wild type MEFs and p53\textsuperscript{-/-} MEFs, respectively, 5 days after Ras\textsuperscript{V12} transduction, and in F) Irbp-Cyclin D1 pineal glands at pre-senescent (PG-PS) and senescent (PG-S) timepoints. Y-axis represents Cdk2 transcript levels normalized to Gapdh. Asterisks indicate significant p-values < 0.05. G) Western blotting for the indicated proteins in wild-type (WT), p53\textsuperscript{-/-}, and p53ER\textsuperscript{K14Tam} MEFs, treated with 4-hydroxytamoxifen (4OH-Tam) or vehicle, and treated with 10 μM Etoposide (Eto) or vehicle, as indicated. Cells were collected 48 hours after treatment for wild-type control cells (lanes 1 and 2), and at day 7 for the remaining cells (lanes 3 to 8). GAPDH is used as a loading control. H) Western blotting for the indicated proteins in Rb f\textsuperscript{o}x/f\textsuperscript{o}x MEFs, after Cre-mediated recombination as indicated, and transduction with Ras\textsuperscript{V12} as indicated. TUBULIN is used as a loading control.

Figure 3: Ectopic CDK2 expression results in bypass of senescence. A) Representative immunofluorescence staining for BrdU (middle), corresponding DAPI nuclear stain (left), and SABG staining (right), in wild-type MEFs (upper panel) and BJs fibroblasts (lower panel) 14 days after irradiation and transduction with the control GFP-expressing plasmid (GFP), or the CDK2-expressing plasmid (CDK2), as indicated. Quantitation of
BrdU-positive cells as percentage of total cells is shown on the right. B) Representative immunofluorescence staining for BrdU (middle), corresponding DAPI nuclear stain (left), and SABG staining (right), in wild-type MEFs after Ras-induced senescence (RIS), and transduction with control (GFP) or both Ras\textsuperscript{V12} and CDK2 expressing plasmids, as indicated. Transduction at post-senescent (RIS post-S CDK2) and pre-senescent (RIS pre-S CDK2) stages are shown as indicated. Lower panel shows quantitation of BrdU-positive cells as percentage of total cells. Asterisks indicate significant p-values < 0.05. C) Western blotting for the indicated proteins in wild-type MEFs 7 days after transduction with Ras\textsuperscript{V12} alone or in combination with CDK2, as indicated. C denotes control cells transduced with backbone plasmid. D) Western blotting for the indicated proteins in human BJ fibroblasts 14 days after treatment; C denotes control cells transduced with backbone plasmid, S-Et denotes Etoposide (Et)-treated senescent cells, transduced with CDK2-expressing plasmid at a post-senescent (lane 3) and pre-senescent (lane 4) stages. E) Cell density assessment by Crystal Violet staining in wild-type MEFs. Upper panel: MEFs transduced with control empty vector (GFP), Ras\textsuperscript{V12} alone, or with constitutively expressed CDK2 (Ras\textsuperscript{V12}/CDK2), as indicated. Lower panel: Control untreated MEFs (C), after irradiation (IR), and both IR and transduction with constitutively expressed CDK2 (IR/CDK2).

Figure 4: CDK2 repression is necessary for senescence induction. A) Representative SABG staining (left) in wild-type MEFs 7 days after irradiation and transduction with either control plasmid (GFP), dominant negative CDK2-DN plasmid (CDK2DN), or treatment with the CDK2 inhibitor CVT313, as indicated. Right panel shows quantitation
of percentage BrdU-positive cells. Asterisks indicate significant p-values < 0.05. B) Western blotting for the indicated proteins in wild-type MEFs 4 days after transduction with $Ras^{Y12}$ expressing plasmid alone (RAS), or both $Ras^{Y12}$ and dominant negative $CDK2-DN$ expressing plasmids (RAS/CDK2DN) as indicated. C) Representative SABG staining (left) in human BJ fibroblasts 7 days after irradiation and transduction with control plasmid (GFP), dominant negative $CDK2-DN$ plasmid (CDK2DN), or treatment with the CDK2 inhibitor CVT313, as indicated. Right panel shows quantitation of percentage BrdU-positive cells. D) Western blotting for the indicated proteins in BJ fibroblasts 7 days after Etoposide (Eto) treatment, transduced with dominant negative $CDK2-DN$ plasmid (CDK2DN), or treated with the CDK2 inhibitor CVT313 as indicated. E) Representative immunofluorescence staining for BrdU (middle), corresponding DAPI nuclear stain (left), and SABG staining (right) in wild-type MEFs 7 days after Etoposide-induced senescence (S-Et) and treatment with the CDK2 inhibitor CVT313, CDK4 inhibitor NSC625987, and pan-CDK inhibitor Flavopiridol as indicated. F) Percentage of BrdU-positive BJ or MEF cells, as indicated, 7 days after Vehicle (Vh) or CVT313 treatment, without stress stimulus. G) Representative immunofluorescence staining for BrdU (middle), corresponding DAPI nuclear stain (left), and SABG staining (right) in wild-type MEFs 14 days after CVT313 treatment without oncogene expression or DNA damage stimulus.

Figure 5: CDK2 repression leads to senescence in a $p53$ and $Rb$-dependent manner in cultured fibroblasts. A) Representative immunofluorescence staining for BrdU (middle), corresponding DAPI nuclear stain (left), and SABG staining (right) in control (left panel)
and irradiated (right panel) $p53^{-/-}$ MEFs, 7 days after transduction with plasmid expressing GFP, CDK2DN, or treatment with CVT313, as indicated. B) Percentage of BrdU-positive cells in the conditions described in (A), as indicated. C) Representative immunofluorescence staining for BrdU (middle), corresponding DAPI nuclear stain (left), and SABG staining (right) in Rb flox/flox MEFs after Cre-mediated recombination, followed by irradiation, and treatment for 7 days with CVT313 or Vehicle (Vh), as indicated. Percentage of BrdU-positive cells is shown on the right. D) Cell density assessment by Crystal Violet staining, in $p53^{-/-}$ MEFs after Ras$^{V12}$ transduction or irradiation, then treatment with vehicle (Vh) or CDK2 inhibitor (CVT313), as indicated. E) Representative staining for BrdU (middle), corresponding DAPI nuclear stain (left), and SABG staining (right), in explanted pineal cells from 10-day old Irbp-Cyclin D1, $p53^{-/-}$ mice, 7 days after treatment with the CDK2 inhibitor CVT313 or vehicle control (Vh), as indicated. Lower panel shows quantitative assessment of percent BrdU-positive cells. F) Representative staining as in (E) but in explanted pineal cells from 10-day old Irbp-Cyclin D1, $p53^{+/+}$ mice. Lower panel shows quantitative assessment of percent BrdU-positive cells. Asterisks indicate significant p-value < 0.05.

**Figure 6:** Pharmacologic inhibition of CDK2 in vivo promotes cellular senescence.

A) Western blotting for the indicated proteins in Irbp-Cyclin D1 (D1) and Irbp-Cyclin D1, $p53^{-/-}$ (D1$p53^{-/-}$) pineal gland lysates after treatment with either vehicle (Vh) or CVT313 (CVT). pH1: phospho-Histone 1, HSC70 is a loading control. B) Representative immunostaining for Ki67, and H3K9me3 and corresponding DAPI staining, as indicated, in Irbp-Cyclin D1 pineal sections at postnatal days (P) 12, 16, and 20, as indicated, after
treatment with vehicle (Vh) or CVT313 (CVT) starting at P10, as indicated. Quantitative analysis of Ki67-positive pinealocytes per high power field is shown on the right. Asterisk indicates a significant p-value < 0.05. C) Representative immunostaining for the indicated proteins in pineal glands of Irbp-Cyclin D1, p53-/- mice treated for 10 days with vehicle (Vh) or CVT313 (CVT), as indicated. CVT-1 and CVT-2 indicate two different mice treated similarly with CVT313. D) Quantitative analysis of positively-stained pinealocytes for the indicated proteins, in 6 different Irbp-Cyclin D1p53-/- mice treated for 10 days as indicated. Numbers are an average of at least 5 sections, and are normalized to vehicle-treated controls. E) Dual immunofluorescence for BrdU (green) and H3K9me3 (red) in control Irbp-Cyclin D1 (D1) and Irbp-Cyclin D1p53-/- (D1p53-/- (1) and D1p53-/- (2)) pineal glands, 10 days after treatment with CVT313. F) Kaplan Meier survival curve depicting time to tumor formation in CVT313-treated Irbp-Cyclin D1, p53-/- mice (grey line) versus untreated controls (black line); p < 0.001. Arrow depicts the period of treatment (P10-P20).
References:


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Figure 5

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