Cleavage of CDK Inhibitor p21\textsuperscript{Cip1/Waf1} by Caspases Is an Early Event during DNA Damage-induced Apoptosis*

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Activation of the p53-mediated DNA damage response induces either G\textsubscript{1} cell cycle arrest or apoptosis. The G\textsubscript{1} cell cycle arrest is in part caused by the p53-dependent transcriptional activation of the CDK inhibitor, p21\textsuperscript{Cip1/Waf1}. We report here that human p21 protein is rapidly induced but selectively cleaved during the apoptotic response to \textgamma-irradiation. Such an event occurred early, well before the morphological appearance of apoptosis. Ectopic expression of p53 in tumor cells alone could induce p21 expression, followed by p21 cleavage and apoptosis. The cleavage of p21 could be reproduced in extracts prepared from irradiated cells or by recombinant caspase-3, suggesting that a caspase-like activity is responsible for this cleavage. p21 binds independently to both CDK2 and proliferation cell nuclear antigen (PCNA). Our studies indicated that p21 cleavage by the caspase-like activity specifically abolished its interaction with PCNA, suggesting that p21 cleavage may interfere with normal PCNA-dependent repair. Our data suggest that p21 may serve as a critical checkpoint regulator for both cell cycle arrest and apoptosis during the p53-mediated DNA damage response. Manipulation of the checkpoint regulators involved in cell cycle arrest and apoptosis may thus provide a novel strategy to cancer therapy.

DNA damage agents are mutagens that induce genetic lesions and cause cancer. Mammalian cells respond to DNA damage signals by activating cell cycle checkpoints which arrest the cell cycle in both G\textsubscript{1} and G\textsubscript{2} phases or by inducing programmed cell death (apoptosis) (1, 2). It is well established that the p53 tumor suppressor is responsible for both DNA damage-induced G\textsubscript{1} cell cycle arrest and apoptosis (3). The DNA damage-induced G\textsubscript{1} cell cycle arrest is in part caused by the p53-dependent transcriptional activation of p21\textsuperscript{Cip1/Waf1} (4), a potent inhibitor of the cell cycle kinases (cyclin/CDKs) that binds to various cell cycle kinases (5, 6). \textit{In vivo}, a major fraction of p21 also associates with the proliferation cell nuclear antigen (PCNA),\textsuperscript{3} a replication and repair factor (7, 8). The binding of p21 has been shown to inhibit the replication, but not repair, activity of PCNA \textit{in vitro} (9, 10). Although it remains unclear the \textit{in vivo} significance of the association between p21 and PCNA, the co-localization of p21 with PCNA at the DNA repair foci, and the fact that p21 can associate with active cell cycle kinases suggest that p21 may play a role during the DNA damage response (8, 10, 11).

Activation of p53 also induces apoptosis and dysfunction of p53 leads to cellular resistance to genotoxic stress such as DNA damage (3). However, it is still unclear how p53 induces apoptosis in certain cells but causes cell cycle arrest in other cells. Several observations suggest that failure to express sufficient levels of p21 converts the normal cell cycle arrest into apoptotic cell death (12, 13). In human cancer cells that normally respond to DNA damage by cell cycle arrest, deletion of p21 genes by homologous recombination leads to the loss of cell cycle checkpoint control and cells die through apoptosis (12). The expression of p21 is also controlled by mitogenic growth factors (13). Growth factor withdrawal reduces p21 expression. DNA-damaged cells cultured under growth factor starvation conditions die through apoptotic death instead of normal cell cycle arrest (15). Conversely, it has been reported that ectopic expression of p21 prevents the p53-mediated apoptosis in human melanoma cells (16).

Genetic and biochemical studies indicate that programmed cell death or apoptosis is triggered by activation of the members of the CED-3/caspase protease family (17, 18). These proteases preferentially cleave protein substrates at certain aspartic acid residues. Activation of caspases during apoptosis converts the inactive, pro-enzyme form of caspases into the active, processed form which in turn cleaves downstream substrates, leading to the appearance of apoptotic morphologies such as condensation of nucleoplasm, blebbing of nuclear and cytoplasmic membranes, and fragmentation of cells to form apoptotic bodies, which are accompanied by biochemical events such as DNA fragmentation (2). In mammals, a number of caspases have been identified. Among them, caspase-3 (formerly C3P32, Yama, or apopain) has been implicated in playing a critical role during apoptosis (19, 20). So far, a number of caspase substrates have been identified. These include poly-(ADP-ribose)/polymerase, nuclear lamin, DNA-dependent protein kinase (DNA-PK), and the U1 RNA-associated 70-kDa protein (21–24). However, it remains unclear whether these proteins are the critical substrates that cause the manifestation of apoptotic morphology and the eventual apoptotic death of the cells.

To investigate how cells control the conversion between cell cycle arrest and apoptosis during DNA damage response, we analyzed the molecular events of cell cycle regulation during \textgamma-irradiation. We report here that in cells that are prone to apoptosis, p21 is rapidly induced by DNA damage but is selectively cleaved by a caspase-like activity. p21 thus appears to be a critical checkpoint target for both cell cycle arrest and apoptosis.
Experimental Procedures

Cell Culture and Metabolic Labeling—The ML-1 (human myeloblastic leukemia) cells were a gift from Dr. David Beach (Cold Spring Harbor Laboratory) and were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, WI38 (human lung fibroblasts), HCT116 (human colon carcinoma), and DLD-1 (human colon adenocarcinoma) cells were purchased from ATCC. They were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C. For metabolic labeling of cells with [35S]methionine, ML-1 cells were irradiated with a 137Cs irradiator at various doses and then cultured in the methionine-deficient medium supplemented with 10% dialyzed fetal bovine serum (Life Technologies, Inc.) in the presence of 250 μCi/ml [35S]methionine (25). For the pulse and chase experiments, the cells were labeled for 2 h in medium containing [35S]methionine. The labeling medium was then removed and the cells were washed twice with warm RPMI 1640 medium. The chase was performed in the regular RPMI 1640 medium in the presence of 1 μM methionine.

Antibodies and Immunological Procedures—The rabbit polyclonal antibodies raised against full-length human p21 and cyclin A were described previously (8). The CDK2 antibody was raised against the 12 amino acid residues at the carboxyl-terminal as described (7). The rabbit polyclonal antibody against human p27 was raised against a fusion protein (GST-p27), between glutathione S-transferase and human p27 using the protocols as described previously (7, 25). All the antibodies were gifts from Dr. David Beach.

Control or irradiated cells were harvested and immunoprecipitated in a buffer containing 0.5% Nonidet P-40, 150 mM NaCl, 50 mM Tris, pH 7.4, 100 mM sodium fluoride, and 10 μg/ml each of the following protease inhibitors: trypsin inhibitor, aprotinin, and leupeptin, as well as 1 mM benzamidine for 3 h at 4 °C (25). Western blot analysis of various proteins was conducted using the ECL method (Amersham) as described before (25). The kinase assay using histone H1 as the in vitro substrate was conducted as described (8).

Adenovirus Purification and Infection—The construction of adenoviruses encoding lacZ, p21, and p53 at the E1 locus under the cytomegalovirus promoter control has been described previously (16, 26, 27). The viruses were amplified using 293 cells as the host. The viruses were purified through two rounds of CsCl density gradient centrifugation according to Graham and Prevec (28). For infection, 5 × 10^6 cells were grown to log phase and infected with adenoviruses at 10 plaque forming units/cell. Twenty-four or 48 h post-infection, the cells were harvested and analyzed by flow cytometry analysis using propidium iodide staining as described (29). The protein level and cleavage of p21 in the adenovirus-infected cells were analyzed using immunoprecipitation and Western blot analyses as described above.

Extract Preparation and Assays for p21 Cleavage—Non-irradiated and 8 gray irradiated ML-1 cells were collected 8 h post-irradiation. They were suspended in a buffer containing 10 mM Hepes, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol, 10 μg/ml each of the following protease inhibitors: trypsin inhibitor, aprotinin, and leupeptin, as well as 1 mM benzamidine (30). Cells were homogenized with a Dounce homogenizer with a loose-type pestle. The cytosolic fraction was obtained after spinning at 100,000 × g and was stored at −80 °C until use. For in vitro translation, the cloned p21 cDNA in Bluescript-II SK- (Stratagene) was transcribed and translated with T3 RNA polymerase in the T3-coupled TNT rabbit reticulocyte lysate system (Promega) in the presence of [35S]methionine. Five microliters of the in vitro translated p21 protein was incubated with 50-μl extracts (40 μg of total protein) or caspase-3 (2 μg) for 30 min at 30 °C. The protein products were isolated by immunoprecipitation. The GST-caspase-3 DNA construct was kindly provided by Dr. Emad Alnemeri (Thomas Jefferson University). It was expressed in Escherichia coli (BL21 strain) and affinity purified by glutathione-Sepharose beads (Pharmacia Biotech Inc.). The caspase inhibitor, AC-DEVD-CHO, was obtained from Bachem and used at 100 nM. The site-directed mutagenesis for the p21 mutants was conducted using the method as described before (8).

Results

Association of p14 with Cyclin A/CDK2 and p21 Complexes in γ-Irradiated Cells—We used the ML-1 cells, a human myeloblastic leukemia cell that contains a functional p53, to investigate the DNA damage response (31). γ-Irradiation of ML-1 cells induced cell cycle arrest (31). Microscopic examination revealed that a substantial fraction of the cells underwent nuclear fragmentation and cell membrane blebbing, morphological changes typically associated with apoptotic cells (Fig. 1A). The apoptotic response was damage dose-dependent and occurred in a time dependent fashion (Figs. 1B and 3A). After a latent period, apoptotic cells, about 30–50% of the total cell population, appeared at 5–6 h post-irradiation (Fig. 1B). Further incubation up to 12 h or more, however, did not significantly increase the fraction of the apoptotic cells. To monitor the molecular events that may be associated with the cyclin-CDK and p21 complexes in the irradiated ML-1 cells, the non-irradiated and irradiated cells were labeled with [35S]methionine. The cyclin-CDK and p21 complexes were then isolated by immunoprecipitation with their respective antibodies. The proteins that associated with cyclin-CDK and p21 complexes were resolved in an SDS-polyacrylamide gel and compared between DNA damage treated and non-treated samples. Strikingly, a 14-kDa protein was found to specifically associate with the cyclin A, CDK2, and p21 complexes after irradiation (Fig. 2A). The specific association of this protein was confirmed by its disappearance from the CDK2 complexes if a competing CDK2 antigenic peptide was included during immunoprecipitation (Fig. 2A). Because of these interesting features, we have further characterized p14.
To determine the identity of p14, we tested whether p14 is a pre-existing protein or a new protein that is induced by DNA damage. To distinguish these possibilities, a pulse and chase experiment was performed. Both control and irradiated cells were pulse labeled for 2 h with [³⁵S]methionine. After removal of the [³⁵S]methionine-containing medium, the cells were re-cultured in regular medium and harvested at different time points (chase). The labeled proteins that associated with cyclin A, CDK2, and p21 complexes were isolated by immunoprecipitation and analyzed for their kinetics of disappearance or appearance.

Comparison of the patterns of the proteins associated with the CDK2∥p21 complexes revealed that p14 was not present during the initial pulse labeling period (first 2 h) after irradiation. However, although the labeled p21 and other proteins were disappearing during the chasing period, p14, in contrast, started to appear in the cyclin A∥CDK2 and p21 complexes during subsequent hours of chasing (Fig. 2B). As observed before, the association of p14 occurred only in the irradiated cell samples, suggesting that p14 might come from a protein that is labeled during the initial pulse labeling period in irradiated cells.

p14 Is a Cleavage Product of p21 after DNA Damage—To identify the source of p14, we blotted the immunoprecipitated p21 and cyclin A∥CDK2 complexes with p21, CDK2, and cyclin A-specific antibodies. As expected, p21 was rapidly induced after irradiation (Fig. 3A). In addition, the p21 antibody could detect the induction of an additional protein of 14 kDa in the irradiated cell samples. p14 became detectable between 2 and 3 h after γ-irradiation, which paralleled with the p21 induction profile (Fig. 3A). To determine whether the presence of p14 is associated with the apoptotic process rather than cell cycle arrest, the p21 complexes from WI38 human fibroblasts and ML-1 cells were compared after γ-irradiation. WI38 cells respond to DNA damage by cell cycle arrest but not apoptosis (32). Although we could clearly detect the induction of p21 in WI38 cells after irradiation, p14 was present only in irradiated ML-1 cells (Fig. 3A). To further examine whether p14 is associated with apoptosis, we tested its potential presence in apoptotic cells induced by other means. ML-1 cells undergo apoptotic death once they reach high cell density. p14 was also detectable in the ML-1 high density-induced apoptotic cells but not in the actively growing cells (Fig. 3A). These studies suggest that p14 might be a proteolytic product of p21 in cells that are prone to apoptotic death. Since p14 is induced much earlier than any detectable apoptotic phenotypes such as nuclear blebbing, the cleavage of p21 appears to be an early event during DNA damage-induced apoptosis.
the DNA damage-induced apoptosis. Interestingly, we found that the cleavage of p21 is quite selective, since other cell cycle regulators such as CDK2, CDK4, CDC2, cyclin A, and p27, were not detectably cleaved after irradiation (Fig. 3B, and data not shown).

We have examined the kinase activity of CDK2 complexes after irradiation using the histone H1 as the in vitro substrate. The kinase activity of CDK2 became inhibited 3 h after γ-irradiation (Fig. 3B). This might be due to the fact that only a fraction of p21 is cleaved and there is substantial increase in total amounts of p21 after γ-irradiation (Fig. 3A). In addition, we found that the transcription of cyclin A and cyclin B became inhibited 6–8 h after the DNA damage treatment (data not shown).

**Induction of p21 Cleavage by p53-induced Apoptosis**—Since ML-1 is a p53 positive cell line (31) and p53 induces both cell cycle arrest and apoptosis, we tried to determine whether the expression of p53 alone can induce p21 cleavage. To deliver and express p53 efficiently, we used a recombinant adenovirus that encodes p53. We also used adenoviruses that contain *lacZ* or p21 genes as the control. Two well characterized human colon carcinoma cell lines, HCT116 and DLD1, were used in these experiments. HCT116 cells contain a functional p53 which have been shown to respond to p53 expression by the cell cycle arrest (32). DLD1 cells are deficient in p53 activity and respond to p53 expression by apoptotic death (33). While infection of HCT116 or DLD1 cells with adenovirus encoding *lacZ* or p21 did not affect cell morphology, expression of p53 in DLD1 cells caused a fraction of these cells to become rounded up and detached from the plate within 24–48 h post-infection. Such an apoptotic cell death was confirmed by flow cytometry analysis (Fig. 4A). As reported before (33), p53 expression in HCT116 cells only caused the G1 cell cycle arrest (Fig. 4A). Analysis of p21 from these adenovirus-infected HCT116 and DLD1 cells revealed that while both p21- and p53-containing adenoviruses induced similar high level expression of p21, p14 was present only in the p53 adenovirus-infected DLD1 cells (Fig. 4B). These data are consistent with our previous findings in WI38 and ML-1 cells that p14 is likely to associate with the p53-mediated apoptotic response during the DNA damage treatment. The elevation of the p21 level alone is not sufficient to induce p14.

**p21 Cleaved by a Caspase Like Activity after γ-Irradiation**—To determine the protease that cleaves p21 after γ-irradiation, we prepared cell extracts from the control and γ-irradiated ML-1 cells. To assay for p21 cleavage activity, *in vitro* translated and [35S]methionine-labeled p21 was incubated with the cell extracts. The reaction products were recovered by immunoprecipitation with p21 antibodies and analyzed by protein gel electrophoresis. In these experiments, the cleavage of p21 could be recapitulated in the γ-irradiated extract but not in the control extract (Fig. 5A). As shown in Fig. 5B, the *in vitro* cleaved p14 product migrated precisely as that of p14 isolated from the *in vivo* irradiated samples. Since one prominent event during apoptosis is the activation of caspase proteases (30), we tested whether p21 could be cleaved by known caspases such as caspase-3, a close homologue of CED-3. A recombinant fusion protein between the glutathione S-transferase and caspase-3 (formerly CPP32) (GST-caspase-3) was expressed in bacteria and isolated using glutathione-Sepharose beads. Incubation of *in vitro* translated p21 with caspase-3 produced a p14 which is identical to the one obtained from either *in vivo* cell samples after γ-irradiation or from the irradiated cell extract (Fig. 5, A and B). Partial V8 protease mapping of p14 obtained from various sources confirmed that they were identical (Fig. 5C). In addition, we also found that p21 cleavage in the irradiated cell extract could be inhibited by the caspase-3 inhibitor, AC-DEVD-CHO (30) (Fig. 5A), suggesting a caspase-like activity in the γ-irradiated cells is responsible for the p21 cleavage.

**p21 Cleavage Affects Its Binding to PCNA in Vitro**—To examine the biological effect of the p21 cleavage, we tried to determine the precise location of the caspase cleavage site. Inspection of the human p21 protein sequence revealed that it contained a potential caspase-3 cleavage motif between aspartic acids 109 and 112 (AEED*HVD*LSL, the first D* is aspartic acid residue 109 and the second is 112) (20). To test whether these aspartic acids are the recognition and cleavage sites of caspase-3, we converted aspartic acid 109 or 112 individually into alanine (D109A or D112A) by site-directed mutagenesis (8). Using the *in vitro* cleavage assays, our data indicated that both D109A and D112A mutants abolished the p21 cleavage by caspase-3 (Fig. 6A). To further analyze the caspase cleavage site, we generated truncated p21 mutants that have deleted the regions after aspartic acid 109 (Asp109) or 112 (Asp112). These truncated p21 derivatives were *in vitro* translated and their electrophoretic mobilities were compared with that of p14. Our results indicated that aspartic acid 112 is the caspase cleavage site (Fig. 6A). Since it has been shown that caspase-3 preferentially cleaves at an aspartic acid if a negatively charged amino acid residue is present near its amino-terminal region (20), the effect of D109A mutant on p21 cleavage is likely caused by the alteration of caspase recognition site on p21.

p21 can bind to both CDK and PCNA independently. Such interactions have been shown to reside on two separate regions of p21 (34). The CDK recognition domain has been mapped to

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**Fig. 4.** p14 is induced during the p53-mediated apoptosis. A, DLD1 and HCT116 cells were infected with adenoviruses encoding *lacZ* (Ad-*lacZ*), p21 (Ad-p21), or p53 (Ad-p53) at 10 plaque forming units/cell. Cells were harvested and analyzed by flow cytometry analysis at 24 (top) or 48 h (bottom) post-infection. Sample order: Ad-*lacZ*, front; Ad-p21, middle; and Ad-p53, back. B, DLD1 and HCT116 cells were infected with adenoviruses as described in A. Forty eight hours after infection, p21 complexes were isolated by anti-p21 immunoprecipitation. The immunoprecipitated proteins were blotted with the p21 antibody.
the amino-terminal half of p21 while the PCNA-binding domain is at the carboxyl-terminal region. To determine the potential significance of p21 cleavage, we examined whether such a cleavage affects its binding to either PCNA or CDK2. p21 and the p21–112Z mutant derivative were expressed in bacteria. The fusion proteins were purified by glutathione-Sepharose beads. The purified GST-p21, GST-p21–112Z, and the control GST proteins were incubated with PCNA or CDK2 that was obtained from in vitro translation. The PCNA and CDK2 proteins that bound to the GST p21 proteins were then recovered from the reactions by glutathione-Sepharose beads. Our studies indicated that while GST-p21 could bind to both PCNA and CDK2 in these assays, the GST-p21–112Z protein could only bind to CDK2 and failed to associate with PCNA.

Fig. 5. p21 is cleaved by a caspase like activity. A, in vitro translated p21 (p21 input) was incubated with either ML-1 extracts prepared from non-irradiated (control extract) or irradiated cells (IR extract). It was also treated with recombinant GST S-transferase or GST-caspase-3 (GST-Cas-3) as indicated. The effect of the caspase inhibitor, AC-DEVD-CHO, was also examined (–/- inhibitor). The reaction products were isolated by immunoprecipitation with p21 antibodies and separated in a protein gel. The proteins were visualized by autoradiography. B, comparison of p14 proteins from in vivo and in vitro samples. The p21 complexes were isolated from non-irradiated (control) and irradiated ML-1 (IR) cells after labeling with [35S]methionine for 5 h. The mobility of p14 was compared with p14 obtained from the in vitro irradiated cell extract (IR extract) or from the caspase-3 cleavage reaction as indicated. C, V8 partial protease mapping. p14 obtained from either IR extract, caspase-treated, or in vivo irradiated ML-1 cell samples were compared by the partial V8 protease mapping method as indicated. The p15 protein from the in vivo p21 immunoprecipitation was used as a control (Fig. 2A).

Fig. 6. Cleavage of p21 by caspase abolishes its binding to PCNA. A, mutational analysis. Left, p21 and p21 mutants, containing either the conversion of aspartic acid 109 or 112 to alanine (D109A or D112A), were in vitro translated. They were treated with recombinant caspase-3 and the cleavage products were analyzed. Right, p21 and p21 truncation mutants, containing a translational stop codon at the end of either aspartic acid 109 or 112 (109Z or 112Z), were in vitro translated. They were treated with caspase-3 and the products were resolved in a protein gel. B, PCNA and CDK2 were in vitro translated in the presence of [35S]methionine. They were incubated with 2 μg of purified GST, GST-p21, and GST-p21/112Z proteins at 30 °C for 30 min. CDK2 or PCNA that associated with the GST-p21 or p21 mutant was isolated by glutathione-Sepharose beads. The proteins were separated in a protein gel and visualized by autoradiography. C, recombinant cyclin A/CDK2 complexes were incubated in the presence of increasing amounts of GST-p21 or GST-p21/112Z (0, 0.5, 1.5, and 4.5 μg) as indicated. The inhibition of the kinase activity was assayed using the histone H1 as the substrate.
mapped position of p21 cleavage, which is located at the carboxyl region of p21. In addition, we have also analyzed the effects of GST-p21 and GST-p21/112Z on the cyclin A/CDK2 activity. Both the wild type p21 and p21/112Z mutant could inhibit the in vitro kinase activity of cyclin A/CDK2 (Fig. 6C). These data suggest that one direct consequence of p21 cleavage is the loss of its interaction with PCNA, although we cannot rule out the possibility that in vivo the cleavage of p21 by the caspase-like activity may potentially affect p21 stability or localization during the DNA damage response.

**DISCUSSION**

DNA damage induces both cell cycle arrest and apoptosis (1). Many lines of evidence indicate that failure to express p21 can lead to the apoptotic cell fate in response to genotoxic stress (12, 15, 33). It has been postulated that the imbalance of the cell cycle signals or failure to arrest the cell cycle may trigger the apoptotic program (1). Indeed, ectopic expression of positive cell cycle regulators such as E2F, CDC25, Myc, or viral oncoprotein E1A causes many cells to die through apoptosis (14, 35). Our finding that p21 is induced in DNA-damaged cells but selectively cleaved by a caspase-like activity prior to the appearance of apoptosis is consistent with the previous observations that the insufficient expression of p21 during DNA damage response may cause apoptosis. Although we show that a direct effect of p21 cleavage is to abolish its interaction with PCNA, the truncation of p21 during apoptosis may cause other effects such as changes in the p21 stability or cellular/nuclear localization. The cleavage of p21 may thus alter its effective inhibitory level in the cell, leading to a failure in the cell cycle arrest in response to DNA damage.

Cells respond to DNA damage by arresting the cell cycle at G1 or G2 phase, a process that allows the time for repairing the DNA lesions (31). It has been shown that p21 associates with PCNA and such an association affects PCNA replication function. Consistently, p21 is normally absent from the replicating nucleus (9, 11). However, p21 does not appear to inhibit the repair activity of PCNA (10). In fact, it has been reported that p21 becomes co-localized with PCNA in the nucleus at the repair foci during the DNA damage response, suggesting p21 may perform a function during the DNA repair process (11). Interestingly, it has been shown that p21-associated cyclin-CDK complexes can exist in both active and inactive states (8). One possibility is that p21 may bring its associated active cyclin-CDK complexes to the repair sites through its interaction with PCNA. If this is the case, the cleavage of p21 by caspase-like activity may interfere with DNA repair, causing prolonged presence of DNA lesions that may trigger apoptosis.

Our data, together with previous reports (12, 13, 33), suggest that p21 is a critical checkpoint target protein for both cell cycle arrest and apoptosis in response to DNA damage. Manipulation of the p21 levels during DNA damage response may thus provide a novel strategy to the cancer therapy.

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