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# Regulation of Bax activation and apoptotic response to UV irradiation by p53 transcription-dependent and -independent pathways

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**Abstract**

The Trp53 tumor suppressor gene product (p53) functions in the nucleus to regulate proapoptotic genes, whereas cytoplasmic p53 directly activates proapoptotic Bcl-2 proteins to permeabilize mitochondria and initiate apoptosis. Here, we demonstrate that both p53 transcription-dependent and -independent pathways contribute to UV-induced apoptosis. First we show that Pifithrin-α, a small molecule inhibitor of p53 transcriptional activity, delays Bax translocation and cell death by UV irradiation. Then using CHX (cycloheximide) to prevent new protein expression in response to p53, we also find that Bax translocation and cell death by UV irradiation are delayed. Furthermore we find that overexpression of Bcl-$x_L$, an inhibitor of cytoplasmic p53 after UV irradiation, prevents cell death. Finally, we observe that Pifithrin-α and CHX effectively inhibit PUMA expression by UV irradiation. Taken together, these data indicate that the nuclear p53 promotes PUMA expression, which then displaces cytoplasmic p53 from Bcl-$x_L$, allowing p53 to induce mitochondrial permeabilization, thereby triggering apoptosis.


*Keywords:* Bax; Bcl-$x_L$; p53; PUMA; UV irradiation; Apoptosis

## 1. Introduction

The p53 tumor suppressor protein, which is stabilized and activated by a variety of genotoxic and non-genotoxic stress, plays a central role in the chemosensitivity and radiosensitivity of cancer cells by inducing cell cycle arrest and apoptosis [1–3]. It has been shown that p53 triggers apoptosis by inducing mitochondrial outer-membrane permeabilization through transcription-dependent and -independent mechanisms [4]. Numerous genes are regulated by nucleus p53, such as those encoding death receptors for example, Fas (CD95)and proapoptotic Bcl-2 proteins (for example, Bax, Bid, Noxa, and PUMA) [5–10]. In parallel, cytoplasm p53 can directly activate the proapoptotic protein Bax to promote mitochondrial outer-membrane

*Abbreviations*: YFP, DsRed, GFP, yellow, red, and green fluorescent protein; Bak, Bcl-2 antagonist/killer; Bax, Bcl-2-associated X protein; BH3, Bcl-2-homology domain-3; Bid, BH3-interacting-domain; MOMP, mitochondrial outer-membrane permeabilization; PUMA, p53-upregulated modulator of apoptosis; MEFs, mouse embryonic fibroblasts; UV, Ultraviolet.

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permeabilization (MOMP) [11–13]. Since the role of p53 in apoptosis induction varies between cell types and death signals, the functional importance of p53 in UV-induced apoptosis of human cancer cells remains controversial [12,14–16].

Bcl-2 family proteins play a critical role in regulating apoptosis initiation through the mitochondria [17]. The anti-apoptotic Bcl-2 family proteins such as Bcl-2 and Bcl-$x_L$ possess four conserved BH domains (BH1–4) and prevent the release of apoptogenic molecules from mitochondria. In contrast, proapoptotic Bcl-2 family proteins, which can be further divided into the Bax subfamily (includes Bax, Bak, and Bok multidomain proteins) or the BH3-only subfamily (such as Bid, Bim, and PUMA), induce mitochondrial outer-membrane permeabilization. It is believed that the BH1–3 proteins Bax and Bak are death effectors that are absolutely required for the mitochondrial pathway of apoptosis [18,19]. The BH3-only proteins, including PUMA and at least eight additional proapoptotic proteins, display sequence homology with other members only within an α-helical BH3 domain, which is essential for apoptosis induction [20]. BH3-only proteins function through multidomain Bcl-2 family members to induce apoptosis, either by antagonizing antiapoptotic proteins or by directly activating proapoptotic proteins [18,21].

PUMA is normally expressed at a low level but is markedly induced after cells are exposed to DNA-damaging agents, such as chemotherapeutic drugs and ionizing radiation [9,22]. PUMA plays an essential role in p53-dependent and -independent apoptosis induced by a variety of stimuli [23]. Deletion of PUMA in HCT116 colon cancer cells abrogated apoptosis induced by p53, the DNA-damaging agent adriamycin, and hypoxia [24]. PUMA-knockout mice recapitulate major apoptotic deficiencies observed in p53-knockout mice [25,26]. It has been shown that PUMA can couple the nuclear and cytoplasmic proapoptotic functions of p53 by displacing p53 from Bcl-$x_L$, thereby allowing p53 to induce mitochondrial permeabilization [27].

Our previous studies demonstrated that Bax translocation by UV irradiation is a Bid-independent event and inhibited by overexpression of Bcl-$x_L$ in ASTC-a-1 cells [28]. To gain further insights into the molecular mechanisms underlying UV-induced apoptosis, we investigate Bax translocation, cell death, and PUMA expression by UV irradiation in the presence or absence of CHX or Pifithrin-α. In addition, we also investigate cell death by UV irradiation when overexpression of Bcl-$x_L$. The results defined UV-mediated apoptosis through both p53 transcription-dependent and -independent pathways.

## 2. Materials and methods

### 2.1. Material

Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO (Grand Island, NY). CHX and Pifithrin-α (p53 inhibitor) were purchased from BioVision (Mountain View, CA, USA). Lipofectamine™ Reagent was purchased from Invitrogen (Carlsbad, CA, USA). DNA Extraction kit was purchased from Qiagen (Valencia, CA, USA). pGFP-Bax was kindly supplied by Dr. R.J. Youle [29], pYFP-Bcl-$x_L$ was kindly supplied by Dr. A.P. Gilmore [30]. pDsRed-Mit was kindly supplied by Dr. Y. Gotoh [31]. Other chemicals were mainly from Sigma (St. Louis, MO).

### 2.2. Cell culture and treatments

The human lung adenocarcinoma cell line (ASTC-a-1) was obtained from Department of Medicine, Jinan University. They were cultured in DMEM supplemented with 15% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 mg/ml) in 5% $CO_2$ at 37 °C in humidified incubator. Transfections were performed with Lipofectamine™ 2000 reagent according to the manufacturer's protocol. The medium was replaced with fresh culture medium after 5 h. Cells were examined at 24–48 h after transfection. For UV treatment, medium was removed and saved, cells were rinsed with PBS and irradiated, and medium was restored. The UV light source was a UV device (AIRTECH, Suzhou, China), UV light (254 nm) was delivered at a fluorescence rate of 0.4 mw/cm$^2$ for 5 min, and observed at 0 h (control), 3, 6, 9, 12 h after irradiation. For experiments with the inhibitors, cells were pretreated with Pifithrin-α (20 μM) or CHX (50 μg/ml) 1 h before UV irradiation. The inhibitors were kept in the medium throughout the experimental process.

### 2.3. Time-lapse confocal fluorescence microscopy

GFP, YFP, and DsRed refer to green, yellow, and red fluorescent protein. GFP, YFP, and DsRed fluorescence were monitored confocally using a

commercial laser scanning microscope (LSM 510/ ConfoCor 2) combination system (Zeiss, Jena, Germany) equipped with a Plan-Neofluar 40 × / 1.3 NA Oil DIC objective. Excitation wavelength and detection filter settings for each of the fluorescent indicators were as follows: GFP fluorescence was excited at 488 nm with an argon ion laser and emission was recorded through a 500–550 nm band pass filter. YFP fluorescence was excited at 514 nm with an argon ion laser and emission was recorded through a 560 nm long pass filter. DsRed fluorescence was excited at 543 nm with a helium–neon laser and emitted light was recorded through a 560 nm long pass filter.

For time-lapse imaging, culture dishes were mounted onto the microscope stage that was equipped with a temperature-controlled chamber (Zeiss, Jena, Germany). During control experiments, bleaching of the probe was negligible.

### 2.4. GFP-Bax translocation assay

To monitor GFP-Bax translocation in living cells, ASTC-a-1 cells were co-transfected with pGFP-Bax and pDsRed-Mit. Using Zeiss LSM 510 confocal microscope, we imaged the distribution pattern of GFP-Bax and DsRed-Mit simultaneously during UV-induced apoptosis. Bax redistribution was assessed by the matching fluorescence of GFP-Bax and DsRed-Mit emission. The cells exhibiting strong punctate staining of GFP, which overlapped with the distribution of DsRed, were counted as the cells with mitochondrially localized Bax.

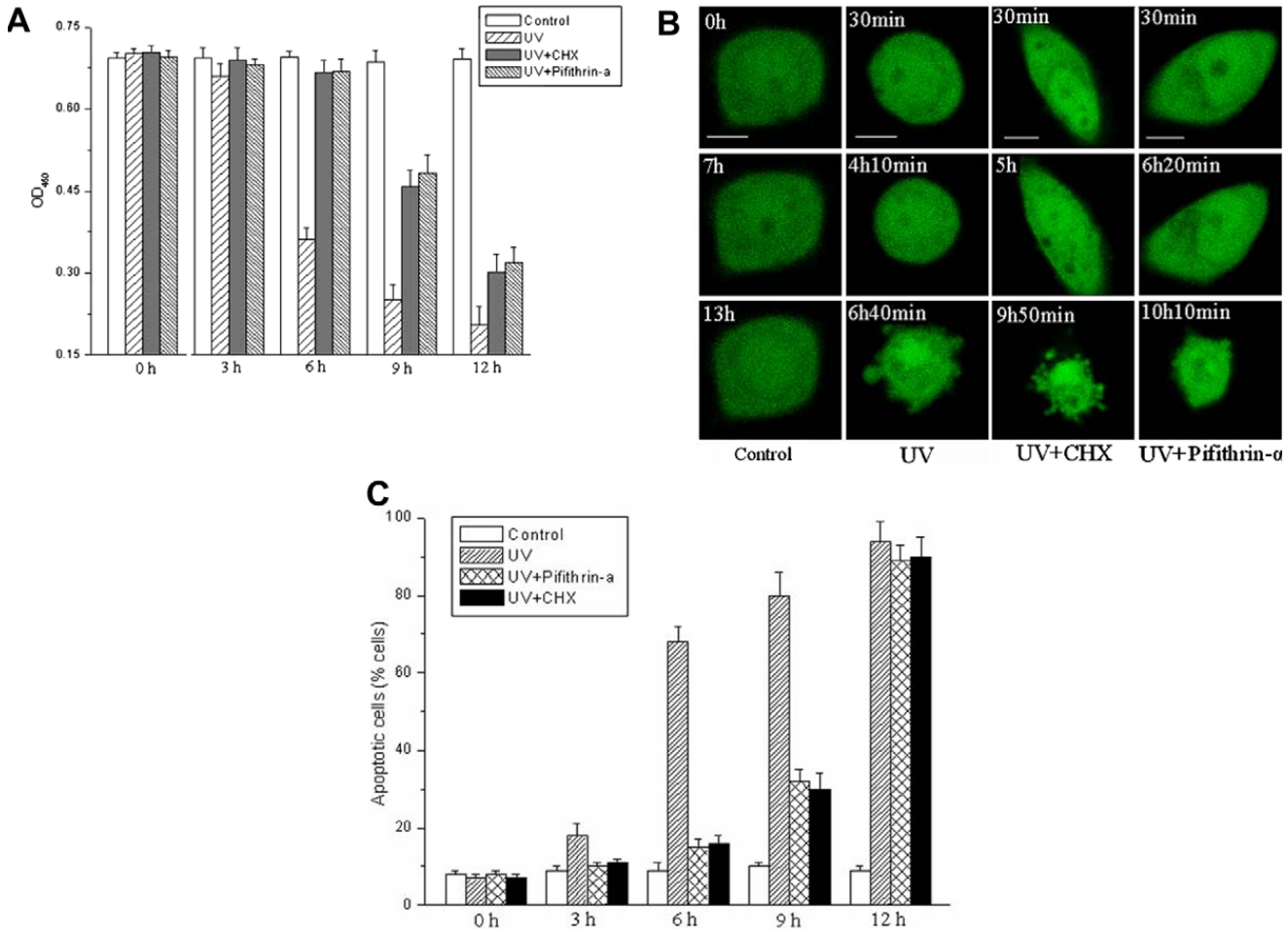


Fig. 1. UV irradiation induces apoptosis in ASTC-a-1 cells. (A) Cell viability was assessed by the CCK-8 assay at 0, 3, 6, 9, and 12 h after 120 mJ/cm$^2$ UV irradiation in the presence or absence of CHX or Pifithrin-α. (B) Cell apoptosis was analyzed using GFP labeling after 120 mJ/cm$^2$ UV irradiation. (C) Statistical analysis of cells transfected with GFP after different treatments. Error bars are SEM from four independent experiments.

### 2.5. Confirmation of cell apoptosis

ASTC-a-1 cells were cultured in 96-well microplate at a density of $5 \times 10^3$ cells/well for 24 h. The cells were then divided into five groups and exposed to UV irradiation at fluence of 120 mJ/cm$^2$. Cell cytotoxicity was assessed with CCK-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. $OD_{450}$, the absorbance value at 450 nm, was read with a 96-well plate reader (DG5032, Hua dong, Nanjing, China), and the $OD_{450}$ is inversely proportional to the degree of cell apoptosis.

### 2.6. RNA isolation and reverse transcription-PCR amplification

Total RNA was extracted using the Trizol reagent (Invitrogen). Reverse transcription-PCR (RT-PCR) analyses were done with the One-Step RT-PCR kit (Takala, Dalian, Liaoning Province, China). PUMA was amplified using the following primers: 5′-ATGGCGGACGACCTCAAC-3′ and 5′-AGTCCCATGAAGAGATTGTACATGAC-3′. Total RNA (0.2 μg) for each sample was used in an RT-PCR reaction. PCR was done with 25 reaction cycles. PCR products were resolved on a 1% agarose

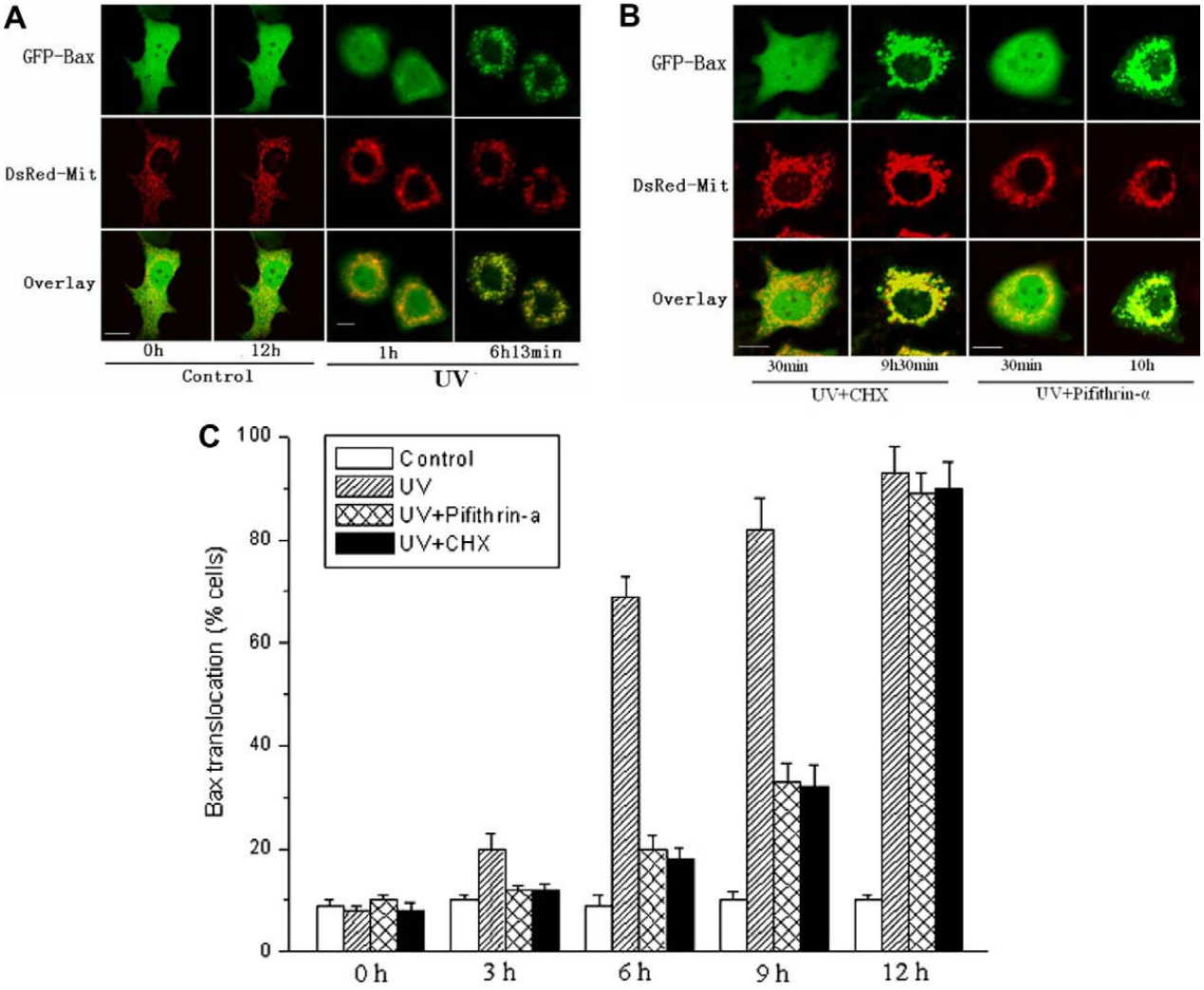


Fig. 2. Dynamics of GFP-Bax translocation during UV-induced apoptosis. (A and B) Typical confocal images of ASTC-a-1 cells co-transfected with GFP-Bax and DsRed-Mit in different conditions. The two panels of GFP-Bax and DsRed-Mit are shown separately and are merged to show the overlay. GFP-Bax localization at mitochondria was determined based on the overlap of GFP-Bax and DsRed-Mit fluorescence images. Similar results were obtained from three-independent experiments. Scale bar: 10 μm. (C) Quantification of cells showing mitochondrial GFP-Bax. At the indicated time points, the percentage of cells showing Bax translocation to mitochondria was assessed by counting the number of cells exhibiting mitochondrial Bax. Data were collected from $n = 150$–200 cells per treatment in 10–15 randomly selected image frames from $n = 3$ independent experiments. Data represent the means ± SEM.

gel stained with ethidium bromide. β-Actin was amplified as an internal control.

## 3. Results

### 3.1. Cells apoptosis induced by UV irradiation is delayed by Pifithrin-α and CHX

Cell apoptosis was analyzed using cell counting kit-8. The $OD_{450}$ value, an indicator of cell apoptosis, was measured. Our previous results demonstrated that the effects of UV irradiation on apoptosis of ASTC-a-1 cells were dose-dependent [28]. CHX (cycloheximide) is an inhibitor of protein biosynthesis in eukaryotic organisms, produced by the bacterium *Streptomyces griseus*. Cycloheximide exerts its effect by interfering with the translocation step in protein synthesis. Pifithrin-α is a small molecule inhibitor of p53 transcriptional activity. It has been proposed that the use of Pifithrin-α in conjunction with chemotherapeutic and radiation therapies for cancer will reduce the side effects of these treatments in normal tissue that still contains wild type p53 [32]. To observe the effects of CHX (cycloheximide) and Pifithrin-α (p53 inhibitor) on UV-induced apoptosis, we added CHX (50 μg/ml) or Pifithrin-α (20 μM) to cells 1 h before UV irradiation. Cell apoptosis was analyzed using cell counting kit-8 at 0 h (control), 3, 6, 9, 12 h after 120 mJ/cm$^2$ UV irradiation in the presence or absence of CHX or Pifithrin-α. The results showed that cell apoptosis was delayed by several hours in the presence of CHX or Pifithrin-α (Fig. 1A).

To further confirm the effects of CHX (cycloheximide) and Pifithrin-α (p53 inhibitor) on UV-induced apoptosis, ASTC-a-1 cells were transiently transfected with GFP to label the morphology of intact cells, the percentage of cells showing apoptosis was assessed by apoptotic morphology. Data were collected from $n = 150$ to 200 cells per treatment in 10–15 randomly selected image frames from $n = 4$ independent experiments. Data represent means

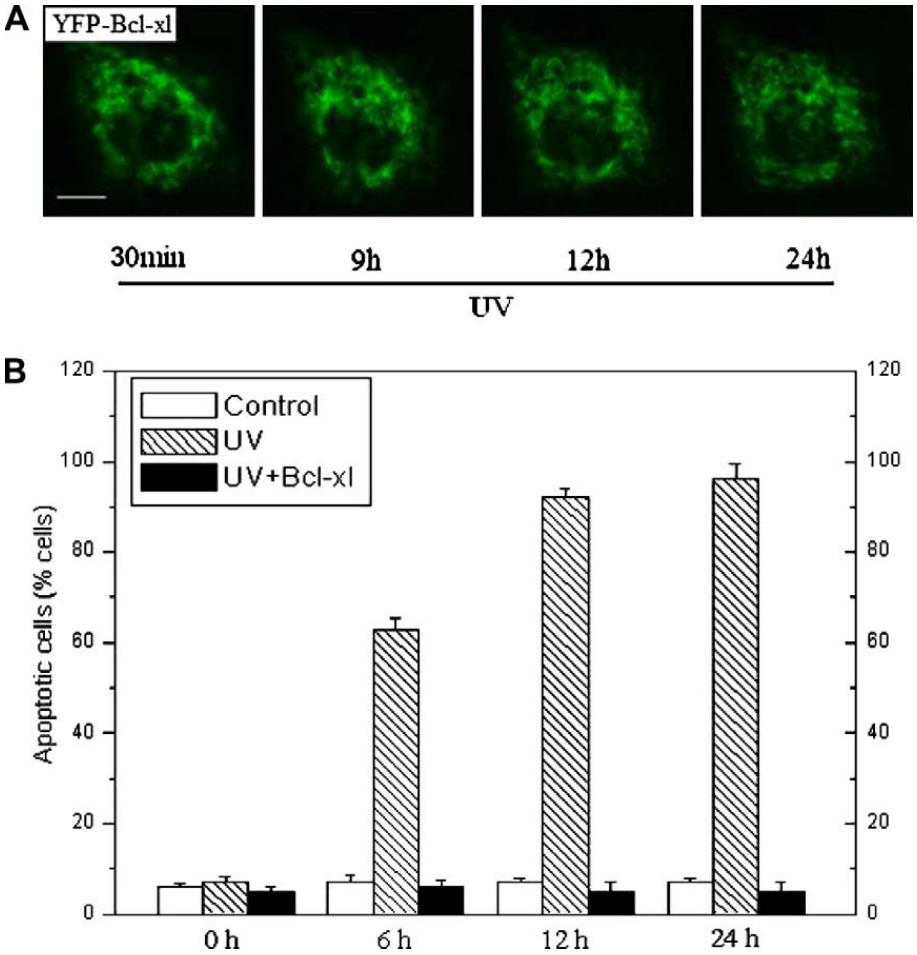


Fig. 3. Bcl-$x_L$ prevents UV-induced apoptosis. (A) Typical confocal images of ASTC-a-1 cells transfected with YFP-Bcl-$x_L$ after UV irradiation. Scale bar: 10 μm. (B) ASTC-a-1 cells transfected with YFP-Bcl-$x_L$ were exposed in different conditions for indicated times and assessed by counting the number of cells exhibiting apoptosis. Error bars are SEM from several hundred cells in three-independent experiments.

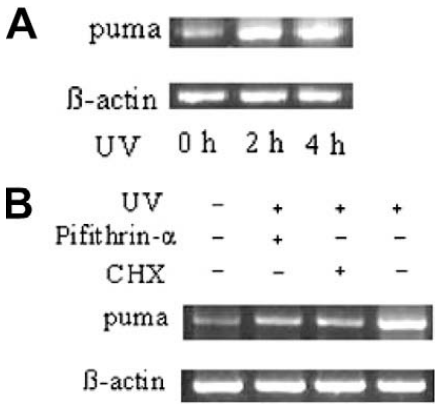


Fig. 4. mRNA expression level analysis of p53 target genes PUMA by reverse transcription-PCR (A) Cells were collected at 0, 2, 4 h after UV irradiation. (B) Cells were collected at 2 h after UV irradiation in the presence or absence of CHX or Pifithrin-α. Total RNA was collected and equal amounts of total RNA (0.2 μg) were added to each reaction mixture. β-Actin was amplified as an internal control.

±SEM. As shown in Fig. 1B, in the presence of CHX or Pifithrin-α, cell apoptosis induced by UV irradiation was delayed. These results were further confirmed by the statistical analysis (Fig. 1C).

### 3.2. Bax translocation by UV irradiation are delayed by Pifithrin-α and CHX

Bax exists in the cytosol of healthy cells and translocates to the mitochondria during apoptosis. For real-time detection of GFP-Bax translocation from the cytosol to the mitochondria during UV-induced apoptosis, we transiently co-transfected GFP-Bax and DsRed-Mit (a marker for mitochondria) into cells, after transfection, the cells were incubated for 48 h, followed by different treatments as indicated, then performed with the LSM microscope . As shown in Fig. 2A, GFP-Bax had a diffuse distribution in the whole cell for more than 12 h without treatment. However, GFP-Bax translocation started at 5–6 h after UV irradiation [28].

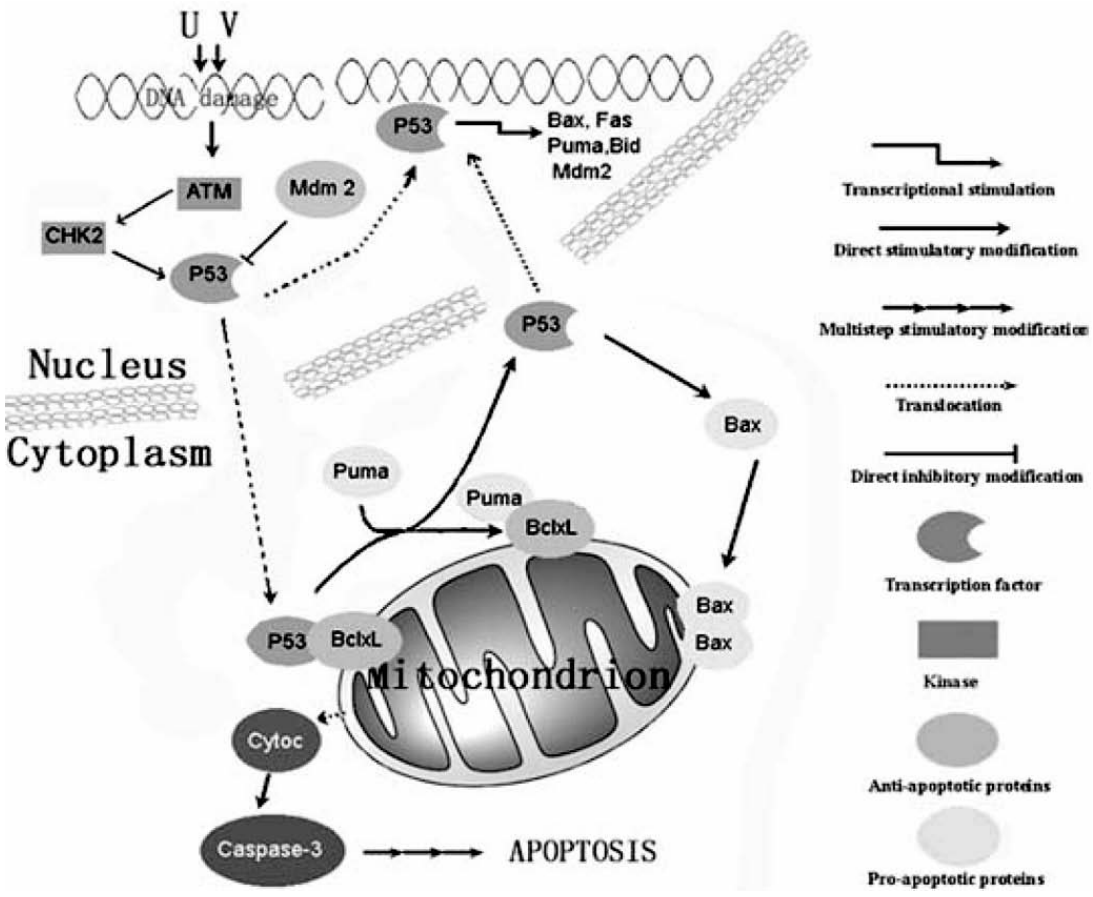


Fig. 5. A proposed model of UV-induced apoptosis. UV irradiation activates the ATM-Chk2-p53 pathway, after p53 stabilization, p53 accumulates in the nucleus to directly regulate PUMA expression. Likewise, p53 accumulates in the cytoplasm and directly binds to Bcl-$x_L$. Once p53-dependent expression of PUMA occurs, PUMA binds to Bcl-$x_L$, which releases p53 to directly activate Bax and induce MOMP.

To investigate the effects of CHX and Pifithrin-α on GFP-Bax translocation by UV irradiation, we added CHX or Pifithrin-α to cells 1 h before UV irradiation. As shown in Fig. 2B, GFP-Bax translocation by UV irradiation was delayed by about 4 h in the presence of Pifithrin-α or CHX. These results were further confirmed by the statistical analysis (Fig. 2C).

### 3.3. Bcl-$x_L$ prevents UV-induced apoptosis

It has been suggested that the interactions between proapoptotic and antiapoptotic Bcl-2 family members play an important role in apoptosis initiation [17,33–36]. PUMA interacts with antiapoptotic Bcl-2 and Bcl-$x_L$ proteins through its BH3 domain [24]. Therefore, to investigate whether Bcl-$x_L$ prevents UV-induced apoptosis, we treated ASTC-a-1 cells transfected with YFP-Bcl-$x_L$ with UV irradiation, then the real-time monitoring of YFP-Bcl-$x_L$ redistribution was performed on LSM microscope. As shown in Fig. 3A, the cells did not exhibit characteristics of apoptosis. These results were also confirmed by statistical analysis (Fig. 3B).

### 3.4. Pifithrin-α and CHX inhibited PUMA expression by UV irradiation

PUMA is induced at the transcriptional level in response to DNA damage and other stimuli. It is reported that PUMA plays an essential role in apoptosis induced by a variety of stimuli in several tissues and cell types [23]. So we investigated PUMA expression in different conditions. As shown in Fig. 4A, PUMA expression increased at 2 h after UV irradiation then remained unchanged.

To investigate the effects of CHX and Pifithrin-α on PUMA expression by UV irradiation, we added CHX or Pifithrin-α to cells 1 h before UV irradiation. As shown in Fig. 4B, PUMA expression at 2 h after UV irradiation in the presence of CHX or Pifithrin-α significantly decreased.

## 4. Discussion

The basis for p53 striking apoptotic and tumor suppressor activity lies in its gene's pleiotropism, which involves transcription-dependent and transcription-independent functions. p53 responds to a broad range of death stimuli by rapid stabilization and activation. One important mechanism through which p53 mediates its biological response is transcriptional activation of proapoptotic target genes and transrepression of prosurvival proteins. PUMA was initially identified as downstream targets of p53 and subsequently shown to play an important role in apoptosis [8,23]. So, in this study, using reverse transcription PCR, we found that PUMA expression increased at 2 h after UV irradiation then remained unchanged (Fig. 4A). CHX and Pifithrin-α can effectively inhibit PUMA expression(Fig. 4B).

Bax has been shown to be necessary for UV-induced apoptosis. Recent studies have demonstrated that purified or recombinant p53 has the ability to activate Bax to oligomerize in lipid membranes and cause permeabilization [12]. It is also reported that Bax activation by active Bid [37,38] or BH3 peptides from Bid or Bim is essential and sufficient to permeabilize vesicles composed of mitochondrial lipids in the absence of other proteins [39,40]. It was demonstrated that $Bid^{-/-}$ MEFs are less susceptible than $Bid^{+/+}$ MEFs to the DNA damage [10]. So, the regulatory mechanism of Bax translocation by UV irradiation has been unclear. In this study, we demonstrated that both p53 transcription-dependent and transcription-independent pathways were involved in Bax translocation and cell death by UV irradiation (Fig. 1 and Fig. 2).

The tumor suppressor p53 can induce apoptosis by activating gene expression in the nucleus, or by directly permeabilizing mitochondria in the cytoplasm. Our studies support a model of p53-dependent, UV-induced apoptosis that includes both nuclear and cytoplasmic functions of p53 (Fig. 5). UV irradiation causes DNA damage, which causes constitutive activation of the ATM-Chk2-p53 pathway, then p53 accumulates in the nucleus to directly regulate the expression of proapoptotic genes, such as Bax and PUMA. p53 also accumulates in the cytoplasm, directly binds to Bcl-$x_L$, and awaits a secondary death signal. Once p53-dependent expression of PUMA occurs, PUMA binds to Bcl-$x_L$, releasing p53 to directly activate Bax and induce MOMP. This may explain why Bcl-$x_L$-deficient animals display sensitivity to DNA damage, whereas PUMA deficiency promotes resistance to numerous p53-dependent apoptotic stimuli [26,41,42].

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$x_L$ plasmid, and we also thank Richard J. Youle for kindly providing the pGFP-Bax plasmid.

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