

# The Prosurvival Activity of p53 Protects Cells from UV-Induced Apoptosis by Inhibiting c-Jun NH<sub>2</sub>-terminal Kinase Activity and Mitochondrial Death Signaling

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## ABSTRACT

The cytoprotective function of p53 recently has been exploited as a therapeutic advantage for cancer prevention; agents activating the pro-survival activity of p53 are shown to prevent UV-induced damages. To explore the mechanisms of p53-mediated protection from UV-induced apoptosis, we have established stable clones of H1299 lung carcinoma cells expressing a temperature-sensitive p53 mutant, tsp53<sup>V143A</sup>. At the permissive temperature of 32°C, the tsp53<sup>V143A</sup>-expressing cells were arrested in G<sub>1</sub> phase without the occurrence of apoptosis; consistent with this is the preferential induction of genes related to growth arrest and DNA damage repair. Previous expression of functional tsp53<sup>V143A</sup> for ≥18 hours inhibited the release of proapoptotic molecules from mitochondria and protected the cells from UV-induced apoptosis; moreover, it suppressed the activation of c-Jun NH<sub>2</sub>-terminal kinase (JNK) signaling and relieved the effect of UV on p53 target gene activation. p53 associated with JNK and inhibited its kinase activity. Using the p53-null H1299 cells, we showed that inhibition of JNK blocked the UV-elicited mitochondrial death signaling and caspase activation. Our results suggest that the ability of p53 to bind and inactivate JNK, together with the activation of the p53 target genes related to cell cycle arrest and DNA damage repair, is responsible for its protection of cells against UV-induced apoptosis.

## INTRODUCTION

Loss of p53 function is the most common genetic alteration in cancer; >50% of human cancers have deleted or mutated *p53* gene. p53 is a tumor suppressor that prevents cells from transformation and undergoing tumorigenesis and has long been regarded as a key therapeutic target for cancer management. p53 responds to a variety of cellular stresses, including DNA damage, oncogene activation, and hypoxia, by exerting protective and apoptosis-enhancing functions (1, 2).

p53 is a sequence-specific transcription factor that activates genes involved in growth arrest and apoptosis-promoting molecules (1, 3–5). A number of proapoptotic genes, such as *Bax* (6), *CD95/Fas* (7), *Killer/DR5* (8), *Noxa* (9), *p53AIP1* (10), and *PUMA* (11), are direct p53 transcriptional targets whose expression is up-regulated by p53; moreover, p53 is involved in the down-regulation of the antiapoptotic protein Bcl-2 (12). Aside from its role in promoting apoptosis, p53 has been shown to participate in DNA damage repair. It acts as a chromatin accessibility factor and mediates UV-induced global chromatin relaxation, which is an essential first step for global genomic repair (13). p53 also is shown to up-regulate the transcription of DNA repair genes, such as ribonucleotide reductase p53R2 (14), UV-damaged DNA binding protein 2 (DDB2; ref. 15), and alkyltransferase (16).

Some p53 target genes are known to encode for cell cycle regulatory proteins; the best characterized among these is p21<sup>Waf1</sup>. p21<sup>Waf1</sup> inhibits cyclin-dependent kinases (CDKs) and is vital in mediating the p53-induced G<sub>1</sub> and G<sub>2</sub> arrest (1, 17). p53 also increases the expression of 14-3-3 $\sigma$ , which sequesters cyclin B1/CDK1 complexes outside the nucleus, thereby helping to maintain a G<sub>2</sub> block (18). It recently has been shown that Reprimo, whose expression leads to a G<sub>2</sub> phase block, is activated by p53 (19). The functions of p53 in cell cycle arrest and DNA damage repair are thought to prevent the replication of damaged DNA and to protect cells from damage-induced apoptosis. Inactivation of p53 allows cells with genomic damage to escape from cell cycle checkpoint control and damage-induced apoptosis and increases the chance of being transformed.

p53 plays a major role in apoptosis induction in cells undergoing DNA damage; it is conceivable that cells harboring wild-type p53 are more sensitive to chemotherapeutic drugs. Numerous strategies have been developed to try to restore p53 function in tumors that, when combined with therapeutic agents, may suppress cell growth and reduce tumor size (20, 21). Conversely, although activation of p53 has been associated with apoptosis, depending on the microenvironment of cells and levels of DNA damage, p53 could exert cytoprotective effects against cell death. Because the pro-survival events induced by DNA-damaging agents may contribute to the resistance of drug cytotoxicity, several studies have taken advantage of the cytoprotective function of p53 to selectively kill the p53-compromised cancer cells. Blagosklonny *et al.* (22) showed that pretreatment of cancer cells with cytostatic doses of DNA-damaging drugs could protect the cells against Taxol-induced death; the protection depended, in part, on the expression of p53 and its downstream target p21<sup>Waf1</sup>. Raj *et al.* (23) also showed that adeno-associated virus selectively induced apoptosis in cells that lacked active p53, whereas cells with intact p53 were arrested in G<sub>2</sub> phase. Thus, the protective pathway of p53/p21 can be exploited as an alternative strategy for cancer therapy.

UV light is the main cause of damage to skin cells and the development of skin cancer. Irradiation of cells by UV leads to the formation of a variety of DNA lesions that can further proceed to DNA double-strand breaks and ultimately trigger apoptosis (24). Conversely, substantial evidence indicates that UV can induce apoptosis through activating the membrane receptors in a DNA damage-independent manner. Thus, exposure of cells to UV light has been shown to induce the clustering of receptors for epidermal growth factor, tumor necrosis factor (25), and Fas ligand (26), which ultimately results in the stimulation of caspase activities and initiation of apoptosis.

One of the major UV-responsive signaling pathways elicited by membrane receptors is the mitogen-activated protein (MAP) kinase cascade (27). Mammalian cells contain three distinct MAP kinases: extracellular signal-regulated protein kinases (ERKs), p38 kinase, and the stress-activated c-Jun NH<sub>2</sub>-terminal kinases (JNKs; ref. 28). The activation of MAP kinases may prompt their translocation to the nucleus, where they phosphorylate target transcription factors such as c-Jun and ATF2, enhance their transcription activities, and increase the expression of stress-responsive genes (29). It has been shown that

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inhibition of JNK and/or p38 kinase activation by genetic inactivation, the use of dominant inhibitory mutants, or by treatment with their specific inhibitors confers resistance to cell death induced by UV irradiation (30, 31), suggesting that the MAP kinase cascades play an important role in mediating cellular response to UV.

By executing its prosurvival function to arrest the UV-damaged cells and to repair the damaged DNA or by executing its proapoptotic function to eliminate the severely damaged cells, p53 acts as a guardian in preventing UV-mediated deleterious effects on the genome (32, 33). It is no surprise that UV-mediated p53 gene mutation has been found as one of the determining factors in skin cancer development (33). Therefore, protecting the genomic DNA from UV-induced mutations and maintaining the intactness of the critical genes involved in growth regulation are important issues for cancer prevention. Recent evidence shows that pretreatment of compounds that activate p53-mediated DNA repair pathways can prevent the subsequent UV-induced mutations and apoptosis (32, 34, 35). For example, Seo *et al.* (35) have shown that pretreatment of fibroblasts with selenium-containing compounds, long known as having cancer-preventative functions, can stimulate the DNA-repairing activity of p53 and reduce the sensitivity to UV-induced cell death. Eller *et al.* (34) have shown that pretreatment of skin fibroblasts with thymidine dinucleotide leads to p53 activation, which then induces the expression of genes related to DNA repair (GADD45) and growth arrest (p21<sup>Waf1</sup>), and increases the clonogenic survival of the UV-irradiated cells. Moreover, topical application of thymidine dinucleotide in mice reduces and delays carcinogenesis after UV irradiation (36). These findings suggest that preactivation of p53-mediated survival function is a beneficial strategy to prevent the UV-induced damages and tumorigenesis.

The main goal of this study is to investigate the underlying mechanisms whereby preactivation of p53 protects cells from UV-induced detriments. Delineating the mechanisms involved in the p53-mediated survival pathways may provide useful information for therapeutic approaches to cancer prevention. Friedlander *et al.* (37) have identified a temperature-sensitive p53 mutant, tsp53<sup>V143A</sup>. Using reporter analysis, they showed that wild-type tsp53<sup>V143A</sup> preferentially activated the promoters of genes involved in growth arrest and DNA repair but not apoptosis. In the present study, we have established clones of H1299 human lung carcinoma cells stably expressing tsp53<sup>V143A</sup> and used the cells as a model system to study the protective mechanisms of p53. By hybridization to a p53 target gene array, we have shown that functional tsp53<sup>V143A</sup> preferentially activates the p53 target genes, whose products are involved in growth arrest and DNA damage repair, consistent with its functional display of arresting cells without apoptosis induction. We have shown that expression of functional tsp53<sup>V143A</sup> before UV irradiation protects cells against UV-induced apoptosis, and the protection is mediated, at least in part, by direct binding of p53 to JNK and inhibiting the JNK activity, which is responsible for inducing mitochondrial death signaling. We have for the first time shown that the ability of p53 to inhibit UV-induced cell death is through negatively regulating JNK activity and that the temperature-sensitive human p53<sup>V143A</sup> expression system is a useful model to study the cytoprotective function of p53.

## MATERIALS AND METHODS

**Materials.** Fluorogenic caspase-3 (VII), caspase-9 (I) substrates, and caspase-9 inhibitor Z-LEHD-FMK were from Calbiochem (La Jolla, CA), and JNK inhibitor SP600125 was from BIOMOL International (Plymouth Meeting, PA). Monoclonal antibodies for p53 (Ab-1), p21<sup>Waf1</sup> (Ab-1), and Bax (Ab-1) were from Oncogene Research Products (San Diego, CA); that for poly(ADP-ribose) polymerase (PARP; C-2-10) was from Novus-Biologicals of Abcam

(Littleton, CO); anti-FLAG M2 monoclonal antibody was from Sigma Chemical Co. (St. Louis, MO); and antibodies for Mdm2 (SMP14) and cytochrome *c* (7H8.2C12) were from BD Pharmingen (San Diego, CA). The anti-Smac/Diablo antibody (78-1-118) was from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibodies to p53, JNK, and apoptosis-inducing factor (AIF) and phospho-specific antibodies to c-Jun (Ser<sup>63</sup>), ATF2 (Thr<sup>71</sup>), JNK (Thr<sup>183</sup>/Tyr<sup>185</sup>), p38 kinase (Thr<sup>180</sup>/Tyr<sup>182</sup>), and ERK1/2 (Thr<sup>202</sup>/Tyr<sup>204</sup>) were from Cell Signaling Technology (Beverly, MA). Antibody against cytochrome oxidase subunit IV (COX IV; 20E8-C12) was purchased from Molecular Probes (Eugene, OR). Glutathione *S*-transferase (GST)-p53 fusion protein and antibodies to DDB2 (S-16), c-Jun (H-79), and JNK2 (D-2) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell Culture and Establishment of Stable Transfectants.** For transfection,  $5 \times 10^5$  H1299 lung carcinoma cells were seeded in 60-mm dishes for 24 hours and transfected with 3  $\mu$ g of plasmid DNA pRC-CMV or pCMV-p53<sup>V143A</sup> (38) using the Lipofectamine PLUS reagent (Invitrogen, Carlsbad, CA). Two days after transfection, cells were trypsinized and seeded in 60-mm dishes; the cells were cultured in Roswell Park Memorial Institute (RPMI) medium containing 0.5 mg/mL G418 for 10 days; and G418-resistant colonies were pooled or further cloned. H1299-neo and H1299-tsp53<sup>V143A</sup> cells were maintained in RPMI medium containing 10% fetal bovine serum and 50  $\mu$ g/mL of gentamicin in 5% CO<sub>2</sub> at 38°C. Temperature shifting was performed by transferring subconfluent cells to the pre-equilibrated incubator at 32°C. Two days after seeding, medium was aspirated off and irradiated with 30 J/m<sup>2</sup> of UV-C light at room temperature. Immediately after irradiation, medium was added back, and the cells cultured as stated.

**Analysis of p53 Target Gene Expression.** A detailed description is presented as online supplementary information.

**Colony Formation Assay.** For growth suppression assay, ~500 cells of H1299-neo or H1299-tsp53<sup>V143A</sup> were plated into 60-mm dishes, and the cells were maintained at 38°C or 32°C; medium was changed every 3 days. Colonies were stained with crystal violet (1 mg/mL) for visualization 10 to 14 days after plating.

For clonogenic survival assay, H1299-tsp53<sup>V143A</sup> or neo control cells were cultured for 24 hours at 38°C; they then were divided into two parts. One set of cells was maintained at 38°C (38/32), and the other was shifted to 32°C (32/32). After 24 hours, the cells were irradiated with 30 J/m<sup>2</sup> of UV light and immediately seeded in 60-mm dishes ( $4 \times 10^4$  per dish) and incubated for another 24 hours at 32°C. The cells were shifted to 38°C, and colonies were scored after 9 to 10 days by staining with crystal violet.

**Flow Cytometry.** Trypsinized cells were resuspended in PBS at a density of  $10^6$  cells/mL. Cells then were fixed by addition of EtOH to a final concentration of 70%. The EtOH-fixed cells were kept up to 1 week at 4°C before use. Cells were pelleted and resuspended in an isotonic buffered solution containing propidium iodide (1 mg/mL). Cell cycle distribution and sub-G<sub>1</sub> fraction were determined by flow cytometry using the Becton Dickinson FACScan system (Franklin Lakes, NJ). Apoptosis was scored 48 or 72 hours after UV irradiation by assessing the fraction of cells with a sub-G<sub>1</sub> DNA content.

**Western Blot Analysis.** For preparation of cell extract, cells were washed with cold PBS, scraped in PBS, and then lysed on ice for 30 minutes in lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L Na<sub>2</sub>EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium PP<sub>i</sub>, 1 mmol/L  $\beta$ -glycerophosphate, 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, and 1  $\mu$ g/mL leupeptin]. The cell extract was obtained by centrifugation at  $10,000 \times g$  at 4°C for 10 minutes. For Western blot analysis, 50 to 100  $\mu$ g of protein were separated by electrophoresis on a 10 to 12% SDS-PAGE and blotted onto a Hybond-Cextra membrane (Amersham Biosciences, Piscataway, NJ). Membranes were blocked for 1 hour in PBS containing 5% nonfat milk powder and 0.1% Tween 20, incubated for 14 to 16 hours at 4°C with the primary antibody, washed three times with PBS containing 0.1% Tween 20, and incubated for 1 hour with a horseradish peroxidase-coupled secondary antibody (Amersham Biosciences). After washing with PBS containing 0.1% Tween 20 (three times for 10 minutes each), blots were developed using a chemiluminescence detection system (Amersham Biosciences).

**Preparation of Cytosolic Extract.** The cytosolic fraction was prepared according to the instruction manual of Mitochondrial Fractionation Kit (Active Motif, Carlsbad, CA). Briefly, cells were harvested, washed twice with ice-cold PBS, resuspended in ice-cold cytosolic buffer, and then stood on ice for

15 minutes. The cells were homogenized on ice with a Dounce homogenizer (20 strokes), and unbroken cells and nuclei were removed by centrifugation at  $2,000 \times g$  for 10 minutes at  $4^{\circ}\text{C}$ . The postnuclear supernatant was further spun at  $10,000 \times g$  for 20 minutes at  $4^{\circ}\text{C}$  to separate the cytosolic fraction from contaminating with mitochondrial. The supernatant was centrifuged at  $18,000 \times g$  for 30 minutes at  $4^{\circ}\text{C}$  to remove any residual mitochondria. The purity of cytosolic extracts was assessed by the absence of COX IV.

**Coimmunoprecipitation.** Cells were lysed on ice for 30 minutes in lysis buffer and centrifuged at  $10,000 \times g$  for 10 minutes in a microcentrifuge tube. For immunoprecipitation, 1 mg of extract was precleared with 25  $\mu\text{L}$  of protein A/G beads for 1 hour at  $4^{\circ}\text{C}$  to reduce nonspecific binding and reacted overnight at  $4^{\circ}\text{C}$  with 1 to 2  $\mu\text{g}$  of antibodies that previously were incubated with protein A/G beads for 1 hour at  $4^{\circ}\text{C}$ . The beads were pelleted down and washed extensively to eliminate nonspecific binding. The presence of p53, JNK, and phosphorylated JNK in the precipitates was detected by immunoblot analysis.

**Caspase Activity Assay.** Cells were washed once with PBS and lysed in the reporter lysis buffer (Promega, Madison, WI). The lysates were spun in a microcentrifuge tube for 10 minutes at  $4^{\circ}\text{C}$ , and the supernatant was collected. Aliquots of protein (100 to 200  $\mu\text{g}$ ) were diluted to 250  $\mu\text{L}$  with reporter lysis buffer and incubated with an equal volume of  $2\times$  caspase reaction buffer [40 mmol/L PIPES (pH 7.2), 200 mmol/L NaCl, 20 mmol/L dithiothreitol, 2 mmol/L EDTA, 0.2% CHAPS, and 20% sucrose] containing 100  $\mu\text{mol/L}$  of fluorogenic caspase-3 or caspase-9 substrate at  $37^{\circ}\text{C}$  for 1 hour. The intensity of emitted fluorescence at 505 nm was measured by a fluorescence spectrometer.

**JNK Activity Assay.** Assay of JNK activity was carried out as described in the protocol provided by New England Biolabs (Beverly, MA). Cell lysates were prepared, and 250  $\mu\text{L}$  of the lysate (250  $\mu\text{g}$  of total protein) were mixed with beads containing 2  $\mu\text{g}$  of c-Jun fusion protein and incubated with gentle rocking at  $4^{\circ}\text{C}$  for 16 hours. The beads were washed twice with 500  $\mu\text{L}$  of lysis buffer and twice with 500  $\mu\text{L}$  of kinase buffer [25 mmol/L Tris-HCl (pH 7.5), 5 mmol/L  $\beta$ -glycerophosphate, 2 mmol/L dithiothreitol, 0.1 mmol/L  $\text{Na}_3\text{VO}_4$ , and 10 mmol/L  $\text{MgCl}_2$ ], and kinase reactions were carried out in the presence of 100  $\mu\text{mol/L}$  ATP at  $30^{\circ}\text{C}$  for 30 minutes. The phosphorylated c-Jun fusion protein was detected by immunoblot analysis using phospho-specific antibodies. To assay for the inhibitory effect of p53 protein on JNK activity, protein lysates prepared from UV-irradiated H1299-neo cells were incubated with GST-p53 fusion protein for 2 hours at  $30^{\circ}\text{C}$  and then at  $4^{\circ}\text{C}$  overnight before measuring the kinase activity.

## RESULTS

**Expression of Functional  $\text{tsp53}^{\text{V143A}}$  Suppresses Cell Growth by Arresting Cells in  $G_1$  Phase.** To elucidate the molecular mechanisms of p53-mediated protection against UV-induced deleterious damages, we generated stable transfectants of the p53-null H1299 human lung carcinoma cells expressing the temperature-sensitive p53 mutant  $\text{tsp53}^{\text{V143A}}$ . Two clones, designated C9 and C14, expressed p53 proteins as shown by Western blot analysis (Supplementary Fig. 1A) and were isolated for further characterization. As shown by immunofluorescence microscopy, most of the  $\text{tsp53}^{\text{V143A}}$  protein was found in the nucleus after 8 hours at the permissive temperature of  $32^{\circ}\text{C}$  (data not shown). When the growth of cells was examined by colony formation assay, it was shown (Supplementary Fig. 1B) at  $32^{\circ}\text{C}$ ; the colony-forming ability of clones C9 and C14, when compared with the H1299 cells transfected with control vector pRc-CMV (H1299-neo), was strongly suppressed by the expression of functional  $\text{tsp53}^{\text{V143A}}$ , whereas no significant differences in colony formation were observed in H1299- $\text{tsp53}^{\text{V143A}}$  versus neo control cells when cultured at  $38^{\circ}\text{C}$ . Analysis by flow cytometry indicated that after 24 hours at  $32^{\circ}\text{C}$ , C9 and C14 H1299- $\text{tsp53}^{\text{V143A}}$  cells were growth arrested in  $G_1$  phase (Supplementary Fig. 1C), whereas no significant increase in sub- $G_1$  cell population was detected, suggesting that expression of functional  $\text{tsp53}^{\text{V143A}}$  did not trigger apoptosis in H1299 cells. These results suggest that functional  $\text{tsp53}^{\text{V143A}}$  sup-

presses colony growth by arresting cells in  $G_1$  phase and not by apoptosis induction.

**Functional  $\text{tsp53}^{\text{V143A}}$  Preferentially Induces Genes Involved in Growth Arrest and DNA Damage Repair.** It previously has been shown that  $\text{tsp53}^{\text{V143A}}$  at  $32^{\circ}\text{C}$  preferentially activated the promoters of genes involved in growth arrest and DNA repair, such as p21<sup>Waf1</sup>, GADD45, and cyclin G1, but not those involved in apoptosis, such as Bax and IGF-BP3 (37). To further examine whether the mRNA expression of the p53 target genes was regulated by functional  $\text{tsp53}^{\text{V143A}}$  in a similar manner, we isolated RNA from H1299-neo and H1299- $\text{tsp53}^{\text{V143A}}$  (clone C14) cells cultured at  $32^{\circ}\text{C}$  for 10 hours and analyzed the expression of p53-responsive genes by hybridizing to a p53 target gene array. Supplementary Table 1 summarizes the ratio of expression for individual transcripts, which represents the mRNA levels of H1299- $\text{tsp53}^{\text{V143A}}$  cells relative to those of H1299-neo control cells. Among 104 p53-regulated genes analyzed, only 8 were up-regulated by functional  $\text{tsp53}^{\text{V143A}}$  expression. These include p21<sup>Waf1</sup> and cyclin G1, which previously have been shown to be involved in mediating the p53-induced cell cycle arrest, and DDB2, p53R2, GADD45, and PCNA, which are implicated in DNA repair. Also induced by functional  $\text{tsp53}^{\text{V143A}}$  expression were Mdm2 and Wip1. Mdm2 is a principal regulator of p53 stability, and Wip1 is a phosphatase reported to participate in the MAP kinase signaling cascade. In contrast, all of the previously reported proapoptotic target genes of p53 were either suppressed or not significantly affected by functional  $\text{tsp53}^{\text{V143A}}$  expression. These results are consistent with those reported using luciferase promoter assay (37), supporting the thought that functional  $\text{tsp53}^{\text{V143A}}$  preferentially transactivates the genes implicated in growth arrest and DNA repair rather than apoptosis.

Northern blot analyses were performed to confirm the array data. As indicated in Supplementary Fig. 2A, expression of p21<sup>Waf1</sup>, Mdm2, DDB2, and GADD45 transcripts was greatly induced in H1299- $\text{tsp53}^{\text{V143A}}$  cells at the early times after shifting to  $32^{\circ}\text{C}$ , whereas PCNA, Bax, PIG3, and Scotin were only modestly induced by functional  $\text{tsp53}^{\text{V143A}}$  expression; the latter three proapoptotic genes were reported to be up-regulated by wild-type p53 in H1299 cells (39–41). Induction of Wip1, p53R2, and cyclin G1 transcripts was observed at later time points after shifting to  $32^{\circ}\text{C}$  (Supplementary Fig. 2B). Western blot analysis was performed to examine the expression of these p53 targets. Elevated levels of p21<sup>Waf1</sup> and Mdm2 proteins could be detected 4 to 6 hours after temperature downshifting; induction of DDB2 protein was detectable only after 8 hours at  $32^{\circ}\text{C}$ , whereas Bax protein was not affected by functional  $\text{tsp53}^{\text{V143A}}$  expression throughout the period analyzed (Supplementary Fig. 2C). These results showed that functional  $\text{tsp53}^{\text{V143A}}$  selectively enhanced the expression of p53 targets implicated in cell growth arrest and DNA damage repair but not apoptosis.

**Previous Expression of Functional  $\text{tsp53}^{\text{V143A}}$  Protects Cells against UV-Induced Apoptosis.** We tested whether previous expression of functional  $\text{tsp53}^{\text{V143A}}$  could protect the H1299 stable clones from UV-induced apoptosis. For this purpose, cells were subjected to various temperature-shifting protocols: The cells were cultured at  $32^{\circ}\text{C}$  for 24 hours for pre-expression of wild-type-like p53 activity before UV irradiation and maintained at  $32^{\circ}\text{C}$  thereafter (32/32), or they were cultured at  $38^{\circ}\text{C}$  for 24 hours and shifted to  $32^{\circ}\text{C}$  immediately after UV treatment (38/32) for post-UV expression of functional  $\text{tsp53}^{\text{V143A}}$ . For control, cells were maintained at  $38^{\circ}\text{C}$  for 24 hours, irradiated with UV, and maintained at  $38^{\circ}\text{C}$  thereafter (38/38). A UV dosage of 30  $\text{J/m}^2$  was used for all of the experiments because it triggered significant cell death in H1299-neo cells. Flow cytometry was performed to monitor the cell cycle distribution; cells with sub- $G_1$  DNA content represent apoptotic cells. As shown in Fig. 1A, UV

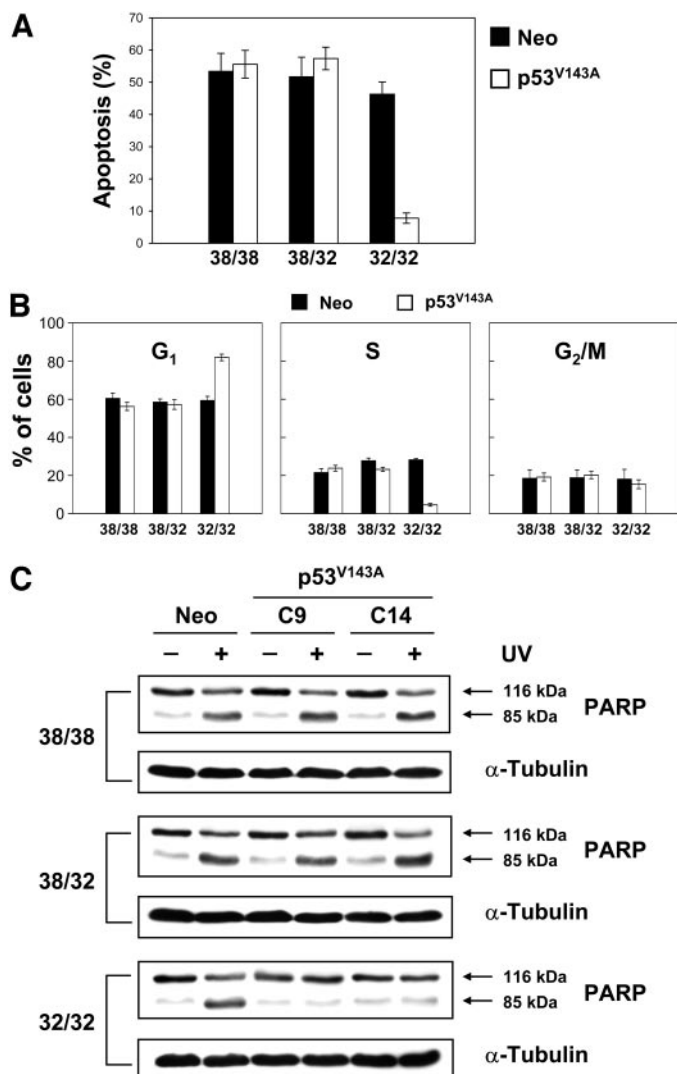


Fig. 1. Previous expression of functional  $\text{tsp53}^{\text{V143A}}$  protects cells against UV-induced apoptosis. **A**, Previous expression of functional  $\text{tsp53}^{\text{V143A}}$  arrests cells in  $G_1$  and suppresses UV-induced apoptosis. H1299-neo and H1299- $\text{tsp53}^{\text{V143A}}$  cells were incubated at  $38^\circ\text{C}$  for 24 hours, and cells were irradiated with UV light ( $30 \text{ Jm}^2$ ) and incubated either at  $38^\circ\text{C}$  (38/38) or  $32^\circ\text{C}$  (38/32) for another 72 hours. Alternatively, cells were cultured at  $32^\circ\text{C}$  throughout the experiment (32/32). The cells were fixed and stained with propidium iodide for flow cytometry analysis. Apoptosis was scored as the percentage of cells with a sub- $G_1$  DNA content (shown in **A**). The percentages of cells in  $G_1$ , S, and  $G_2$ -M are shown in **B**. Results shown in **A** and **B** are the mean  $\pm$  SD from three independent experiments. **C**, Previous expression of functional  $\text{tsp53}^{\text{V143A}}$  inhibits the UV-induced cleavage of PARP. H1299-neo and H1299- $\text{tsp53}^{\text{V143A}}$  (clones C9 and C14) cells were subjected to the three temperature treatment protocols mentioned in **A** as indicated, and protein lysates were prepared and analyzed by Western blot analysis using a specific antibody recognizing PARP ( $M_r$  116,000) and its cleavage fragment ( $M_r$  85,000). Expression of  $\alpha$ -tubulin also is shown as a protein loading control.

induced apoptosis in the H1299-neo and H1299- $\text{tsp53}^{\text{V143A}}$  cells (C14) when the cells were maintained at  $38^\circ\text{C}$  (38/38), as indicated by the presence of  $>50\%$  of cells with a sub- $G_1$  DNA content. Expression of functional  $\text{tsp53}^{\text{V143A}}$  immediately following UV irradiation (38/32) did not affect the UV-induced apoptosis or cell cycle distribution (Fig. 1A and B), and temperature shifting did not affect the UV-induced apoptosis in control H1299-neo cells (Fig. 1A; 38/32). In contrast, expression of functional  $\text{tsp53}^{\text{V143A}}$  24 hours before UV irradiation (32/32) significantly suppressed the UV-induced apoptosis (Fig. 1A), as indicated by a decrease in the sub- $G_1$  fraction; an increase in the  $G_1$  population was noted concomitant with a decrease in S phase (Fig. 1B). Because PARP cleavage is characteristic of apoptotic cells, we also performed Western blot analysis using anti-

PARP antibody on lysates from the H1299- $\text{tsp53}^{\text{V143A}}$  (C9 and C14) or neo control cells subjected to three temperature treatment protocols. As shown in Fig. 1C, elevated levels of  $M_r$  85,000 PARP cleavage fragment, indicative of apoptosis, were detected in cell lysates from the H1299- $\text{tsp53}^{\text{V143A}}$  and neo-control cells subjected to UV irradiation under the 38/38 and 38/32 protocols. In contrast, reconstitution of wild-type-like p53 activity before UV irradiation (32/32) inhibited the UV-triggered PARP cleavage in H1299- $\text{tsp53}^{\text{V143A}}$  cells (Fig. 1C). These results show that pre-expression of functional  $\text{tsp53}^{\text{V143A}}$  for a 24-hour period protects H1299 cells against UV-induced apoptosis.

**Previous Reconstitution of Wild-Type-Like p53 Activity Suppresses the UV-Triggered Mitochondrial Death Signaling and Caspase Activation.** To elucidate the mechanisms underlying p53-mediated cytoprotection, cells were subjected to all of the three temperature protocols and analyzed by Western blot analysis for the release of proapoptotic molecules, such as cytochrome *c*, AIF, and Smac/Diablo. As shown in Fig. 2A, release of AIF, cytochrome *c*, and Smac/Diablo was observed in the UV-treated H1299-neo cells that underwent all of the three temperature-shifting protocols, consistent with the results from flow cytometry analysis (Fig. 1A), indicating significant apoptosis in these cells. However, previous reconstitution of wild-type-like p53 activity in H1299- $\text{tsp53}^{\text{V143A}}$  cells for 24 hours before UV irradiation (32/32) completely suppressed the release of all of the three mitochondria-derived proapoptotic molecules (Fig. 2A).

We next examined whether UV-mediated caspase activities were inhibited by previous functional  $\text{tsp53}^{\text{V143A}}$  expression (32/32). As indicated in Fig. 2B, UV stimulated caspase-9 and -3 activities in H1299-neo and H1299- $\text{tsp53}^{\text{V143A}}$  cells cultured at  $38^\circ\text{C}$  (38/38); preincubation of the H1299- $\text{tsp53}^{\text{V143A}}$  cells at  $32^\circ\text{C}$  for 24 hours (32/32) completely inhibited the UV-induced caspase-9 and -3 activities. In the presence of caspase-9 inhibitor, it was shown that the UV-activated caspase-3 activity was dose-dependently inhibited (Supplementary Fig. 3), indicating that caspase-3 activation lies downstream of caspase-9. No significant increase in caspase-8 activity was observed after UV irradiation (data not shown), suggesting that death receptor signaling pathways were not involved in the UV-triggered apoptosis in H1299 cells. Collectively, these data show that UV-induced apoptosis is mediated by mitochondrial death signaling that results in caspase-9 and -3 activation; previous reconstitution of wild-type-like p53 activity abrogates the UV-induced mitochondrial death signaling and protects cells from UV-induced apoptosis.

To examine the minimal time of functional  $\text{tsp53}^{\text{V143A}}$  expression required for protection against UV-induced apoptosis, H1299- $\text{tsp53}^{\text{V143A}}$  and the neo control cells were cultured at  $32^\circ\text{C}$  for various times before UV irradiation; caspase activities were examined on day 3 after UV treatment. As shown in Fig. 2C, at least 18 hours of preincubation were required for functional  $\text{tsp53}^{\text{V143A}}$  to significantly protect cells against UV-induced apoptosis. Time course analysis indicated that the ability of functional  $\text{tsp53}^{\text{V143A}}$  to protect against UV-induced apoptosis correlates with the extent of p53-induced  $G_1$  arrest (compare Fig. 2C with D), suggesting that the protective effect of functional  $\text{tsp53}^{\text{V143A}}$  may require the establishment of a previous  $G_1$  arrest.

**Previous Expression of Functional  $\text{tsp53}^{\text{V143A}}$  Relieves the UV-Mediated Suppression on the Activation of p53 Target Genes and Promotes Post-UV Clonogenic Survival.** Mechanisms underlying p53-mediated protection against UV-induced apoptosis were further explored by analyzing the expression of the p53 target genes. On the basis of our observation that the time window of functional  $\text{tsp53}^{\text{V143A}}$  expression is crucial to determine the outcome of UV irradiation, we compared the activation of p53 target genes in cells expressing wild-type-like p53 activity before (32/32) and after (38/

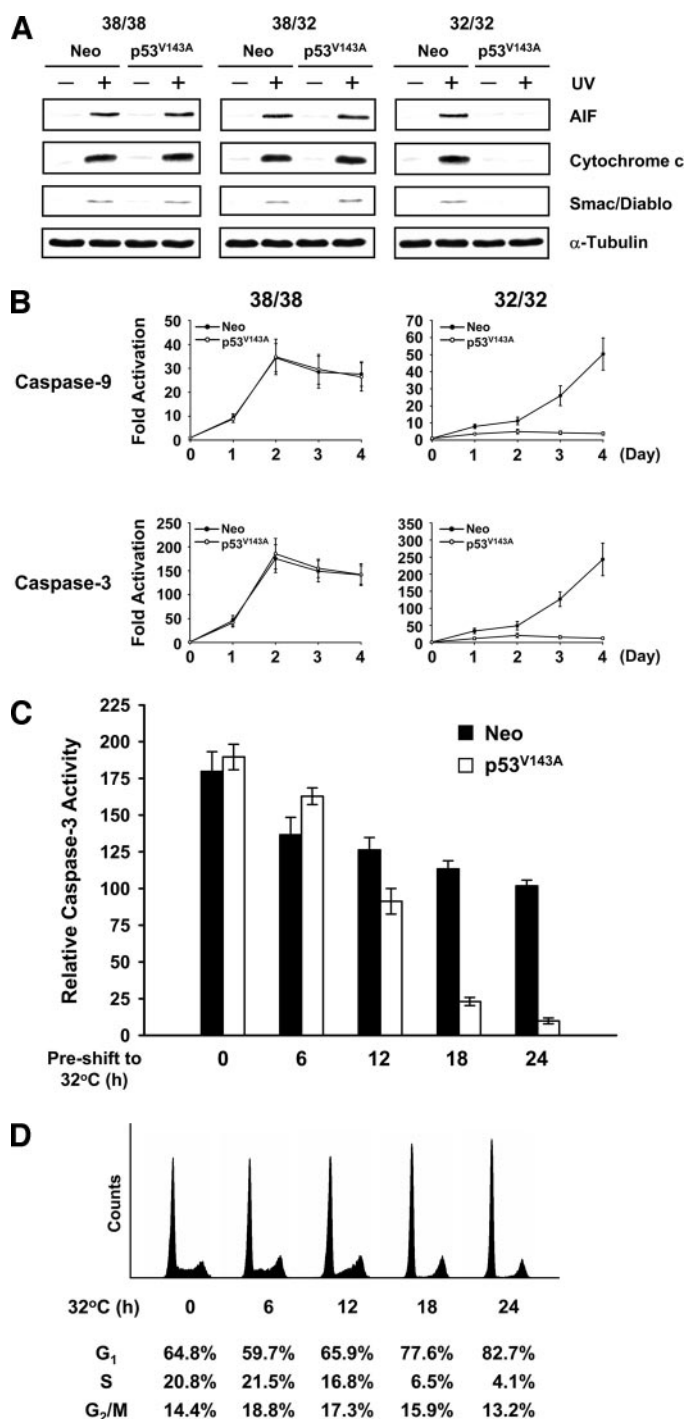


Fig. 2. Expression of functional tsp53<sup>V143A</sup> before UV treatment suppresses the UV-induced mitochondrial death signaling and caspase activation. **A**, Pre-UV expression of functional tsp53<sup>V143A</sup> suppresses the UV-triggered release of mitochondrial proapoptotic molecules. H1299-neo and H1299-tsp53<sup>V143A</sup> cells were subjected to the temperature shifting protocols with or without UV irradiation as described in Fig. 1A; cytosolic proteins were prepared as described in Materials and Methods and analyzed by Western blot analysis using antibodies specific to AIF, cytochrome c, and Smac/Diablo. The amount of  $\alpha$ -tubulin also is shown for equal protein loading. **B**, UV-activated caspase-9 and -3 activities are inhibited by pre-UV functional tsp53<sup>V143A</sup> expression. H1299-neo and H1299-tsp53<sup>V143A</sup> cells were subjected to the temperature treatment protocols (38/38 or 32/32) as indicated; lysates were prepared at the indicated times after UV irradiation and reacted with the fluorogenic substrates of caspase-9 and caspase-3. The caspase activity is shown as fold of activation relative to the nonirradiated control. The bars shown represent mean  $\pm$  SD from three independent experiments. **C**, analysis of the time required for pre-UV functional tsp53<sup>V143A</sup> expression to protect against UV-induced apoptosis. H1299-neo and H1299-tsp53<sup>V143A</sup> cells were precultured at 32°C for the indicated times and irradiated with UV light. Cells were maintained at 32°C for an additional 3 days and harvested for caspase-3 activity assay. Values represent the mean  $\pm$  SD from three independent experiments. **D**, flow cytometry analysis of the cell

cycle distribution of H1299-tsp53<sup>V143A</sup> cells cultured at 32°C for the indicated times. The percentages of cells in each cell cycle phase are shown. A significant increase of cells in G<sub>1</sub> phase was observed concomitant with a decrease in S phase after cells were incubated at 32°C for  $\geq$ 18 hours.

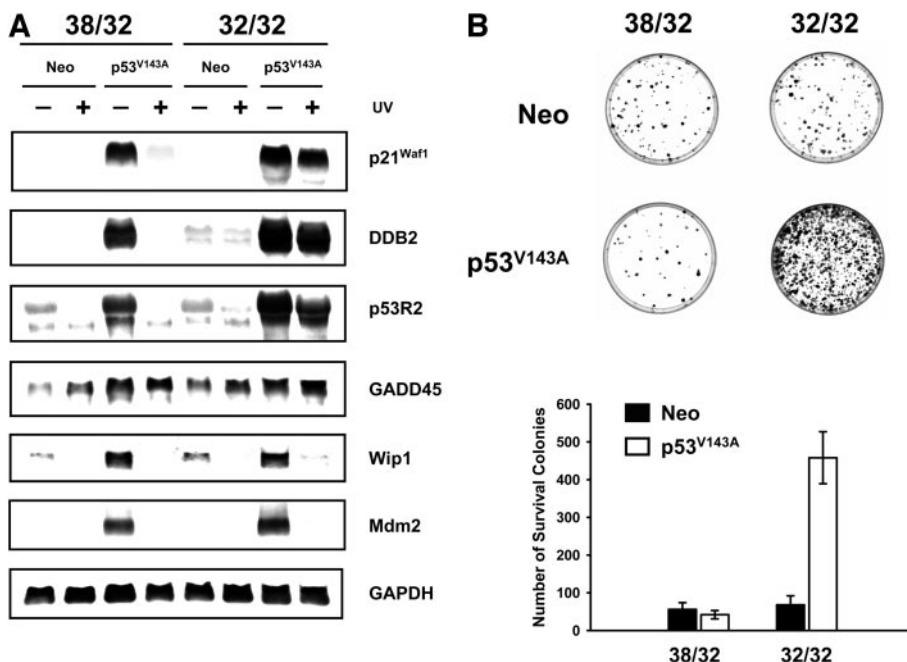
32) UV treatment; these temperature-shift protocols resulted in cell survival or apoptosis, respectively (Figs. 1A and C and 2A). The H1299-tsp53<sup>V143A</sup> and control neo cells were cultured at 38°C or 32°C for 24 hours, and the cells were UV irradiated and incubated at 32°C for an additional 8 hours and analyzed by Northern blot analysis for the expression of p53 target genes. As shown in Fig. 3A, UV irradiation before functional tsp53<sup>V143A</sup> expression (38/32) inhibited the p53-mediated induction of its target genes, such as p21<sup>Waf1</sup>, DDB2, and p53R2; however, pre-UV expression of functional tsp53<sup>V143A</sup> (32/32) suppressed the UV-mediated inhibition of the p53 target genes. This suppression appeared to be specific because the levels of Wip1 and Mdm2 were not restored by previous expression of functional tsp53<sup>V143A</sup> (Fig. 3A). The transcript levels of p53 targets also were manifested in their protein expression as indicated by Western blot analysis; UV treatment before (38/32), but not after (32/32), functional tsp53<sup>V143A</sup> expression significantly suppressed the p53-induced p21<sup>Waf1</sup> and DDB2 protein levels (Supplementary Fig. 4).

To test whether the cells escaped from UV-induced apoptosis because of pre-expression of functional tsp53<sup>V143A</sup>-retained reproductive potential, the post-UV clonogenic survival assay was performed on the H1299-neo and H1299-tsp53<sup>V143A</sup> cells subjected to the temperature-shifting protocols (38/32 and 32/32). At 32°C, the H1299-tsp53<sup>V143A</sup> cells were growth arrested as a result of functional tsp53<sup>V143A</sup> expression; therefore, for clonogenic assay cells were shifted back to 38°C after various temperature treatment protocols. As indicated in Fig. 3B, H1299-tsp53<sup>V143A</sup> cells that had functional tsp53<sup>V143A</sup> restored before UV irradiation (32/32) increased their clonogenic survival to 6.5-fold that of control neo cells. In contrast, the H1299-neo and H1299-tsp53<sup>V143A</sup> cells were low in clonogenic activity when subjected to 38/32 temperature-shifting protocols (Fig. 3B). These results suggest that pre-UV expression of functional tsp53<sup>V143A</sup> not only protects cells against UV-induced apoptosis but also rescues them from UV-mediated suppression on long-term survival.

**Previous Expression of Functional tsp53<sup>V143A</sup> Blocks the UV-Stimulated JNK Signaling.** To explore the role of MAP kinase family proteins in the p53-mediated protection against UV-induced apoptosis, the phosphorylation states of the kinases and their substrates were examined. H1299-tsp53<sup>V143A</sup> and control neo cells were preincubated at 32°C (32/32) or 38°C (38/38) for 24 hours; cells were UV irradiated; and the activation of kinases was examined at 0, 2, 4, and 8 hours after UV irradiation by Western blot analysis using the phospho-specific antibodies against JNK, p38 kinase, and ERK. As shown in Fig. 4A, levels of phospho-ERK1/2 were not affected by UV treatment nor were they affected by previous expression of functional tsp53<sup>V143A</sup>. Similarly, no significant changes in phospho-p38 levels were observed except for a slight reduction in phospho-p38 levels at 0 and 2 hours after UV irradiation in H1299-tsp53<sup>V143A</sup> cells cultured at 32°C (32/32; Fig. 4A). The decreased phospho-p38 levels in these cells may be attributed to the p53-mediated induction of Wip1 (Supplementary Fig. 2B), a phosphatase known to dephosphorylate p38, whereas the subsequent recovery of phospho-p38 levels at 4 and 8 hours may result from UV-mediated suppression of the *Wip1* gene expression (Fig. 3A). In contrast, increased phosphorylation of JNK was observed in UV-irradiated H1299-neo and H1299-tsp53<sup>V143A</sup> cells cultured at either 38°C (38/38) or 32°C (32/32); as a control, total JNK protein levels remained unaffected (Fig. 4A). Analysis of c-Jun phosphorylation indicated (Fig. 4A) that UV treatment signifi-

cycle distribution of H1299-tsp53<sup>V143A</sup> cells cultured at 32°C for the indicated times. The percentages of cells in each cell cycle phase are shown. A significant increase of cells in G<sub>1</sub> phase was observed concomitant with a decrease in S phase after cells were incubated at 32°C for  $\geq$ 18 hours.

Fig. 3. Pre-UV expression of functional  $\text{tsp53}^{\text{V143A}}$  eliminates the UV-mediated suppression on expression of p53 target genes related to growth arrest and DNA repair and increases clonogenic survival of UV-irradiated cells. A, effects of UV irradiation on the activation of p53 target gene expression. H1299- $\text{tsp53}^{\text{V143A}}$  and neo control cells were cultured at 38°C (38/32) or 32°C (32/32) for 24 hours, and cells were irradiated with (+) or without (–) UV light and then incubated at 32°C for another 8 hours. Total RNA was prepared and analyzed by Northern blot analysis using specific  $^{32}\text{P}$ -labeled cDNA probes for the selected genes. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression was included as a loading control. B, Expression of functional  $\text{tsp53}^{\text{V143A}}$  before UV treatment restores clonogenic survival of UV-irradiated H1299 cells. H1299- $\text{tsp53}^{\text{V143A}}$  and neo control cells were preincubated at 38°C (38/32) or 32°C (32/32) for 24 hours and subjected to UV irradiation. Cells were trypsinized immediately after UV treatment, and  $4 \times 10^4$  cells were plated in a 60-mm dish. After 24 hours of incubation at 32°C, the cells were shifted to 38°C and cultured at the same temperature for 8 to 10 days. Colonies formed were visualized by staining with crystal violet, and results from one representative experiment are shown here (top). The statistic data presented (bottom) are the mean  $\pm$  SD from three independent experiments.



cantly increased c-Jun phosphorylation in H1299-neo cells incubated at either the restrictive (38/38) or the permissive (32/32) temperature; however, expression of functional  $\text{tsp53}^{\text{V143A}}$  for 24 hours (32/32) significantly delayed and attenuated the UV-stimulated phosphorylation of c-Jun, suggesting that UV-induced JNK signaling pathway was suppressed by previous expression of p53.

We next examined whether the timing of functional  $\text{tsp53}^{\text{V143A}}$  expression was critical in suppressing the UV-triggered JNK signaling. Cells were cultured at 38°C or 32°C for 24 hours; they were irradiated with UV and immediately shifted to 32°C for 2 hours, and the activation of JNK and its substrates c-Jun and ATF2 was analyzed. As shown in Fig. 4B, expression of functional  $\text{tsp53}^{\text{V143A}}$  immediately after UV irradiation (38/32) had no significant effects on the UV-induced phosphorylation of JNK, c-Jun, and ATF2, whereas expression of functional  $\text{tsp53}^{\text{V143A}}$  before UV irradiation (32/32) blocked the UV-stimulated phosphorylation of c-Jun and ATF2 without affecting JNK phosphorylation (Fig. 4B). Control experiment with JNK1/2 antibody indicated that the amount of JNK remained unchanged during these treatments (Fig. 4B).

To examine the discrepancies between the phosphorylation states of JNK and its substrates c-Jun and ATF2 in cells that had previous functional  $\text{tsp53}^{\text{V143A}}$  expression (Fig. 4B), we analyzed JNK activity using *in vitro* kinase assay with GST-c-Jun fusion protein as a substrate. Cells were subjected to the UV treatment before (38/32) or after (32/32) functional  $\text{tsp53}^{\text{V143A}}$  expression; lysates prepared 2 hours after UV irradiation were assayed for JNK activity. As shown in Fig. 4C, UV-stimulated JNK activity was reduced to the unstimulated levels in H1299- $\text{tsp53}^{\text{V143A}}$  cells preshifted to 32°C (32/32), consistent with the observation that c-Jun and ATF2 were not phosphorylated *in vivo* under such circumstances (Fig. 4B). Collectively, these results clearly show that reconstitution of the wild-type-like p53 activity in cells before UV irradiation suppresses UV-stimulated JNK activity and inhibits JNK signaling.

**p53 Associates with and Inactivates JNK.** The possibility that p53 suppressed JNK activity by binding with the enzyme was examined by coimmunoprecipitation. The  $\text{tsp53}^{\text{V143A}}$  cells preincubated at 32°C for various time periods were irradiated with UV; lysates prepared 2 hours later were reacted with p53-specific antibody, and the

immunoprecipitates were analyzed for the presence of total and phosphorylated JNK by Western blot analysis. As shown in Fig. 5A, p53 antibody precipitated JNK in the absence of UV irradiation or pre-temperature downshifting; however, a threefold increase in p53-JNK complex formation was observed in UV-irradiated cells that were preincubated for  $\geq 18$  hours at 32°C for wild-type-like p53 expression. This result was confirmed when JNK2 antibody was used to precipitate the protein complex (Fig. 5B).

The aforementioned results suggest that p53 inhibits JNK activity by binding to the enzyme. To verify this hypothesis, we assayed for the JNK activity *in vitro* in the presence of increasing amounts of p53 protein. Cell lysates prepared from UV-irradiated H1299-neo cells were used as a source of JNK; they were reacted with GST-c-Jun in the presence of increasing amounts of recombinant GST-p53 fusion protein, and phosphorylation of GST-c-Jun was determined using phospho-c-Jun-specific antibody. As shown in Fig. 5C, p53 dose-dependently inhibited the ability of JNK to phosphorylate c-Jun *in vitro*, whereas control GST did not have any detectable effect on JNK activity. These results indicate that p53 interacts with and inhibits JNK activity.

**JNK Signaling Pathway Participates in UV-Induced Apoptosis in H1299 Cells.** We next explored the role of JNK in the UV-induced apoptosis. For these studies, the H1299-neo cells that are p53 null were used. Cells were pretreated with various doses of JNK inhibitor SP600125 (42) 30 minutes before irradiated with UV. As shown in Supplementary Fig. 5, exposure to 20  $\mu\text{mol/L}$  of SP600125 was sufficient to suppress the UV-stimulated c-Jun phosphorylation. However, despite the abolishment of UV-stimulated JNK activity, pretreatment with SP600125 for 30 minutes was not able to significantly inhibit the UV-mediated activation of caspase-9 and -3 (data not shown). It has been reported that at least 2-day incubation was required for SP600125 to suppress cell proliferation (43); our data indicated that SP600125 treatment for 48 hours inhibited the proliferation of H1299 cells (data not shown). The H1299-neo cells were pretreated with SP600125 for 48 hours, irradiated with UV, and incubated for an additional 48 hours for caspase assay. As shown in Fig. 6A (top), SP600125 suppressed the UV-induced caspase-9 and -3 activities to  $\sim 40\%$  of control ( $P < 0.005$  and  $P < 0.001$  for caspase-9

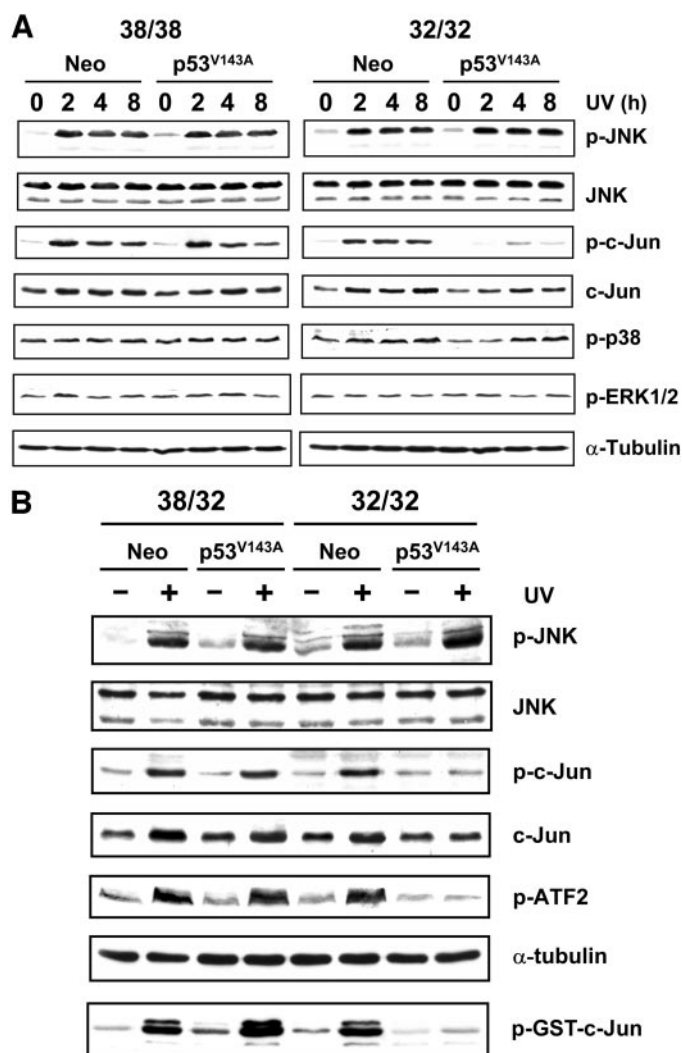


Fig. 4. Expression of functional  $\text{tsp53}^{\text{V143A}}$  before UV irradiation inhibits the UV-activated JNK signaling. **A**, Previous reconstitution of wild-type-like p53 activity selectively inhibits the UV-induced phosphorylation of c-Jun but not its upstream kinase JNK. H1299- $\text{tsp53}^{\text{V143A}}$  and neo control cells were subjected to the temperature-shifting protocols (38/38 and 32/32); cells were irradiated with UV, and extracts were prepared at the indicated time points after UV treatment. Western blot analysis was performed on the extracts using specific antibodies against phosphorylated JNK, c-Jun, p38 kinase, and ERK1/2. Total JNK, c-Jun, and  $\alpha$ -tubulin proteins also were determined using antibodies specific to these three proteins. **B**, Pre-UV, but not post-UV, expression of functional  $\text{tsp53}^{\text{V143A}}$  suppresses UV-induced phosphorylation of c-Jun and ATF2. H1299-neo and H1299- $\text{tsp53}^{\text{V143A}}$  cells were preincubated at 38°C (38/32) or 32°C (32/32) for 24 hours, and the cells were irradiated with UV light and incubated at 32°C for another 2 hours. Cell lysates were prepared and analyzed by Western blot analysis using specific antibodies against phosphorylated forms of JNK, c-Jun, and ATF2. Expression of JNK and c-Jun was examined with JNK1/2 and c-Jun antibodies, respectively. Expression of  $\alpha$ -tubulin is shown as a loading control. **C**, Previous expression of functional  $\text{tsp53}^{\text{V143A}}$  suppresses UV-induced JNK activity. H1299- $\text{tsp53}^{\text{V143A}}$  and neo control cells were subjected to the same temperature protocols as described in **B**; lysates were prepared and analyzed for JNK activity by *in vitro* kinase assay using GST-c-Jun as substrate, and the extent of phosphorylation was analyzed by immunoblot analysis using specific antibody against phosphorylated c-Jun.

and -3, respectively). Western blot analysis showed that during the experimental period, JNK activity remained suppressed (Fig. 6A, bottom).

The role of JNK in UV-triggered mitochondrial death signaling next was examined. H1299-neo cells with or without previous SP600125 treatment were UV irradiated; cytosolic fractions were prepared at different times after UV irradiation and analyzed by Western blot analysis for the presence of AIF, cytochrome *c*, and Smac/Diablo. As shown in Fig. 6B, pretreatment with SP600125

resulted in >90% inhibition of UV-induced release of AIF and Smac/Diablo and in 65% that of cytochrome *c*.

Although SP600125 has been shown to have little effect toward other kinases at the concentration used in this study (42), the possibility exists that prolonged exposure to the drug may increase its chance of affecting other cellular components; therefore, the inhibition on apoptosis may not be a direct consequence of JNK inhibition. To exclude this possibility, we conducted the dominant-negative experiment to specifically inhibit JNK. Because the predominant isoform in H1299 cells is JNK2, inhibition of JNK by dominant-negative JNK2 (DN-JNK2) was performed to verify the role of JNK2 in the UV-induced apoptosis. The p53-null H1299-neo cells were transfected with DN-JNK2 or its vector control pcDNA3 for 24 hours and plated in medium containing 0.5 mg/mL of G418 for 5 days. The cells were UV irradiated, and caspase activities were assayed after 48 hours. As indicated in Fig. 6C (top), expression of DN-JNK2 resulted

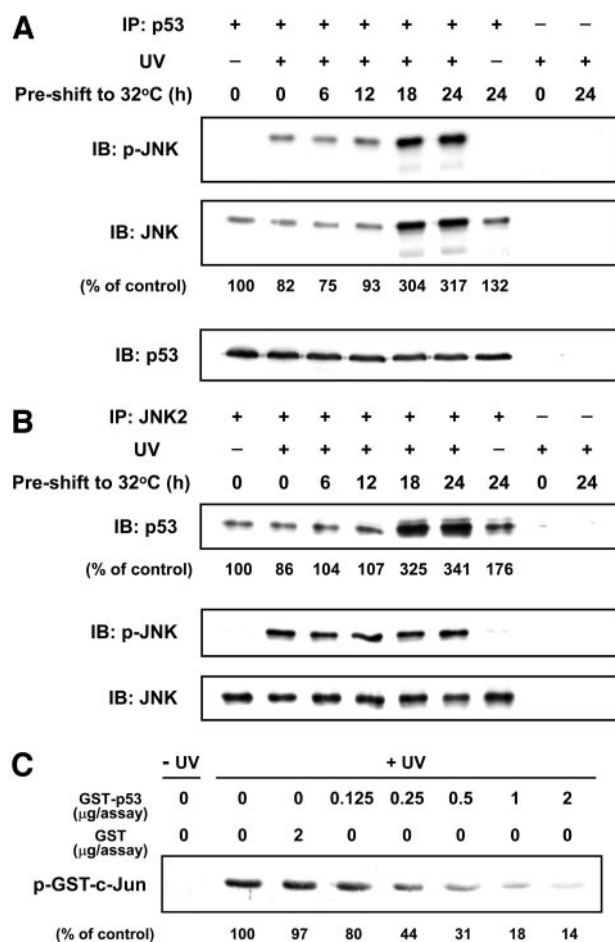


Fig. 5. p53 binds and inactivates JNK. **A** and **B**, p53 associates with JNK *in vivo*. **A**, H1299- $\text{tsp53}^{\text{V143A}}$  cells were preincubated at 32°C for the indicated times; cells were treated with (+) or without (-) UV irradiation and further incubated at 32°C for another 2 hours, and lysates were prepared and immunoprecipitated (IP) with (+) or without (-) p53 antibody (Ab-1). The immunoprecipitates were analyzed for the presence of p53, JNK, and phospho-JNK by immunoblot (IB) analysis using specific antibodies. The amount of JNK2 coprecipitated with p53 was quantitated by computer-assisted densitometry and shown as the percentage of control at the bottom. **B**, Cells were treated, and lysates were analyzed as described in **A** except that anti-JNK2 antibody was used in the immunoprecipitation reaction. The amount of p53 coprecipitated with JNK2 was quantitated by computer-assisted densitometry and shown as the percentage of control. **C**, p53 inhibits JNK activity *in vitro*. H1299-neo cells were treated with (+UV) or without (-UV) 30 J/m<sup>2</sup> of UV light to stimulate the endogenous JNK activity. Cells were harvested after 2 hours, and lysates were prepared for JNK activity assay in the presence of the indicated amounts of GST or GST-p53 fusion protein. The phosphorylated GST-c-Jun levels were detected by Western blot analysis and quantitated by densitometry analysis and are presented as the percentage of control at the bottom.

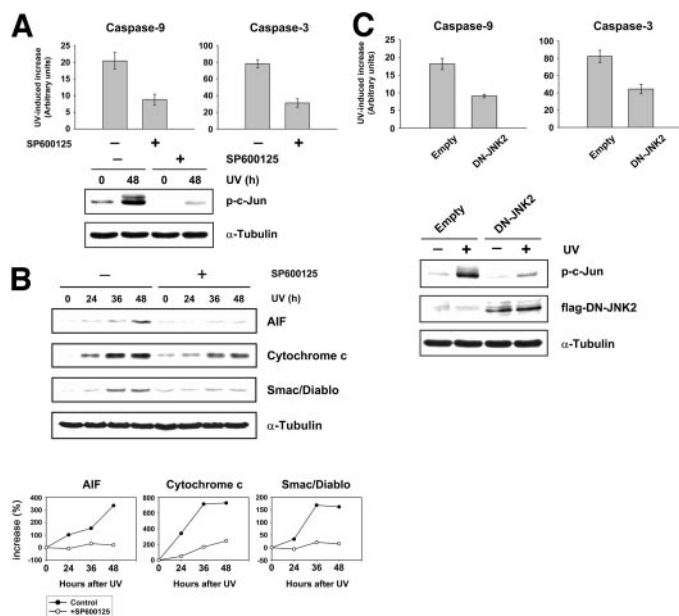


Fig. 6. JNK is required for UV-stimulated release of the mitochondrial proapoptotic molecules and caspase activation. **A**, effect of SP600125 on the UV-induced activation of caspase-9 and -3. H1299-neo cells were treated with either vehicle (–) or 20  $\mu$ M SP600125 (+) for 48 hours at 37°C before UV irradiation. Two days after UV irradiation, cells were harvested and assayed for the activities of caspases-9 and -3 as described in Materials and Methods. Results presented (top) are the mean  $\pm$  SD from three independent experiments. Western blot analysis (bottom) confirmed the effectiveness of SP600125 in inhibiting JNK activity, and probe with phospho-c-Jun antibody indicated that c-Jun phosphorylation was blocked. Expression of  $\alpha$ -tubulin is shown for equal protein loading. **B**, effect of SP600125 on UV-triggered release of mitochondria-derived proapoptotic molecules. H1299-neo cells were treated with either vehicle (–) or 20  $\mu$ M SP600125 (+) for 48 hours at 37°C before UV irradiation. Cytosolic extracts were prepared at the indicated time points after UV treatment and subjected to immunoblot analysis (top) using specific antibodies for AIF, cytochrome *c*, Smac/Diablo, and  $\alpha$ -tubulin. Expression levels were quantitated by computer-assisted densitometry and are presented as percent increase over the control (in the absence of UV treatment; bottom). **C**, effect of DN-JNK2 expression on the UV-induced caspase activation. H1299-neo cells were transfected with pcDNA3–DN-JNK2 or its vector control pcDNA3; after 24 hours, cells were cultured in medium containing 0.5 mg/mL G418 for 5 days. The cells were exposed to 30 J/m<sup>2</sup> UV light, and caspase activities were measured 48 hours after irradiation. Western blot analysis using lysate prepared 2 hours after UV irradiation was performed with phospho-c-Jun–specific antibody and antibody against FLAG for the expression of transfected DN-JNK2.

in 50% inhibition of caspase-9 ( $P < 0.001$ ) and 46% inhibition of caspase-3 ( $P < 0.005$ ). Western blot analysis confirmed that the FLAG-tagged DN-JNK2 was expressed in the transfectant (Fig. 6C, bottom). Analysis of JNK activity showed that the UV-stimulated c-Jun phosphorylation was significantly inhibited by the expression of DN-JNK2 (Fig. 6C, bottom). Collectively, these results clearly show that JNK2 plays a major role in mediating the activation of mitochondrial death-signaling pathways by UV.

## DISCUSSION

The ability of p53 to transactivate many of its target genes is central to its role as a tumor suppressor. Accumulating evidence indicates that the selectivity of p53 to regulate the prosurvival and proapoptotic genes is affected by growth environment, the type of stress used, the cellular context, and the target gene structure (3, 4, 44). In this study, we have established H1299 lung carcinoma cell clones stably expressing the temperature-sensitive p53<sup>V143A</sup>. We show that expression of functional p53<sup>V143A</sup> differentially activates the prosurvival aspect of p53 functions; cells are arrested in G<sub>1</sub> phase without signs of apoptosis. Analysis of the expression profile of the p53<sup>V143A</sup>-modulated genes by performing a p53 target gene array shows that functional p53<sup>V143A</sup> preferentially activates genes related to cell cycle arrest

and DNA repair but not apoptosis. The discriminating transcriptional activity of p53<sup>V143A</sup> correlates with its functional display, suggesting that transactivation of distinct target genes by p53 is important in cell fate determination. Using the H1299–p53<sup>V143A</sup> cells as a model, we show that previous restoration of wild-type–like p53 activity protects cells from the subsequent UV irradiation, and inhibition of JNK activity may contribute to the p53-mediated inhibition of mitochondrial death signaling that leads to apoptosis. Although our conclusion is derived from studies of H1299 cells overexpressing a mutant of p53 that restores wild-type–like activity at permissive temperature, protection from UV-induced apoptosis by previous activation of wild-type p53 recently has been reported (34, 35). For example, by comparing the viability of the p53<sup>+/+</sup> and p53<sup>–/–</sup> mouse embryonic fibroblast cells (MEFs) irradiated with UV, it was shown that p53 protects the cells from UV-induced apoptosis; moreover, pre-UV treatment of MEFs with selenomethionine activates p53 and further increases the survival of UV-irradiated p53<sup>+/+</sup> cells, whereas the p53<sup>–/–</sup> cells remain UV sensitive with or without selenomethionine pretreatment (35). These results suggest that the protection against UV radiation is not unique to overexpressed p53<sup>V143A</sup>, which retains preferentially the prosurvival activity of p53.

We have shown that the timing of functional p53<sup>V143A</sup> expression is important in cellular response to UV irradiation; p53 expressed before, but not immediately after, UV treatment significantly protects the cells from apoptosis and restores clonogenic survival. Our results indicate that UV irradiation suppressed the subsequent p53-mediated activation of target genes; however, previous expression of functional p53<sup>V143A</sup> specifically attenuates the suppressive effect of UV on p53-mediated induction of p21<sup>Waf1</sup>, DDB2, and p53R2 (Fig. 3A). It is possible that the elevated level of p21<sup>Waf1</sup> allows the p53<sup>V143A</sup>-expressing cells to maintain a sustained G<sub>1</sub> growth arrest (Fig. 1B) and prevents the UV-damaged cells from replication. DDB2 and p53R2, implicated in DNA damage repair, also may help to prevent the formation of UV-induced double-stranded DNA breaks and chromosomal aberrations that would otherwise lead to cell death (24). The fact that pre-UV expression of functional p53<sup>V143A</sup> restores clonogenic survival (Fig. 3B), a sign indicating that the cells may have repaired the UV-induced DNA damage and recovered from damage-induced replication crisis, supports the notion that p53-mediated DNA repair plays a role in protecting cells against UV-induced deleterious effect.

JNK has been shown to be involved in the UV-triggered mitochondrial death pathways such as cytochrome *c* release and caspase activation (31, 45). Using the p53-null H1299 cells, we show that SP600125, despite being able to effectively block the activity of JNK, only partially suppresses the UV-induced release of cytochrome *c* and caspase activity, suggesting the existence of JNK-independent pathways in UV-mediated apoptosis. Pre-UV expression of wild-type–like p53 inhibits UV-induced JNK activity and completely blocks mitochondrial death signaling and caspase activation. These results suggest that inhibition of the JNK-dependent and the JNK-independent death pathway contributes to the p53<sup>V143A</sup>-mediated cytoprotection against UV-induced cell death. A more definitive support to this conclusion would be provided by culturing the H1299–p53<sup>V143A</sup> cells with or without SP600125 and subjecting the cells to the 32/38 temperature protocol. Comparing the UV-induced cell death in the presence or absence of SP600125 will reveal the participation of JNK-dependent and -independent pathways in the p53-mediated cytoprotection. Because the JNK binding domain has been mapped to amino acids 97 to 116 of p53 (46), using the p53<sup>V143A</sup> mutant that lacks only the JNK binding ability will be another potential strategy to unveil the contribution of the JNK-independent mechanisms in the p53-mediated cytoprotection against UV.



We have shown that the suppressive effect of functional  $\text{tsp53}^{\text{V143A}}$  on JNK signaling is through binding with and inhibiting JNK to phosphorylate downstream targets such as c-Jun and ATF2. The p53-JNK complex formation is significantly increased in UV-irradiated cells that are preincubated at 32°C for >18 hours. Interestingly, this time window coincides with that required for H1299- $\text{tsp53}^{\text{V143A}}$  cells to establish a G<sub>1</sub> arrest (Figs. 2D and 5A and B). Time course analysis of the pre-UV functional  $\text{tsp53}^{\text{V143A}}$  expression indicates that p53-JNK complex formation parallels with the accumulation of G<sub>1</sub>-arrested cells and is inversely correlated with the fractions of apoptotic cells after UV irradiation, supporting our hypothesis that the p53-JNK binding in the G<sub>1</sub>-arrested cells inhibits UV-induced JNK activity and apoptosis triggering. It appears that G<sub>1</sub> arrest before UV treatment may be a prerequisite for p53-mediated protection against UV-triggered apoptosis. This conclusion is consistent with the report by Buschmann *et al.* (47), who found the association of p53 and JNK is cell cycle-regulated and is found predominantly in G<sub>0</sub>/G<sub>1</sub> phase. It was shown that enhancement of p53-JNK complex formation correlates with post-translational modifications and conformational changes of p53, suggesting that cell cycle and stress-elicited changes in the conformation of p53 and JNK may affect the p53-JNK affinity. In the same analogy, the UV- and G<sub>1</sub>-dependent phosphorylation may affect the conformation of p53 and increase its binding to JNK. It is of note that when JNK inhibitor SP600125 was used to explore the role of JNK in UV-induced apoptosis, we found that preincubation of H1299 cells with SP600125 for 30 minutes before UV treatment completely inhibited UV-stimulated JNK activity without inhibiting apoptosis. A prolonged (2-day) preincubation with SP600125 was required to suppress the UV-induced apoptosis. JNK recently has been shown to play a key role in cell proliferation; the JNK1/JNK2 double-knockout fibroblast cells proliferated at a much slower rate than their wild-type counterpart (31), and it is shown that exposure of cells with SP600125 for  $\geq 2$  days also suppresses cell proliferation (43). We found that H1299 cells are growth arrested by DN-JNK inhibition or after 2 days' treatment with SP600125 (data not shown). It is possible that, in cooperation with JNK inhibition, a slowdown of cell proliferation is beneficial, analogous to establishment of G<sub>1</sub> arrest before UV treatment, for cells to escape from UV-induced death. The collective evidence strongly suggests that JNK-targeted apoptosis rescue may be dependent on cell growth arrest.

The interaction between JNK and p53 and its effect on p53 stability previously have been reported (46–48); however, the corresponding consequence on JNK activity has never been described before. Fuchs *et al.* (46) showed that JNK associated with and accelerated the degradation of p53 through ubiquitination in unstressed cells; c-Jun and a peptide corresponding to the JNK binding site on p53 were able to efficiently block JNK-mediated degradation of p53. These data raise the possibility that p53 and c-Jun compete for binding to the same site on JNK, and formation of p53-JNK complex could interfere with the ability of JNK to bind and phosphorylate c-Jun and other substrates like ATF2. Besides p53, p21<sup>Waf1</sup> also has been reported to interact with various JNK isoforms and therefore may play a role in regulating JNK signaling (49). However, we were unable to detect the presence of JNK in the p21<sup>Waf1</sup> immunoprecipitate, and p21<sup>Waf1</sup> was not found in the p53-JNK complex in H1299 cells (data not shown), suggesting that the association between p21<sup>Waf1</sup> and JNK may be cell type specific.

In conclusion, we have used the  $\text{tsp53}^{\text{V143A}}$ -expressing cell line that displays only the antiapoptotic function of p53 as a model to study the molecular mechanisms underlying the p53-mediated cytoprotection against UV irradiation. We show that pre-UV expression of

functional  $\text{tsp53}^{\text{V143A}}$  inhibits the UV-stimulated JNK activity and mitochondria death signaling and protects cells against UV-induced apoptosis. p53 binds and inhibits JNK, and the attenuation of JNK signaling contributes to the protection against UV-induced cell death. We have for the first time shown that inhibition of UV-activated JNK signaling cascade is one of the mechanisms for p53 to suppress the UV-induced apoptosis. Assigning the temperature-sensitive  $\text{p53}^{\text{V143A}}$  mutant with pro-survival function provides a tool to study the p53-based cancer prevention and therapy.

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