

Dual Induction of Apoptosis and Senescence in Cancer Cells by Chk2 Activation: Checkpoint Activation as a Strategy against Cancer

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Abstract

The human checkpoint kinase 2 (Chk2) plays a central role in regulation of the cellular response to DNA damage, resulting in cell cycle arrest, DNA repair, or apoptosis depending on severity of DNA damage and the cellular context. Chk2 inhibitors are being developed as sensitizers for chemotherapeutic agents. In contrast, here we report that direct activation of Chk2 alone (without chemotherapeutic agents) led to potent inhibition of cancer cell proliferation. In the absence of *de novo* DNA damage, checkpoint activation was achieved by increased Chk2 expression, as evidenced by its phosphorylation at Thr⁶⁸, resulting in senescence and apoptosis of cancer cells (DLD1 and HeLa). The Chk2-induced apoptosis was p53 independent and was mediated by caspase activation triggered by loss of mitochondrial potential. The Chk2-induced senescence was also p53 independent and was associated with induction of p21. These results suggest that direct activation of checkpoint kinases may be a novel approach for cancer therapy. (Cancer Res 2005; 65(14): 6017-21)

Introduction

Complex mechanisms have evolved in mammalian cells to maintain genome integrity. Treatment of cells with DNA-damaging agents can lead to either activation of checkpoints that stop DNA replication to allow for DNA repair, or apoptosis, with both mechanisms acting to ensure proper replication of DNA. The human checkpoint kinase Chk2 is thought to play a central role in the response to DNA damage (1). Chk2 is able to receive signals from DNA damage sensors and relay those signals to the cell cycle machinery, resulting in a pause in cell cycle progression or, under certain circumstances, apoptosis (1, 2).

Much of the data regarding the involvement of Chk2 in the DNA damage-apoptosis pathway comes from studying the cellular response to DNA damage insults (3). Agents that cause DNA double-strand breaks (DSB), including ionizing radiation and certain chemotherapeutic drugs, activate Chk2 by phosphorylation at Thr⁶⁸ mediated by the ataxia-telangiectasia-mutated (ATM) kinase (4-6). Thr⁶⁸ phosphorylation is followed by phosphorylation of the p53 tumor suppressor at the Ser²⁰ residue, resulting in stabilization of p53 (7-9), a p53-dependent increase in the cyclin-dependent kinase (CDK) inhibitor p21 and, subsequently, a block in cell cycle progression. These findings suggest that Chk2 may play a pivotal role in the p53-regulated pathways that protect cells from replicating damaged DNA in the presence of DNA damage insults.

In addition to exogenous DNA-damaging insults, critical telomere erosions can generate an endogenous "DNA damage" signal that leads to Chk2 activation. Most human somatic cells can undergo only a limited number of population doublings *in vitro*. This exhaustion of proliferative potential, termed senescence, can be triggered when telomeres cannot fulfill their normal protective functions. Senescence is a p53-dependent growth arrest state that is thought to represent an innate defense against tumor progression.

It seems that Chk2 forms a central component of DNA damage response pathways to both telomere erosion and DNA damage insults. The therapeutic exploration of DNA damage response pathways thus far has been focused on developing checkpoint kinase inhibitors (2). The rationale is that checkpoint kinase inhibitors should suppress DNA damage repair, thereby enhancing apoptotic or growth arrest responses to chemotherapy or radiation. Inhibitors of Chk2 and other checkpoint kinases are now in pre-clinical and clinical development as sensitizers for chemotherapy (1, 2, 10).

It is unknown how cancer cells respond to checkpoint kinase activation in the absence of DNA-damaging agents or critical telomere attrition. Cancer cells are characterized by genomic instability and constitutive oncogene activation. The checkpoint response machineries are underfunctioning but preserved in cancer cells (1). We hypothesize that direct Chk2 activation might force cancer cells to respond optimally to checkpoint activation in the setting of endogenous chromosomal/DNA damage and inability to exit the cell cycle, which may induce checkpoint-mediated cell death or growth arrest. Here, we report that direct activation of Chk2 alone can lead to both senescence and apoptosis in human cancer cells without use of chemotherapeutic agents and that these effects are independent of the effects of the functional p53 protein. These results suggest that direct checkpoint activation may be explored as a novel strategy in the discovery of targeted cancer therapeutics.

Materials and Methods

Cell culture and expression vectors. DLD1 and HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin, and fungizone. Chk2-inducible cell lines were maintained in the aforementioned medium supplemented with 10 µg/mL Blasticidin and 500 µg/mL Zeocin. Chk2 induction was done by addition of 1,000 ng/mL tetracycline to growth medium unless specified otherwise. To create tetracycline-inducible Chk2 expression vector pcDNA4/TO-Chk2, the coding region of Chk2 was amplified from the full-length Chk2 cDNA clone (Invitrogen, Carlsbad, CA) by PCR and inserted into the *HindIII/EcoRI* sites of pcDNA4/TO (Invitrogen). The DLD1-T-Rex and HeLa-T-Rex cell lines were generated by stably transfecting the DLD1 and HeLa cancer cell lines with the regulatory vector pcDNA6/TR (Invitrogen) under selection with

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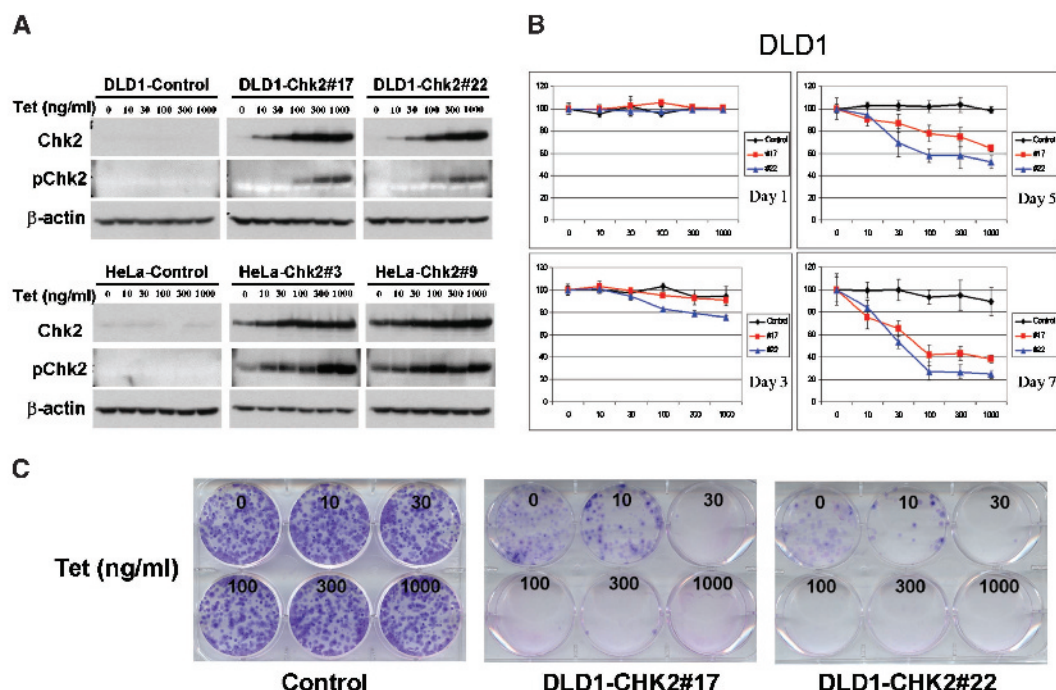


Figure 1. Chk2 expression inhibits cell proliferation. *A*, cells were cultured in DMEM with indicated concentration of tetracycline for 24 hours. The presence of total Chk2, the phospho-Thr⁶⁸ form of Chk2, and actin were assessed by direct immunoblotting of total cellular lysates. Lysates prepared from control cells were used as controls. *B*, cells were incubated with indicated concentrations of tetracycline. MTT assays were performed at the times indicated and the data were plotted. *C*, cells were incubated in medium with indicated concentrations of tetracycline. Cells were cultured for 2 to 3 weeks and colonies were stained with Giemsa.

10 μ g/mL Blasticidin. Chk2-inducible cell lines were generated by stably transfecting the DLD1-T-Rex and HeLa-T-Rex with the expression vector pcDNA4/TO-Chk2 under selection with 500 μ g/mL Zeocin. Inducible expression of the tet-Chk2 was monitored by Western blot, and two clones from each cancer cell line were selected for further analyses (DLD1-Chk2#17, DLD1-Chk2#22, HeLa-Chk2#3, and HeLa-Chk2#9). To inhibit the activity of caspase in cells, Z-VAD (Calbiochem, San Diego, CA) was added to culture medium to a final concentration of 100 μ mol/L.

Flow cytometry analysis. Tet-Chk2 clones were seeded in 6-well tissue culture dishes at 5×10^5 per well and were incubated in the presence or absence of 1,000 ng/mL tetracycline. Cells were harvested after 1 to 7 days of Chk2 induction, and aliquots of cells were subjected to DNA content analysis, Annexin V staining (Roche, Indianapolis, IN), and Pancaspase assays (Chemicon, Temecula, CA) as described previously (11) or as suggested by manufacturers.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and colony formation assays. For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cells were plated in 96-well plates at 5,000 cells per well and incubated in the presence of 0, 10, 30, 100, 300, and 1,000 ng/mL of tetracycline for 1, 3, 5, or 7 day(s). MTT was added to each well (0.5 mg/mL, final concentration) and plates were incubated for 2 hours at 37°C. Medium was aspirated and formazan product was solubilized in 150 μ L of isopropyl alcohol. The absorbance of each well was measured at 492 nm using a microplate reader. For colony formation assay, cells were plated in 6-well plates at 1,000 cells per well and cultured in medium with 0, 10, 30, 100, 300, and 1,000 ng/mL of tetracycline for 2 to 3 weeks. The colonies were stained with Giemsa.

Analysis of senescence. Senescence was assessed by staining for senescence-associated β -galactosidase activity as suggested by the manufacturer (Cell Signaling Technology, Beverly, MA).

Mitochondrial potential. Changes in mitochondrial membrane potential were monitored using DePispher Dye (Trevigen, Gaithersburg, MD). This dye forms red aggregates upon membrane polarization and reverts to

its green monomeric form if the potential is disturbed. The red aggregates have absorption/emission maxima of 585/590 nm and the green monomers of 510/527 nm. Briefly, DLD1-tet-Chk2#17 cells were pretreated with tetracycline for 7 days and were incubated with 10 μ mol/L DePispher at 37°C for 15 minutes, washed twice, and resuspended in PBS. Images with a fluorescein filter and rhodamine filter were taken with a Nikon Eclipse TE300 microscope.

Immunofluorescence. For immunofluorescence, cells were seeded on glass slides and were fixed in 4% paraformaldehyde for 10 minutes and were blocked in PBS containing 5% FBS for 1 hour. The samples were permeabilized with 0.2% Triton X-100 and incubated for 2 hours with primary antibody and for 0.5 hour with secondary antibody. The primary antibodies used were anti-P-Chk2 (Thr⁶⁸; Cell Signaling Technology), anti-P- γ H2AX (Ser¹³⁹; Upstate, Charlottesville, VA), anti-53BP1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-MDC1 (Abcam, Cambridge, MA), anti-P-ATM (Ser¹⁹⁸¹; Rockland Immunochemicals, Gilbertsville, PA), and anti-Phospho-(serine/threonine) ATM/ATR substrate antibody (Cell Signaling Technology). The secondary antibodies used were Rhodamine Red-conjugated goat anti-mouse IgG, Rhodamine Red-conjugated donkey anti-Rabbit IgG, FITC-conjugated donkey anti-mouse IgG, and FITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA). Images were obtained on a Nikon Eclipse TE300 microscope.

Immunoblotting. Cultured cells were harvested and lysed in HKMG buffer (11) by sonication. Samples were centrifuged and the supernatants were collected and mixed with sample loading buffer. Equivalent micrograms of protein from each lysate were electrophoresed on 8% to 15% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. After incubation with primary and secondary antibodies, the immunostained proteins were detected by enhanced chemiluminescence (Amersham, Piscataway, NJ). The primary antibodies used were anti-Chk2, anti- β -actin (Santa Cruz Biotechnology), anti-P-Chk2 (Thr⁶⁸), anti-caspase-3, anti-cleaved-caspase-3 (Asp¹⁷⁵), anti-caspase-9, anti-cleaved-caspase-9 (Asp³³⁰; Cell Signaling Technology), and anti-p21 (Labvision, Fremont, CA).

Results

Increased expression of Chk2 leads to activation of kinase activity. To study the effects of Chk2 expression in p53-deficient cancer cells, we generated a tetracycline-inducible (tet-on) Chk2 expression system in DLD1 and HeLa cancer cell lines, which harbor mutant p53 (DLD1) and undetectable wild-type (wt) p53 due to E6-mediated degradation (HeLa). We selected several DLD1-Chk2 and HeLa-Chk2 clones that behaved similarly in all the assays done. In this study, we choose two representative clones (#17 and #22 for DLD1 cells and #3 and #9 for HeLa cells) from each cancer cell line for further analyses. Western blot analysis showed that addition of tetracycline to the culture medium resulted in Chk2 protein production in a dosage-dependent manner in DLD1-Chk2 and HeLa-Chk2 cell lines (Fig. 1A). Cell lines with vector alone did not induce Chk2 protein production in the presence of tetracycline (Fig. 1A). Next, we examined the phosphorylation status of Chk2 and found that Chk2 protein could undergo phosphorylation at its activation site, Thr⁶⁸ (Fig. 1A). In contrast, we did not detect phosphorylation of Thr⁶⁸ on Chk2 in uninduced or control cells.

Thus, Chk2 protein expression is tightly controlled in our DLD1-Chk2 and HeLa-Chk2 tet-on systems.

Chk2 expression inhibited cell growth. To understand the biological effects of Chk2 expression, we first examined the effect of Chk2 expression on cell proliferation. Increasing concentrations of tetracycline were added to the culture medium of the DLD1-Control and DLD1-Chk2 (clones #17 and #22) cells and MTT and colony formation assays were done. Induction of Chk2 expression was found to suppress cell proliferation in both MTT assays and colony formation assays (Fig. 1B and C). Similar results were obtained in HeLa-Chk2 cell lines (data not shown). In contrast, growth of DLD1-Control cells was not affected by tetracycline addition (Fig. 1B and C). These results suggested that Chk2 expression induces cell cycle arrest and/or cell death.

Chk2 activation leads to dual induction of apoptosis and senescence of cancer cells. To investigate the effect of Chk2 expression on cell cycle progression, we analyzed the cell cycle profiles of DLD1-Chk2 clones (#17 and #22) after Chk2 induction by addition of tetracycline. We found that Chk2 expression resulted in

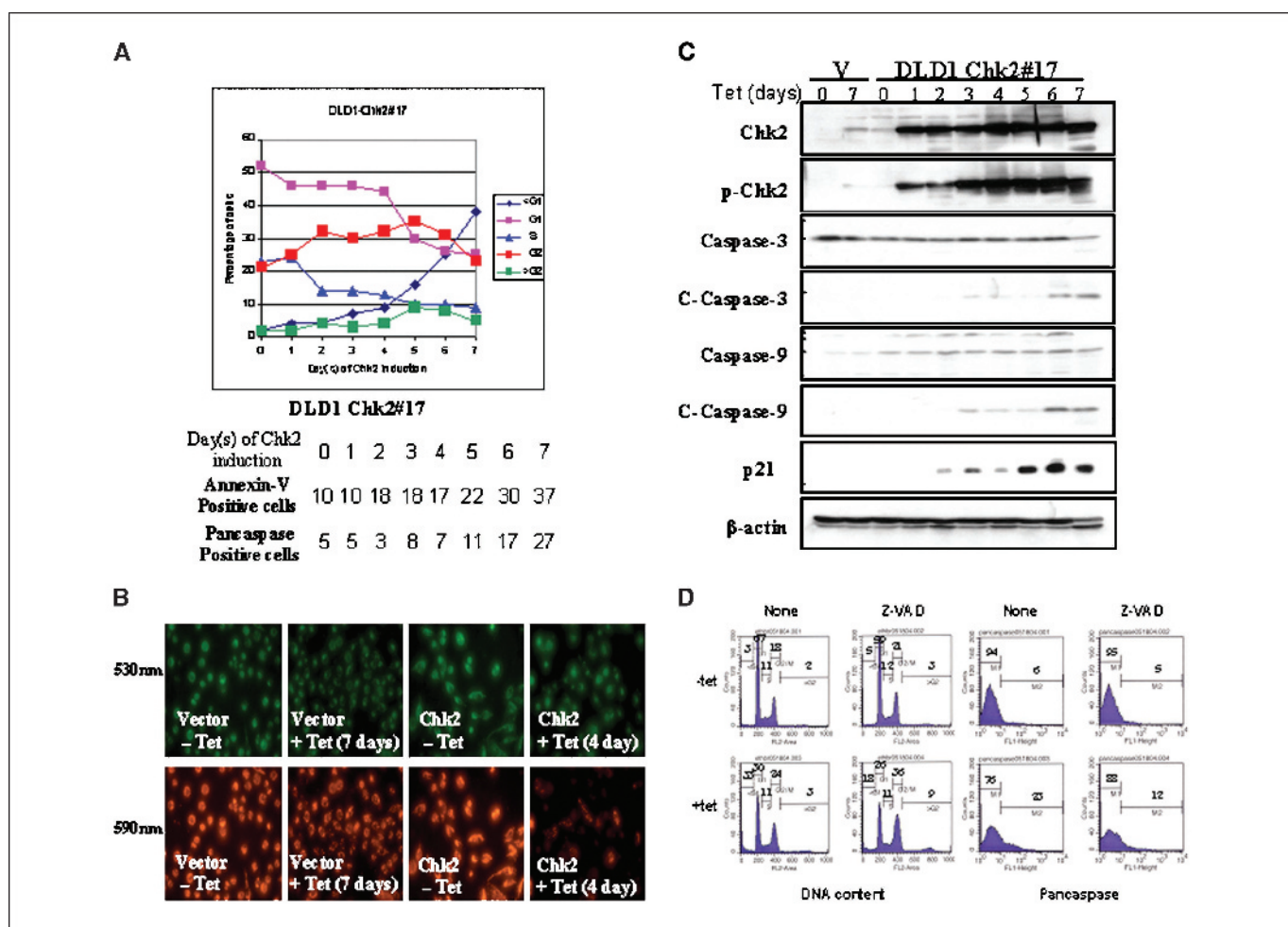


Figure 2. Chk2 expression induces p53-independent apoptosis. **A**, DLD1-tet-Chk2#17 cells cultured in medium containing tetracycline were collected at the indicated times. The DNA content of cells as measured by flow cytometry were plotted (top). The percentages of Annexin V-positive cells and Pancaspase-positive cells were determined by flow cytometric analysis (bottom). **B**, DLD1-tet-Chk2#17 cells were cultured in the presence or absence of tetracycline. Cells were stained with DePsipher dye and the intensity of the monomer (530 nm) and aggregates (590 nm) of DePsipher were monitored by fluorescent microscopy. **C**, DLD1-tet-Chk2#17 cells cultured in medium with tetracycline were collected at the indicated times. Lysates were prepared and subjected to immunoblotting with the antibodies indicated on the left. Lysates prepared from cells with vector alone were used as control. **D**, DLD1-tet-Chk2#17 cells treated with Z-VAD- or mock-treated (None) and cultured in the presence (+tet) or absence (-tet) of tetracycline for 6 days. DNA content and caspase activity of cells were determined by flow cytometric analysis and the percentages of cells undergoing apoptosis are indicated.

an increase in cell populations with sub-G₁ DNA content (~4% to ~40%) and G₂-M DNA content (20% to 30-35%) and a decrease in populations with G₁ DNA content (~52% to ~25%) and S DNA content (~25% to ~9%), compared with uninduced culture or control cells (Fig. 2A; data not shown). These results suggest that Chk2 expression can result in both a G₂-M delay and cell death. To further analyze the Chk2-induced cell cycle arrest and determine whether it was G₂-M transition arrest or mitotic arrest, we stained tetracycline-treated DLD1-Chk2 cells with an anti-phospho-Histone H3 Ser¹⁰ antibody that identifies mitotic cells. We found no increase in phospho-Histone H3-positive cells in tetracycline-treated DLD1-Chk2 cells (data not shown). Taken together, these observations suggest that expression of Chk2 resulted in G₂ arrest and cell death.

Chk2 expression induces apoptosis after G₂-M phase arrest. To distinguish apoptosis and necrosis, tetracycline-treated DLD1-Chk2 cells were subjected to Annexin V staining and Pancaspase

assay. We found that the numbers of Annexin V-positive and Pancaspase-positive cells increased significantly after 2 to 3 days of Chk2 induction (Fig. 2A). In addition, an increase in the active form of caspase-3 and caspase-9 and a decrease in the mitochondrial potential were observed in cells expressing Chk2 (Fig. 2C). Furthermore, Chk2-induced cell death was partially rescued by Z-VAD, a general caspase inhibitor (Fig. 2D). Similar results were obtained in HeLa-Chk2 cells (data not shown). In contrast, no increase of Annexin V-positive or Pancaspase-positive cells, active caspase-3 and caspase-9, or decrease in mitochondrial potential were observed in uninduced or control cells (Fig. 2A-C; data not shown). Collectively, these results indicate that expression of Chk2 resulted in caspase activation and apoptosis.

Chk2 expression induces cellular senescence. In addition to cell cycle arrest and apoptosis, expression of Chk2 resulted in cells assuming a large and flat morphology, with abundant cytoplasmic

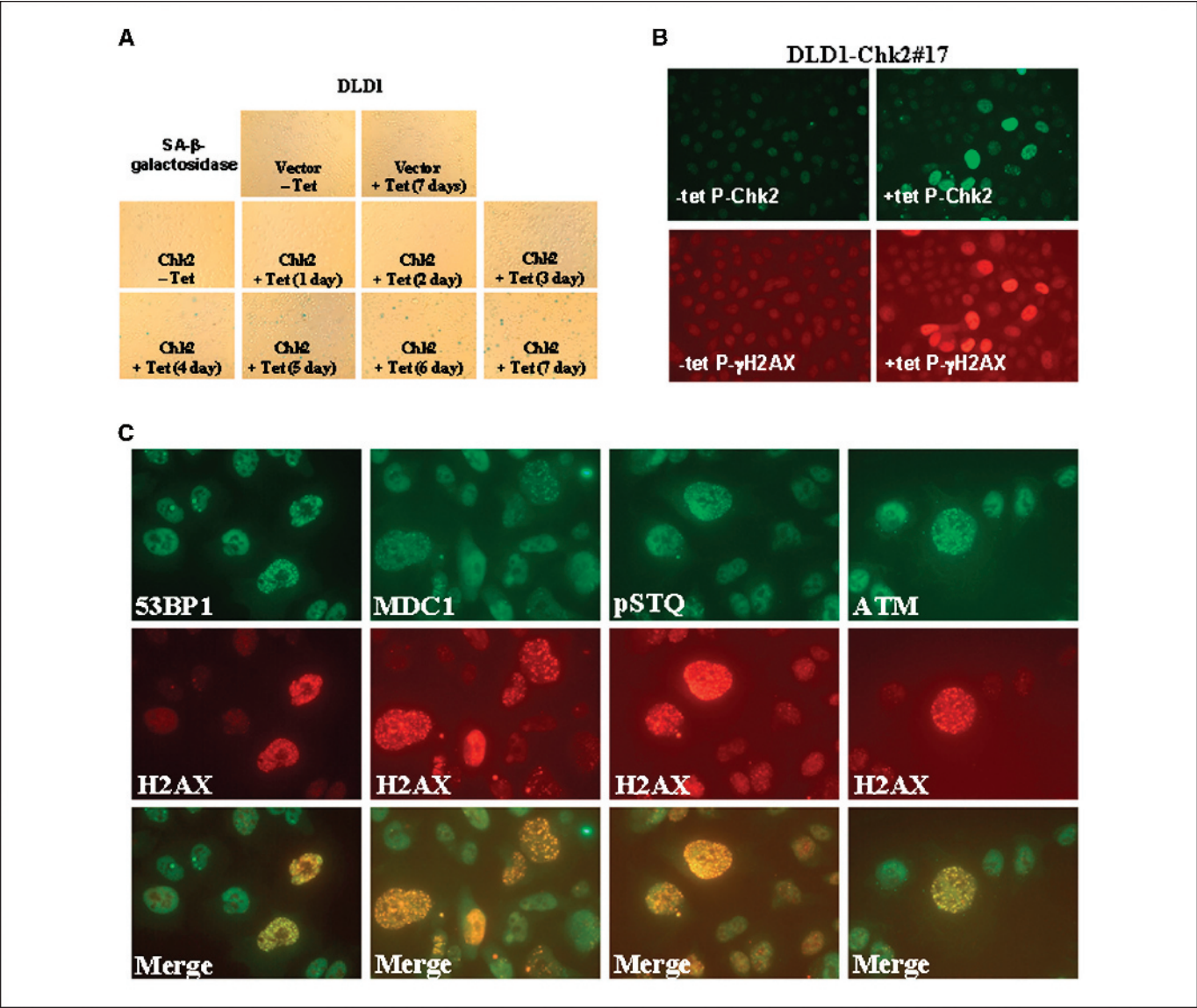


Figure 3. Chk2 expression induces p53-independent senescence. **A**, DLD1-tet-Chk2#17 cells grown in the presence (+tet) or absence (-tet) of tetracycline were subjected to senescence-associated β-gal staining at the times indicated. Pictures of cells were taken after 24 hours. DLD1 cells with vector alone were used as negative controls. **B** and **C**, DLD1-tet-Chk2#17 cells cultured in the presence (+tet) or absence (-tet) of tetracycline were subjected to immunofluorescence analysis with antibodies indicated. The merged images of two different stainings are shown (Merge). DLD1 cells with vector alone were used as negative controls.

vacuolization (data not shown). Similar phenotypic changes have been frequently observed in senescent cells. To assess whether DLD1 cells undergo cellular senescence following Chk2 expression, we analyzed the activity of the senescence-associated β -galactosidase (SA- β -gal), a specific marker of cellular senescence. Approximately 15% of DLD1-Chk2 cells were positive for SA- β -gal staining after 4 days of Chk2 induction, and the percentage was increased further to ~35% after 7 days of induction (Fig. 3A). In addition, Chk2-expressing cells had increased p21 expression (Fig. 2C), which is frequently found in senescent cells. Similar results were obtained following expression of Chk2 in HeLa cells. These data suggest that expression of Chk2 can result in cellular senescence.

Chk2-expressing cells form nuclear foci. The accumulation of γ -H2AX foci has been found in both apoptotic and senescent human cells (12, 13). γ -H2AX foci reveal DNA DSBs and colocalize with DSB repair factors such as ATM, 53BP1, and MDC1. To investigate whether γ -H2AX foci were present in cells following expression of Chk2, we examined the incidence of foci in DLD1-Chk2 cells by immunofluorescence. We found the appearance of γ -H2AX in 10% to 15% of DLD1-Chk2 cells after 3 days of Chk2 induction, and the percentage increased to 25% to 30% after 7 days of Chk2 induction (Fig. 3B; data not shown). In addition, we also found colocalization of ATM, 53BP1, and MDC1 with the γ -H2AX foci (Fig. 3C). Similar results were observed following expression of Chk2 in HeLa cells.

Discussion

In this study, we report that *in the absence of de novo DNA damage*, direct Chk2 activation leads to potent inhibition of survival and growth arrest of cancer cells. Increased expression of Chk2 may result in its activation via oligomerization-mediated autophosphorylation (6), with subsequent cell growth arrest, cell death, and senescence.

Importantly, activation of Chk2 leads to both senescence and apoptosis in cancer cells without functional p53. Apoptosis occurred via the classic external apoptotic pathway with loss of mitochondrial potential and activation of caspase-9 and caspase-3. This was accompanied by the appearance of markers of apoptosis and also accumulation of γ -H2AX foci and phosphorylation, a marker of checkpoint kinase activation (12, 13). The ability of Chk2 to induce

cell senescence is especially intriguing. Usually, DNA damage can cause accelerated senescence via induction of p53 and p21 (14–16). In addition, simultaneous activation of mitogen-dependent kinases and induction of CDK inhibitors such as p21 causes cell senescence (16). Here we show that Chk2 also induces cell senescence associated with p21 in the absence of DNA damage and p53 function.

Chk2 induces both apoptosis and senescence. Inhibition of caspases partially prevented apoptosis; therefore, inhibition of caspases may transform Chk2-induced apoptosis to cell senescence. Currently, it is recognized that apoptosis of tumor cells is a goal of cancer therapy (17, 18). If apoptosis fails, however, induction of tumor cell senescence is an alternative (19). Therefore, activation of Chk2 is a particularly promising anticancer strategy because if the apoptotic program is blocked, cell senescence can be induced as an alternative. These results also suggest that apoptosis and cellular senescence may be interconnected and interconvertible.

Inhibitors of Chk2 and other checkpoint kinases are in preclinical and clinical development as sensitizers for chemotherapy (1, 2, 10). Normally, Chk2 is activated by DNA damage caused by DNA-damaging agents. Our findings suggest that expression of Chk2 in p53-mutated tumor cell lines, in the absence of exogenous DNA-damaging agents, is sufficient to block cell cycle progression that can lead to senescence and apoptosis. These findings suggest an attractive novel concept for cancer therapy. Due to genomic instability and high levels of DNA damage (upstream of Chk2) and constitutive oncogene activation in cancer cells the direct activation of checkpoints can be cytotoxic to cancer cells (20). As we showed here, expression of Chk2 in p53-deficient cancer cells alone resulted in phosphorylation of Chk2 in the absence of DNA damage, leading to apoptosis and senescence. This result suggests that the direct activation of checkpoint kinases/regulators should be explored as a novel therapeutic approach for targeted cancer therapy.

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