

PUMA PROMOTES BAX ACTIVATION IN A FOXO3a-DEPENDENT MANNER IN STS-INDUCED APOPTOSIS

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PUMA (p53 up-regulated modulator of apoptosis, also called Bbc3) was first identified as a BH3-only Bcl-2 family protein that is transcriptionally up-regulated by p53 and activated upon p53-dependent apoptotic stimuli, such as treatment with DNA-damaging drugs or UV irradiation. Recently, studies have shown that PUMA is also up-regulated in response to certain p53-independent apoptotic stimuli, such as growth factor deprivation or treatment with glucocorticoids or STS (staurosporine). However, the molecular mechanisms of PUMA up-regulation and how PUMA functions in response to p53-independent apoptotic stimuli remain poorly understood. In this study, based on real-time single cell analysis, flow cytometry, and western blotting technique, we investigated the function of PUMA in living human lung adenocarcinoma cells (ASTC-a-1) after STS treatment. Our results show that FOXO3a was activated by STS stimulation and then translocated from cytosol to nucleus. The expression of PUMA was up-regulated via a FOXO3a-dependent manner after STS treatment, while p53 had little function in this process. Moreover, cell apoptosis and Bax activation induced by STS were not blocked by Pifithrin- α (p53 inhibitor), which indicated that p53 was not involved in this signaling pathway. Taken together, these results suggest that PUMA promoted Bax activation in a FOXO3a-dependent pathway during STS-induced apoptosis, while p53 was dispensable in this process.

Keywords: PUMA; Bax activation; FOXO3a; STS; apoptosis.

1. Introduction

The tumor suppressor and transcription factor p53 binds to specific DNA sequences in promoter regions of its target genes after DNA damage.¹ Activation of these genes by p53 triggers apoptosis, cell cycle arrest, DNA repair, and other responses.² Despite its critical role in response to DNA damage treatment, p53 is dispensable for certain stress stimuli, such as cytokine withdrawal induced apoptosis in lymphoid cells.^{3,4} In contrast,

the PI3K–Akt signaling pathway has been shown to mediate cell survival under these conditions,^{5,6} possibly through inhibition of FOXO transcription factors. Members of mammalian FOXO family of forkhead transcription factors are critical positive regulators of longevity in species as diverse as worms and flies.^{7–9} It has been reported that activated FOXO3a (one of the FOXO family members) can trigger p53-dependent cell death, and the transcriptional activity of p53 is dispensable for such forms of apoptosis.¹⁰ However, the critical

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mediators downstream of FOXOs still remain unclear.

PUMA (p53 up-regulated modulator of apoptosis), a member of the Bcl-2 family proteins, also known as bbc-3 (bcl-2 binding component 3), was first identified as a BH3-only protein that is transcriptionally up-regulated by p53 and activated upon p53-dependent apoptotic stimuli, such as treatment with DNA-damaging drugs and UV irradiation.^{11–14} However, it has been shown that PUMA is also up-regulated in response to certain p53-independent apoptotic stimuli, such as growth factor deprivation or treatment with glucocorticoids or phorbol ester.¹² Experiments with gene-targeted mice have shown that PUMA is required for apoptosis of lymphoid cells and fibro-epithelium and also certain p53-independent ones, such as cytokine deprivation, treatment with glucocorticoids, or phorbol ester.^{15,16} It was demonstrated recently that the transcription factor FOXO3a increases PUMA expression in response to growth factor deprivation in lymphoid cells and mouse embryonic fibroblasts, suggesting that PUMA together with Bim may have overlapping functions as FOXO3a downstream targets following removal of survival factors.¹⁷ Proapoptotic Bax and Bak are essential regulators of the mitochondrial pathway of apoptosis.¹⁸ Bak resides permanently on the outer mitochondrial membrane (OMM), whereas Bax is normally found in the cytosol of healthy cells and translocates to the OMM during apoptosis.¹⁹ After translocation to mitochondria, Bax induces cytochrome c release either by forming a pore by oligomerization in the OMM, or by opening other channels.²⁰

We have demonstrated that PUMA promotes Bax translocation by both interacting with Bax directly and by competitive binding to Bcl-X_L through a p53-dependent pathway during UV-induced apoptosis,¹⁴ and we have discussed many signaling pathways of cell survival and apoptosis.^{21–25} In order to further study whether PUMA can induce Bax activation and cell apoptosis in a p53-independent manner, in this study, based on real-time single cell analysis, flow cytometry, and western blotting technique, we investigated the function of PUMA in living human lung adenocarcinoma cells (ASTC-a-1) after staurosporine (STS) treatment. And our results suggested that PUMA promoted Bax activation in a FOXO3a-dependent manner after STS treatment, while the transcriptional activity of p53 was dispensable in this process.

2. Methodology

2.1. Materials

Dulbecco's modified Eagle medium (DMEM) was purchased from GIBCO (Grand Island, NY). Pifithrin- α (p53 inhibitor) was purchased from BioVision (Mountain View, CA, USA). LipofectamineTM Reagent was purchased from Invitrogen (Carlsbad, CA, USA). DNA Extraction kit was purchased from Qiagen (Valencia, CA, USA). pGFP-Bax was kindly supplied by Richard J. Youle,²⁶ pDsRed-Mit was kindly supplied by Dr. Y. Gotoh,²⁷ pGFP-FOXO3a was kindly supplied by Link W.²⁸ Other chemicals were mainly from Sigma (St Louis, MO, USA).

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 units/ml), and streptomycin (100 mg/ml) in 5% CO₂ at 37°C in humidified incubator. Transfections were performed with LipofectamineTM 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. Unless otherwise specified, cells were exposed to UV irradiation at a fluence of 120 mJ/cm² and observed at the time indicated. For STS treatment, 1 μ M STS was added in the medium directly.

2.3. Time-lapse confocal fluorescence microscopy

GFP, CFP, 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 \times /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 500–520 nm band 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 and GFP-FOXO3a 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 both the distribution pattern of GFP-Bax and that of 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. For GFP-FOXO3a translocation, ASTC-a-1 cells were transfected with GFP-FOXO3a, using Zeiss LSM 510 confocal microscope, we imaged the distribution of GFP-FOXO3a simultaneously. FOXO3a is localized in the cytosol and little in the nucleus, so we can see the fluorescence in the nucleus changes brighter clearly after STS stimulation.

2.5. *Antibodies and western blotting*

The antibodies used for Western blotting include antibodies against PUMA (Epitomics, CA, USA). At the indicated time after UV irradiation, cells were harvested and washed twice with ice-cold phosphate-buffered saline (PBS, pH 7.4), and lysed with ice-cold lysis buffer (50 mmol/L Tris-HCl pH 8.0, 150 mmol/L NaCl, 1 × TritonX-100, 100 μg/ml PMSF) for 30 min on ice. The lysates were centrifuged at 12,000 rpm for 5 min at 4°C, and the protein concentration was determined. Equivalent samples (30 μg protein extract was loaded on each lane) were subjected to SDS-PAGE on 12% gel. The proteins were then transferred onto nitrocellulose membranes, and probed with indicated antibody, followed by IRDye 800 secondary antibody (Rockland Immunochemicals, Inc.). Detection was performed using the LI-COR Odyssey Infrared Imaging System (LI-COR, Inc., USA).

2.6. *Flow cytometry*

For Flow cytometric analysis (FACS analysis), Annexin-V-FITC conjugate and binding buffer were

used as standard reagents. Flow cytometry was performed on a FACScanto flow cytometer (Becton Dickinson, Mountain View, CA, USA) with excitation at 488 nm. Fluorescent emission of FITC was measured at 515–545 nm and that of DNA-PI complexes at 564–606 nm. Cell debris was excluded from analysis by an appropriate forward light scatter threshold setting. Compensation was used wherever necessary.

2.7. *Immunofluorescence*

For Bax activation, MitoTracker Red (0.5M; Molecular Probes) was added into the medium, and cells were incubated at 37°C for 30 min. Slides were fixed in 4% paraformaldehyde for 15 min at room temperature and then washed five times with PBS. Samples were incubated in blocking buffer (10% BSA in PBS) for 1 h at room temperature, followed by incubation with anti-Bax antibody 6A7 (5 μg/ml in blocking buffer, Abcam, Cambridge, UK) at 4°C overnight. Cells were washed five times for 5 min each, after which Alexa Fluor 488-conjugated rabbit anti-mouse secondary antibody (diluted 1:400 in blocking buffer, Invitrogen, CA, USA) was added for 1 h at room temperature. After five additional washes with PBS, slides were mounted and analyzed by confocal microscopy.

3. Results

3.1. *Cells apoptosis induced by STS treatment are p53-independent*

In order to evaluate the status of cell apoptosis, ASTC-a-1 cells were treated with 1 μM STS, and then cell apoptosis was analyzed by using FACS analysis. Pifithrin-α, an inhibitor of p53, was used to inhibit the transcription activity of p53 in this process. As shown in Fig. 1, cell apoptosis increased obviously after STS treatment compared with the control group. While Pifithrin-α has little effect on the percentage of cell apoptosis meaning the transcriptional activity of p53 is dispensable in STS-induced apoptosis.

3.2. *STS up-regulates the expression of PUMA through activating FOXO3a*

As p53 has little influence in STS-induced apoptosis, then which molecular signaling pathway is involved in this process? It has been reported that

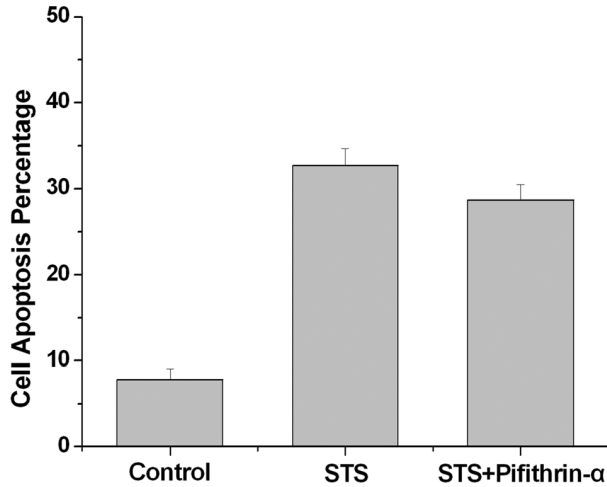


Fig. 1. STS induces apoptosis in ASTC-a-1 cells. Cell apoptosis was analyzed using FACS technique at 6 h after STS treatment. The percentage of apoptosis cells were used to calculate. Bars represent means \pm s.e.m. from at least four independent experiments.

activated FOXO3a (one of the FOXO family members) can trigger p53-independent cell death, and the transcriptional activity of p53 is dispensable for such forms of apoptosis. We then detected whether FOXO3a was activated after STS treatment. ASTC-a-1 cells were transiently transfected with GFP-FOXO3a following with 1 μ M STS treatment. As the result shown in Fig. 2(b), upon STS stimulation, GFP-FOXO3a translocated from cytosol to nucleus, indicating the activation of FOXO3a, at about 45 min after STS treatment. By the contrast, GFP-FOXO3a had a diffuse distribution in the cytosol for more than 120 min in the control group (Fig. 2(b)).

FOXO3a is a transcription factor, the activated FOXO3a will translocated to nucleus to up-regulate the expression of its target genes. It was demonstrated recently that PUMA is one of the downstream targets of FOXO3a (33). To detect whether PUMA was up-regulated by activated FOXO3a during STS-induced apoptosis, western blotting was performed to analyzed the expression of PUMA after UV or STS stimulation in the presence or absence of Pifithrin- α . As a result, PUMA was up-regulated after UV irradiation and STS treatment compared with the control group. Pifithrin- α could inhibit the up-regulation of PUMA induced by UV irradiation, but could not inhibit the up-regulation by STS treatment, which indicated that STS up-regulated the expression of PUMA in a p53-independent way. Of course, FOXO3a may play a

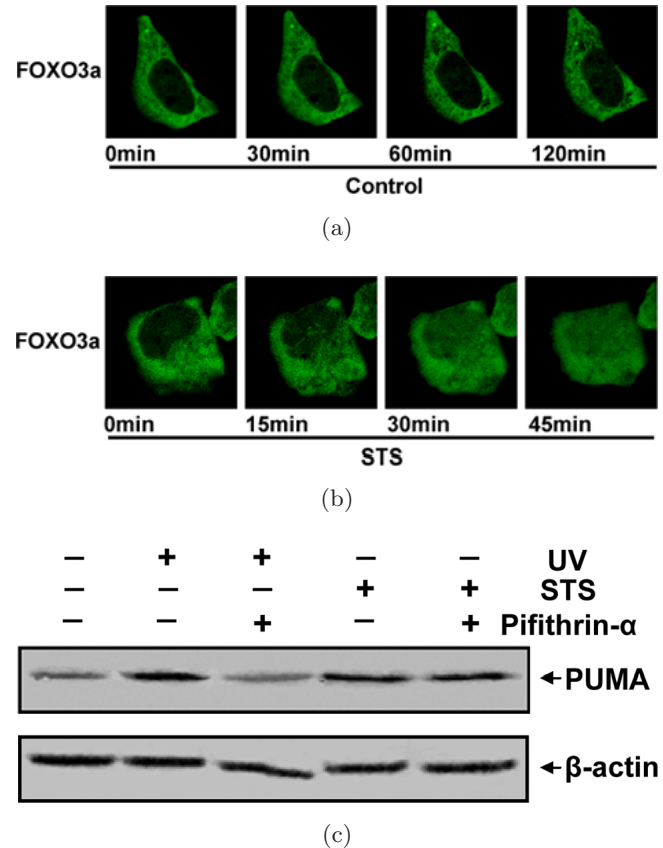


Fig. 2. STS activates FOXO3a and up-regulates the expression of PUMA. (a and b) ASTC-a-1 cells were transiently transfected with GFP-FOXO3a. (a) The GFP-FOXO3a typically displayed a diffuse, cytoplasmic localization in the untreated cell. (b) GFP-FOXO3a translocated from cytosol to nucleus at about 45 min after STS treatment. (c) The expression of PUMA increased after UV or STS treatment. Western blotting was performed to detect the expression of PUMA at 6 h after UV or STS stimulation in the presence or absence of Pifithrin- α .

critical role in this process which should be further studied.

3.3. Real-time detection of the Bax translocation after STS stimulation

To detect the GFP-Bax redistribution during STS-induced apoptosis in real-time, ASTC-a-1 cells were transiently co-transfected with GFP-Bax and DsRed-Mit (a marker for mitochondria), and then treated with 1 μ M STS. As the result shown in Fig. 3(a), GFP-Bax had a diffuse distribution in the whole cell for more than 12 h in the control group. By the contrast, almost all the GFP-Bax translocated from cytosol to mitochondria, indicating the activation of Bax, at about 3 h after STS treatment

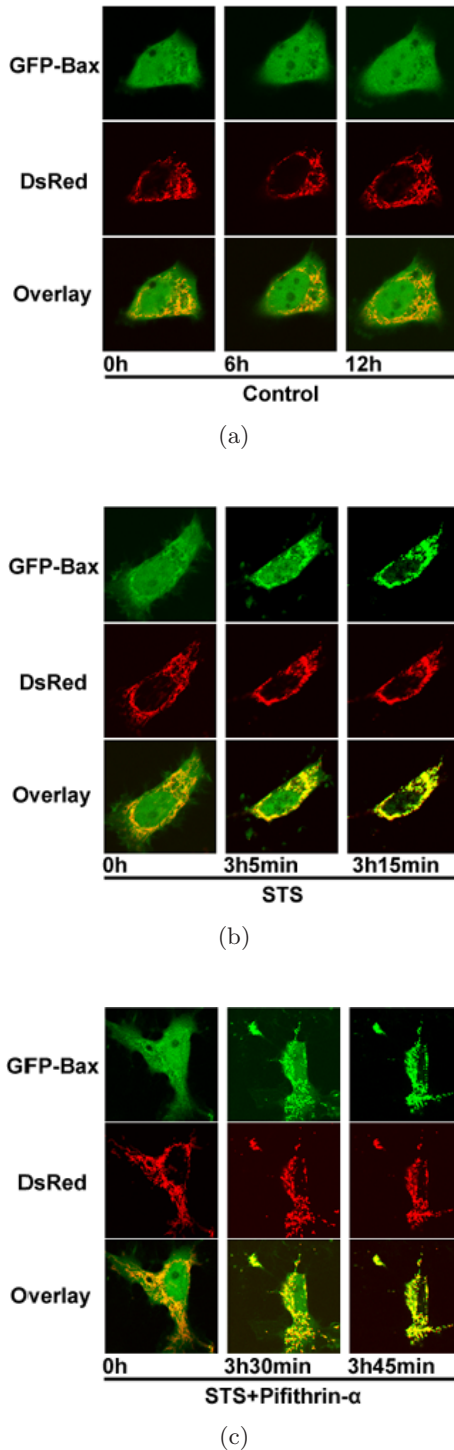


Fig. 3. Spatial and temporal changes in Bax subcellular localization during STS-induced apoptosis. (a–c) ASTC-a-1 cells were transiently co-transfected with GFP-Bax and DsRed-Mit (a marker for mitochondria). (a) The GFP-Bax typically displayed a diffuse, cytoplasmic localization in the untreated cell. (b) GFP-Bax translocated to mitochondria noticeably after STS stimulation at about 3 h. (c) Pifithrin- α was added in 1 h before STS treatment, real-time detection of GFP-Bax redistribution showed GFP-Bax still translocated to mitochondria in the presence of Pifithrin- α .

(Fig. 3(b)). Besides, Pifithrin- α could not inhibit Bax translocation (Fig. 3(c)) suggesting that STS-induced Bax translocation via a p53-independent manner.

3.4. Bax activation induced by STS is independent of p53

Generally speaking, the activation of Bax is inferred by its translocation from cytosol to mitochondria. To directly detect Bax activation, ASTC-a-1 cells were treated with 1 μ M STS, then cells were fixed and subjected to immunofluorescence staining for active Bax by using a conformation-specific anti-Bax monoclonal antibody (6A7) that selectively recognizes only the activated/pro-apoptotic form of Bax, and mitochondria were stained with MitoTracker Red. As the result shown in Fig. 4(a), active Bax was not identified in untreated cells (Control) but was readily detectable in cells

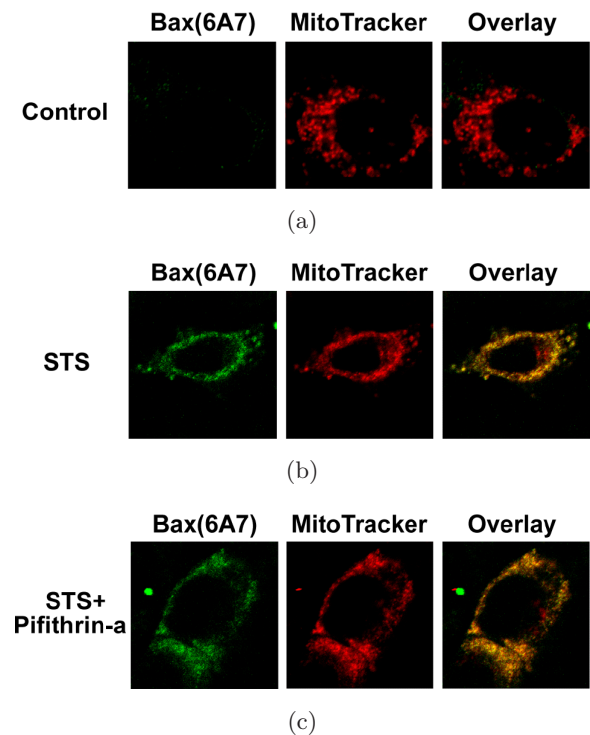


Fig. 4. Mitochondria localization of conformationally activated Bax in cells treated by STS. (a–c) MitoTracker Red (0.5M; Molecular Probes) was added into the medium, and cells were incubated at 37°C for 30 min. Slides were fixed in 4% paraformaldehyde for 15 min at room temperature and then washed five times with PBS. Samples were incubated with antibodies and then analyzed by confocal microscopy. (a) Active Bax was not identified in untreated cells. (b) Cells were treated with 1 μ M STS before immunofluorescence staining, and Bax was activated. (c) Pifithrin- α was added in 1 h before STS treatment, Bax was still activated.

after 3 h of STS treatment, further more the p53 inhibitor Pifithrin- α could not inhibit Bax activation indicating Bax activation induced by STS was independent of p53. The yellow color in overlay indicates the presence of conformationally changed activated Bax co-localized with mitochondria.

4. Discussion

BH3-only Bcl-2 family proteins are evolutionarily conserved and essential mediators of apoptosis initiation in the mitochondria pathway. PUMA was originally identified as a p53 downstream target.^{11–13} However, recently studies reported that cells lacking PUMA are also resistant to several p53-independent death stimuli, and PUMA mRNA levels were up-regulated under these conditions.^{3,4} Additionally, one of the common features shared by some of these stress stimuli that cause p53-independent PUMA up-regulation is that they could attenuate the PI3K–Akt signaling pathway, which in turn modulates the activity of FOXO transcription factors.¹⁷

Members of mammalian FOXO family of fork-head transcription factors are critical positive regulators of longevity in species as diverse as worms and flies.^{29,30} FOXO factors can play a proapoptotic role in neurons or Hematopoietic cells subjected to growth factor or cytokine withdrawal. In this situation, the survival kinase AKT is inactive, and FOXO remains in the nucleus where it induces p27 and possibly other unidentified downstream targets to induce apoptosis.³¹ Recently studies have shown that FOXO3a is activated after cytokine withdrawal and play an important role in cell apoptosis.¹⁷ In our study, based on real-time single cell analysis, we detected the activation of FOXO3a and its translocation from cytosol to nucleus after STS stimulation (Fig. 2(b)), which means, as a transcription factor, FOXO3a regulates its downstream targets in nucleus after activation. Besides, western blot shows that the expression of PUMA was up-regulated when FOXO3a was activated (Fig. 2(c)), and this up-regulation was independent of p53. So activated FOXO3a may play a critical role in PUMA up-regulation, however, how FOXO3a functions in this process needs to be further studied.

Generally speaking, the activation of Bax is inferred by its translocation from cytosol to mitochondria. However, to exclude the situation that

exogenous GFP-Bax is not reflective the function of endogenous Bax, real-time single cell analysis, and immunofluorescence staining were used at the same time to demonstrate Bax activation. Our results shows that GFP-Bax translocated from cytosol to mitochondria (Figs. 3(b) and 3(c)), which consistent with the result that endogenous Bax had a conformation change and was activated (Figs. 4(b) and 4(c)). These results can clearly demonstrate that Bax activation is accompanied by its translocation to mitochondria, suggesting the translocation of Bax means the activation of it. Interestingly, Bax activation after STS treatment was also independent of p53 (Figs. 3(c) and 4(c)) as the up-regulation of PUMA expression.

The function of PUMA in Bax translocation and cell apoptosis after UV stimulation has been well established in our early work.¹⁴ However, the mechanisms of PUMA up-regulation and how PUMA functions in response to p53-independent apoptotic stimuli remain unclear. In this article, our results clearly show that Pifithrin- α could not inhibit Bax translocation and cell apoptosis induced by STS stimulation meaning this process was independent of p53 (Figs. 1, 3, and 4). STS treatment could activate FOXO3a and then up-regulate the expression of PUMA (Fig. 2), the accumulation of PUMA promoted Bax activation and its translocation from cytosol to mitochondria. However, this up-regulation of PUMA was not dependent on the transcriptional activity of p53, but FOXO3a. Taken together, we speculated that PUMA promotes Bax activation in FOXO3a-dependent pathway during STS-induced apoptosis.

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