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ORIGINAL PAPER

YAP accelerates Aβ_{25-35}-induced apoptosis through upregulation of Bax expression by interaction with p73

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Abstract Accumulation of amyloid-β-peptide (Aβ) in the brain is considered as a pathological hallmark of Alzheimer's disease (AD). Previous studies show that p73 is vital for mediating the pathogenic process of AD. Yes-associated protein (YAP) has been shown to positively regulate p73 in promoting apoptosis induced by anti-cancer agents. However, the functional role of YAP and potential relationship between YAP and p73 in AD are unknown. In the present study, we found that YAP accelerated apoptosis in response to Aβ_{25-35} and the nuclear translocation of YAP was involved in cellular signals that regulated the apoptosis. Aβ_{25-35} induced YAP translocation from cytoplasm to nucleus accompanied with the increased phosphorylation on Y357, resulting in the enhancement of interaction between YAP and p73. Moreover, inhibition of YAP expression by small hairpin RNA (shRNA) suppressed apoptosis induced by Aβ_{25-35}. More importantly, p73-mediated induction of Bax expression and activation were in a YAP-dependent manner. Overexpression of YAP accelerated Bax translocation, upregulated Bax expression and promoted caspase-3 activation. Taken together, our findings first demonstrated that YAP accelerated Aβ-induced apoptosis through nucleus translocation, leading to the induction of Bax expression and activation. Our results provided a potential therapeutic strategy for the treatment of AD through inhibiting YAP/p73/Bax pathway.

Keywords Alzheimer's disease (AD) · Amyloid beta (Aβ) · YAP · p73 · Bax

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Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disease which is characterized pathologically by amyloid plaques, neurofibrillary tangles, and neuronal loss [1–4]. Previous studies demonstrate that apoptosis implicates in the pathogenesis of AD [5–7]. Amyloid beta (Aβ) has been found to cause neurotoxicity and cell apoptosis in vivo and in vitro [8–10]. Recent studies show that caspase-8 is activated during Aβ-induced apoptosis [11]. Additionally, Butterfield et al. have found that Aβ induce free radical oxidative stress in the neurodegeneration in AD brain [12]. However, the signal transduction pathways involved in the apoptosis induced by Aβ remain to be clarified.

Yes-associated protein (YAP) is a transcriptional coactivator [13, 14], and previous studies indicate that YAP needs to bind its target transcription factors to stimulate gene expression [15]. Reported YAP target transcription factors include TEAD, p73, Runx2, and the ErbB-4 [13, 16–19]. YAP is conserved in human and mouse/rat [14]. The structures of mouse/rat YAP is almost the same as human YAP [20]. Different YAP protein isoforms fall into two classes according to the C-termini alternative splicing [13, 20, 21]—(i) the full-length YAP (FL-YAP), or YAP proteins, having a COOH-terminal transactivation domain, participate in DNA-damage induced apoptosis [22–24] and (ii) its novel neuron-specific isoforms (YAPΔCs), or short-form proteins, which truncation of COOH-terminal transactivation domain are sustained to suppress neuronal death [20, 25]. However, the role of FL-YAP in neurodegenerative disease has not been demonstrated.

To date, various signaling pathways have been reported to account for Aβ-induced apoptosis [11, 12]. p73 is a reported YAP target transcription factor and YAP has been

shown to positively regulate p73 in promoting apoptosis through mediating the expression of cell death-promoting genes, *bax* or *puma* [22–24]. Interaction between YAP and p73 is critical to p73 transactivation of apoptotic target genes and induction of apoptotic cell death [16, 26, 27]. YAP phosphorylation on tyrosine 357 (Y357) by c-Abl results in stabilization of YAP [28]. More stable YAP protein displays higher affinity to p73 and selectively coactivates p73 proapoptotic target genes [23, 28]. However, the physical interaction between FL-YAP and p73 during AD has not been investigated.

In the present study, we found that YAP accelerated Aβ_{25-35}-induced apoptosis through translocating from cytoplasm to nucleus, which resulted in binding its target transcription factor p73, and increasing the expression and activation of Bax. In addition, we showed that the redistribution of YAP was accompanied by the increased phosphorylation on Y357. Moreover, inhibition of YAP expression by small hairpin RNA (shRNA) prevented cell apoptosis induced by Aβ_{25-35}. Here we reported that YAP protein mediated Aβ_{25-35}-induced apoptosis through interacting with p73. Our results first demonstrated that the YAP/p73 pathway was activated following Aβ_{25-35}-treatment and might play a pathogenic role in AD.

Materials and methods

Materials

Lipofectamine™ 2000 and LTX were purchased from Invitrogen (Carlsbad, CA). Aβ_{25-35} and Aβ_{1-42} were purchased from Sigma-Aldrich (St. Louis, MO), Aβ_{25-35} and Aβ_{1-42} stock solution of 1 mM were prepared in distilled and deionized water and stored at −20°C. Prior to a treatment, peptides were pre-incubated at 37°C for 5 days to promote aggregation and then diluted with medium to desired concentrations (25 μM). NC shRNA and YAP shRNA were synthesized by GenePharma Co., Ltd. (Shanghai, China). Green fluorescent protein expressplasmids EGFP–YAP was kindly supplied by Prof. Subham Basu and Prof. Julian Downward [22]. The pYFP–p73 was a gift from Prof. Hedeki Shimodaira [29, 30].

shRNA

Targeting sequence of YAP–shRNA was described previously [31]. The oligonucleotides for negative control pGPU6/Neo-shNC were synthesized as follows: 5′-GTT CTCCGAACGTGTCACGT-3′. The shRNA was transfected into cells using Lipofectamine™ 2000.

Cell culture and transfection

PC12 cells were cultured in a humidified (5% CO_2, 37°C) incubator in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.), supplemented with 10% fetal bovine serum, 5% horse serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). To obtain neuronally differentiated PC12 cultures, subconfluent cells were differentiated for up to 5 days in DMEM with 5% fetal bovine serum and 2.5 s NGF (100 nM) (Sigma-Aldrich, St. Louis, MO). 293T cells were cultured in a humidified (5% CO_2, 37°C) incubator in DMEM (Life Technologies, Inc.), supplemented with 10% fetal bovine serum. To ensure maximum bioavailability, the medium was refreshed every other day, and cells were plated at appropriate densities according to each experimental protocol. The plasmids GFP (pGFP), EGFP-YAP or YFP-p73 were transfected into PC12/293T cells using Lipofectamine™ 2000.

Primary hippocampal cells

Hippocampi from Sprague-Dawley rats in new-born day one were dissected and primary rat hippocampal cultures were prepared as described previously [8]. Hippocampal cells were seeded in polylysine-coated wells and maintained in Neurobasal medium supplemented with B27 (Invitrogen, Carlsbad, CA) plus 100 U/ml penicillin and 100 μg/ml streptomycin for 5–7 days before the cell treatments. To inhibit glial proliferation, 2 μM Cytosin-Arabinoside (AraC, Sigma-Aldrich, St. Louis, MO) was added on the third day of culture and removed 2 days later. The pEGFP-YAP was transfected into primary hippocampal cells using Lipofectamine™ LTX.

Cell apoptosis assay

Quantification of apoptosis by Annexin-V/PI staining was performed as described previously [32, 33]. Briefly, both floating and attached cells were collected 48 h after YAP-directed shRNA transfected or 24 h after Aβ_{25-35}-treatment. Apoptotic cell death was determined using the BD ApoAlert Annexin-V-FITC Apoptosis Kit (Becton-Dickinson, Biosciences) according to the manufacturer's instructions. Flow cytometry was performed on a BD FACSCanto™ II flow cytometer (Becton-Dickinson, Mountain View, CA).

Time-lapse confocal fluorescence microscopy

GFP and YFP were monitored confocally using a commercial laser scanning microscope (LSM 510/ConfoCor2) combination system (Carl Zeiss, Jena, Germany) equipped with a Plan-Neofluar 40×/1.3 numerical aperture (NA) oil

differential interference contrast (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- to 520-nm band pass filter. YFP fluorescence was excited at 514 nm with an argon ion laser, and emission was recorded through a 535- to 545-nm band pass filter. For time-lapse imaging, culture dishes were mounted onto the microscope stage that was equipped with a temperature-controlled chamber (Carl Zeiss, Jena, Germany). During control experiments, bleaching of the probe was negligible.

GFP–YAP translocation assay

To monitor GFP–YAP translocation in living cells, PC12 cells were transfected with pGFP–YAP. Using an LSM 510 confocal microscope (Carl Zeiss, Jena, Germany), we imaged the distribution pattern of GFP–YAP simultaneously during Aβ-induced apoptosis. YAP redistribution was assessed by the fluorescence of GFP–YAP emission and we analyzed the redistribution by the change of the fluorescence ratio of nucleus and cytoplasm. The cells exhibiting increased relative fluorescence of GFP in the nuclear were counted as the cells which showed YAP translocation.

Fluorescence recovery after photobleaching (FRAP) experiments

For FRAP experiments, primary hippocampal cells were transfected with 400 ng of pGFP–YAP. Cells were selected for the analysis by virtue of their GFP–YAP enrichment in one compartment. FRAP analysis was performed as described previously [34]. After 6 h of Aβ_{25-35}-treatment, photobleaching was performed on a confocal laser scanning microscope (LSM510/ConfoCor2, Zeiss, Jena, Germany). The recovery curves shown are averages of the recovery curves of at least five cells (or three cells for some point bleach experiments).

Fluorescence resonance energy transfer (FRET) analysis

FRET was performed on a commercial laser scanning microscopes (LSM510/ConfoCor2) combination system (Carl Zeiss, Jena, Germany) as described previously [35, 36]. For excitation, the 458-nm line of an argon-ion laser was attenuated with an acousto-optical tunable filter, reflected by a dichroic mirror (main beam splitter HFT458), and focused through a Plan-Neofluar 40×/1.3 NA oil DIC objective (Carl Zeiss) onto the sample. GFP and YFP (a FRET acceptor) emissions were collected through 500- to 520- and 535- to 545-nm band pass filters, respectively. The quantitative analysis of the fluorescence images was performed using Zeiss Rel3.2 image processing software (Carl Zeiss). After background subtraction, the average of fluorescence intensity per pixel was calculated. During control experiments, bleaching of the probe was negligible.

Immunofluorescence (IF)

PC12 were cultured in DMEM, supplemented with 15% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were fixed in 4% paraformaldehyde for 15 min at room temperature and then were washed five times with PBS. Samples were incubated in blocking buffer (10% bovine serum albumin in PBS) for 1 h at room temperature, followed by incubation with anti-YAP antibody, anti-GFP/YFP antibody or anti-Bax antibody 6A7 at 4°C overnight. Cells were washed five times for 5 min each, after which FITC-conjugated secondary antibodies (Proteintech, Group Chicago) were added for 2 h at a room temperature. Nucleus was stained with PI (10 μg/ml). After five additional washes with PBS, slides were mounted and analyzed by confocal microscopy.

YFP–Bax translocation assay

To monitor Bax translocation in living cells, PC12 cells were transfected with pYFP–Bax and the mitochondria were stained with MitoTracker Red (a marker for mitochondria). The pYFP–Bax was described by previous studies [36, 37]. Using an LSM 510 confocal microscope (Carl Zeiss, Jena, Germany), we imaged both the distribution pattern of YFP–Bax and that of MitoTracker Red simultaneously during Aβ-induced apoptosis. Bax redistribution was assessed by the matching fluorescence of YFP–Bax and MitoTracker Red emission. The cells exhibiting strong punctate staining of YFP, which overlapped with the distribution of MitoTracker Red, were counted as the cells with mitochondrially localized Bax.

Antibodies and western blotting

The antibodies used for western blotting include antibodies against YAP (Bioworld Technology, Inc.), Bax (Cell Signaling Technology, Danvers, MA), Bax-antibody 6A7 (Abcam plc, UK), full length/cleaved caspase-3 (Cell Signaling Technology, Danvers, MA), GFP/YFP (GenScript, Piscataway, NJ), anti-Puma antibody (Epitomics, Burlingame, CA) and Histone H3 (Santa Cruz, CA). At the indicated times after Aβ_{25-35} treatment, cells were harvested, washed with ice-cold PBS, pH 7.4, and lysed with ice-cold lysis buffer [50 mM Tris–HCl, pH 8.0, 150 mM

NaCl, 1% Triton X-100, and 100 μg/ml phenylmethylsulfonyl fluoride (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 (40–100 μg of protein extract was loaded on each lane) were subjected to SDS-PAGE on 10 or 13% gel. The proteins were then transferred onto nitrocellulose membranes and probed with the indicated antibodies, followed by secondary antibodies: goat anti-mouse conjugated to Alexa Fluor 680 or goat anti-rabbit conjugated to IRDyeTM800. Detection was performed using the LI-COR Odyssey Infrared Imaging System (LI-COR, Inc. Lincoln, NE).

Cellular fractionation

To prepare cytoplasmic and nuclear fractions, cells were washed in ice-cold PBS, scraped, and homogenized in ice-cold hypotonic buffer (10 mM HEPES pH 7.4; 10 mM KCl; 1.5 mM $MgCl_2$; 1 mM EDTA; 1 mM DTT) containing 100 μg/ml PMSF. Cytoplasmic and nuclear fractions were obtained as described [8]. Then the cellular fractions were analyzed by western blotting.

Immunoprecipitation (IP) and coimmunoprecipitation (Co-IP)

Cells were extracted in lysis buffer (50 mM Tris–HCl [pH 8.0], 150 mM NaCl, 1% TritonX-100, 100 μg/ml PMSF) supplemented with protease inhibitor cocktail set I for 60 min on ice. After centrifugation, the protein samples was incubated with 50% slurry of protein A-Sepharose (Roche Applied Sciences, Indianapolis, IN) for 2 h at 4°C, and this mixed samples was centrifuged at 12,000×*g* for 30 s. The supernatant was incubated with the indicated antibody and subsequently with protein A-Sepharose (50% slurry) at 4°C overnight. The 12,000×*g* pellets were washed three times. After adding the same volume of Tris–glycine SDS sample buffer to the sample, these samples were boiled to remove Sepharose beads. After centrifugation at 12,000×*g* for 1 min, the cells lysates and immunoprecipitates were analyzed by western blotting.

Statistical analysis

All data represent at least three independent experiments and are expressed as the mean ± SEM. Differences between groups were compared using Student's *T* tests by *SPSS* software, and significance was accepted at $P < 0.05$.

Results

$A\beta_{25–35}$ induces YAP translocation from cytoplasm to nucleus

To determine the role of YAP during $A\beta_{25–35}$-induced apoptosis, PC12 cells were transfected with GFP–YAP to examine its subcellular localization. We found that YAP translocated from cytoplasm to nucleus in $A\beta_{25–35}$-treated cells, while YAP predominantly localized in cytosol in control cells (Fig. 1a, top). GFP had a diffuse distribution in the whole cell both in the control and $A\beta_{25–35}$-treated cells (Fig. 1a, bottom). These results were further confirmed by the quantitative analysis of nuclear GFP fluorescence emission intensities (Fig. 1a, right panel). In addition, primary neural cells were transfected with GFP–YAP for FRAP experiments. As shown in Fig. 1b (top panel), $A\beta_{25–35}$ accelerated the rate of the nuclear fluorescence recovery comparing with control cells. Moreover, YAP translocated to nucleus at 6 h after treatment with $A\beta_{25–35}$ in primary neural cells (Fig. 1b, bottom). Similar results were obtained in 293T cells (Fig. 1c). Furthermore, IF analysis showed that GFP–YAP translocated to nucleus in differentiated PC12 cells (Fig. 1d).

To examine the endogenous YAP distribution, PC12 cells were treated with $A\beta_{25–35}$ for 24 h. The location of YAP was determined by IF assay. As shown in Fig. 1e, YAP relocalized to the nucleus comparing with control (Fig. 1e). The same results were found by western blotting (Fig. 1f). Furthermore, the phosphorylation of YAP on Y357 was increased under $A\beta_{25–35}$ (Fig. 1g) and $A\beta_{1–42}$ (Fig. 1h) treatment.

Inhibition of YAP attenuates $A\beta_{25–35}$-induced apoptosis

To further determine the functional role of endogenous YAP in PC12 and 293T cells during $A\beta_{25–35}$-induced apoptosis, the expression of YAP was suppressed by shRNA (Fig. 2a). PC12 and 293T cells were transiently transfected with shNC and YAP–shRNA for 24 h, and then treated with $A\beta_{25–35}$ for another 24 h. Approximately 30% of PC12 cells underwent apoptosis within 24 h under $A\beta_{25–35}$ treatment (Fig. 2b). However, the apoptosis rate was decreased to 20.3% in YAP–shRNA-transfected cells ($P < 0.005$) (Figs. 2b). Similar results were obtained in 293T cells (Fig. 2c).

The interaction between YAP and p73 increases during $A\beta_{25–35}$-induced cell apoptosis

PC12 cells were transiently cotransfected with GFP–YAP and YFP–p73, and treated with $A\beta_{25–35}$. The typical time-course images of GFP–YAP, FRET, and the ratio of FRET/

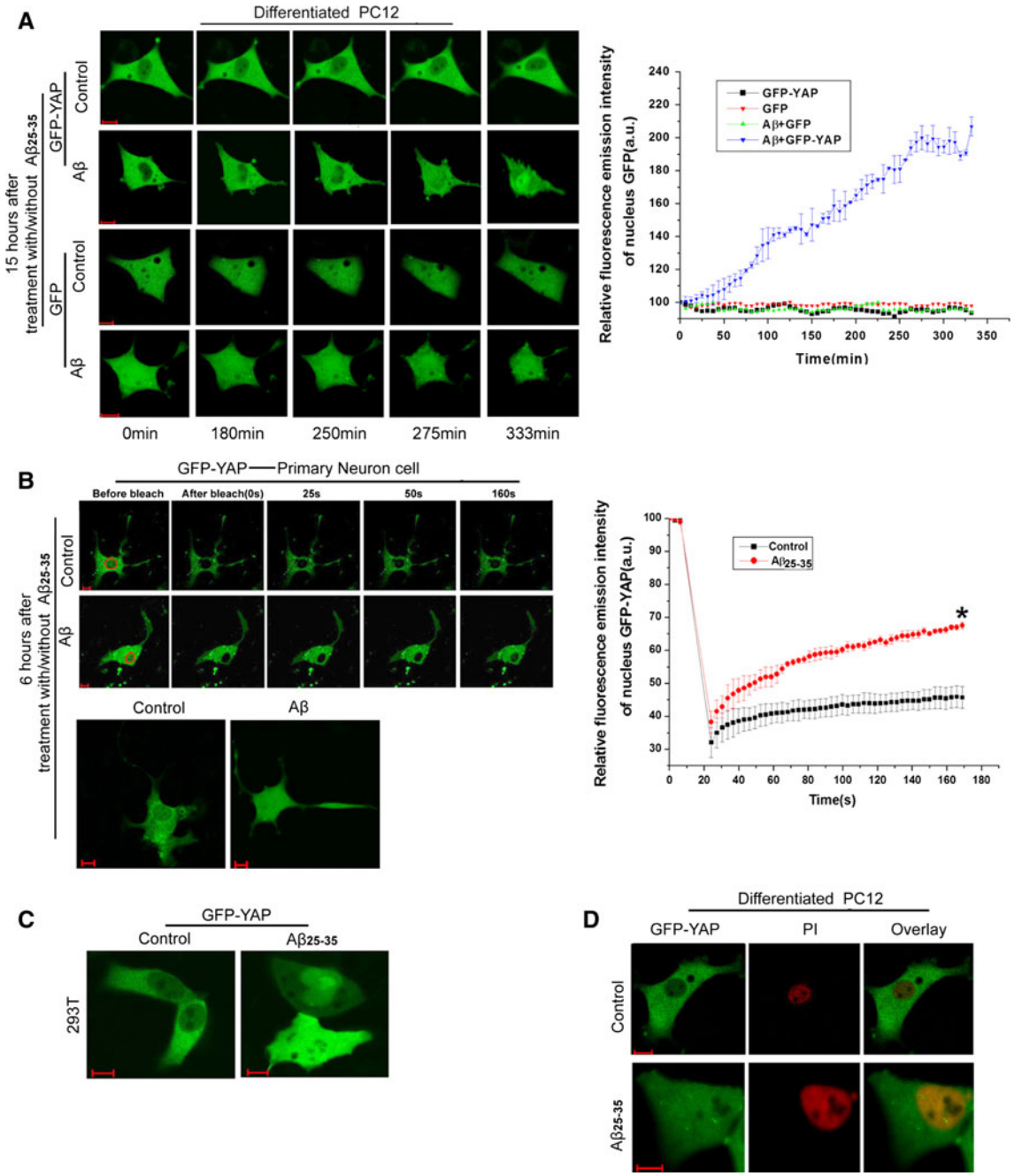


Fig. 1 Induction of YAP translocation from cytoplasm to nucleus in cells exposed to A$\beta_{25–35}$. **a–f** YAP translocated from cytoplasm to nucleus during A$\beta_{25–35}$-induced apoptosis. **a** Differentiated neural PC12 cells transiently transfected with GFP–YAP were treated with A$\beta_{25–35}$ for 15 h, then imaged by confocal microscopy. Quantitative analysis of relative GFP–YAP fluorescence emission intensities of nucleus in PC12 cells was subjected to different treatments. Data represent the mean ± SEM of five independent experiments. **b** Redistribution of YAP in neural cells after A$\beta_{25–35}$ treatment. Primary neural cells were transiently transfected with GFP–YAP and then treated with A$\beta_{25–35}$ for 6 h, FRAP experiments were conducted. Data represent the mean ± SEM of five independent experiments. *$P < 0.0005$, significant from the untreated cells. **c** YAP had redistribution in 293T after treatment with A$\beta_{25–35}$. Representative images were shown. **d** PC12 cells transfected with pGFP–YAP were treated with A$\beta_{25–35}$ and stained nucleus with PI to differentiate from the cytoplasm. Representative images were shown. **e** IF analysis for the localization of endogenous YAP. Statistical analysis of endogenous YAP translocation was conducted and the percentage of cells showing YAP translocation to nuclear was assessed by counting the number of (nuclear + cytoplasmic) cells. Data represented the mean ± SEM and was collected from $n = 150–200$ cells per treatment. *$P < 0.005$, significant from the untreated cells. **f** Western blotting of whole-cell, cytoplamsic and nuclear extracts were designed to examine the level of YAP. Cytoplasmic and nuclear fractions were obtained from the neural PC12 cells after treatment without (−) or with (+) A$\beta_{25–35}$. **g**, **h** Western blotting was performed to detect the phosphorylation of YAP on Y357 at the indicated time after A$\beta_{25–35}$ (**g**) or A$\beta_{1–42}$ (**h**) treatment

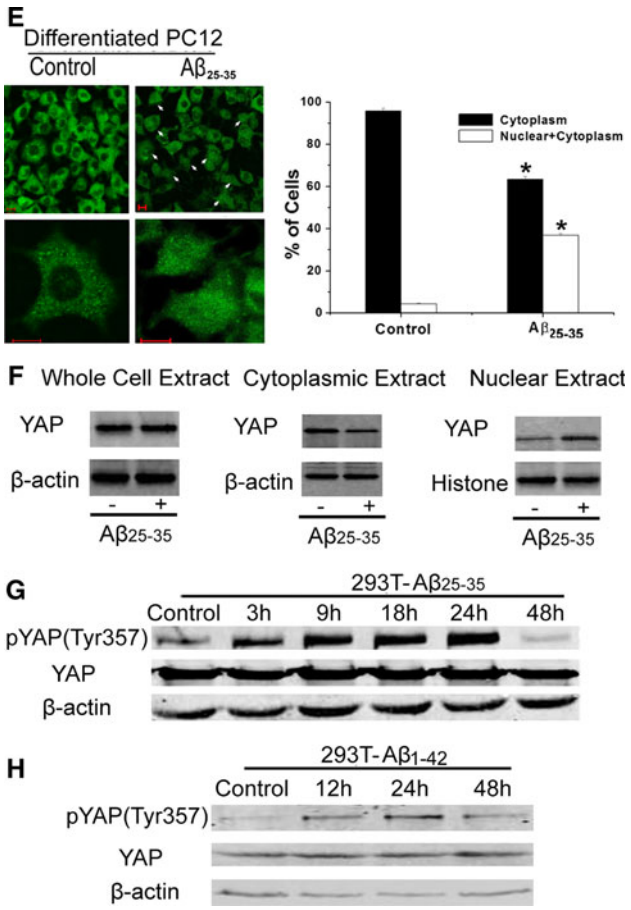


Fig. 1 continued

GFP in cells after A$\beta_{25–35}$ treatment were obtained (Fig. 3a, b). As shown in Fig. 3a, FRET remained unchanged in untreated cells. However, in A$\beta_{25–35}$-treated cells, the emission in the GFP channel decreased. The emission in the FRET channel and the ratio of the FRET/GFP channel increased, indicating that the interaction between YAP and p73 increased (Fig. 3b).

To further confirm the interaction between YAP and p73 during Aβ-induced apoptosis, we transfected YFP or YFP–p73 into 293T cells and examined the YAP–p73 complex by Co-IP. Cells were treated without (−) or with (+) A$\beta_{25–35}$ or A$\beta_{1–42}$. As shown in Fig. 3c and d, we found that YAP–p73 complex increased significantly after treatment with Aβ, while no interaction was detected between YFP control vector and YAP.

Overexpression of YAP accelerates Bax translocation induced by A$\beta_{25–35}$

Bax is an important transcriptional target of p73 [22–24, 27]. To detect Bax redistribution during A$\beta_{25–35}$-induced apoptosis in real-time, PC12 cells were transiently transfected with YFP–Bax or GFP–YAP and treated with A$\beta_{25–35}$. As shown in Fig. 4a, YFP–Bax had a diffuse distribution in the whole cell in the control and GFP–YAP-transfected cells. Under A$\beta_{25–35}$ treatment, YFP–Bax translocated from cytosol to mitochondria (labeled with MitoTracker Red) after 16 h (Fig. 4b, left panels), however, YFP–Bax translocated from cytosol to mitochondria within 13 h in GFP–YAP-transfected cells, indicating that overexpression of GFP–YAP accelerated the translocation of Bax (Fig. 4b, right panels).

Moreover, we used a conformation-specific anti-Bax monoclonal antibody (6A7) which selectively recognized the activated/proapoptotic form of Bax to directly detect Bax activation. PC12 cells were treated with A$\beta_{25–35}$ or A$\beta_{1–42}$, and IF staining were performed to detect active Bax. As shown in Fig. 4c, active Bax was not identified in untreated cells but was readily detectable both in A$\beta_{25–35}$ and A$\beta_{1–42}$-treated cells.

YAP accelerates A$\beta_{25–35}$-induced cell apoptosis

To further determine the involvement of YAP and p73 in A$\beta_{25–35}$-induced cell apoptosis, we examined Bax, the transcriptional target of p73 [16, 22, 23]. We used western blotting to detect the stimulation of endogenous Bax expression by A$\beta_{25–35}$-treatment in transfected cells with GFP–YAP. We found that GFP–YAP overexpression upregulated Bax expression during A$\beta_{25–35}$-induced apoptosis (Fig. 5a). Conversely, depletion of YAP attenuated the Bax expression (Fig. 5b, c), suggesting that A$\beta_{25–35}$-induced expression of endogenous Bax was dependent on YAP.

In addition, we detected the activated Bax in PC12 cells. As shown in Fig. 5c, increased Bax expression was accompanied with Bax activation during A$\beta_{25–35}$-induced apoptosis. Both expression and activation of Bax significantly decreased in YAP–shRNA-transfected cells. These results were further confirmed by IF staining for active Bax using Bax monoclonal antibody (6A7). The active Bax was almost not identified in YAP–shRNA-transfected cells (Fig. 5d). Consistently, knockdown of endogenous YAP significantly decreased Bax activation induced by A$\beta_{1–42}$ (Fig. 5e). We examined Puma expression, another transcriptional target of p73 [24], by western blotting. As shown in Fig. 5f, the expression of Puma was significantly inhibited by YAP–shRNA in response to A$\beta_{25–35}$.

Caspase-3 is activated in Aβ-induced apoptosis in AD [38]. We examined activated caspase-3 by western blotting. As shown in Fig. 5g, the cleaved fragments of caspase-3 increased significantly in GFP–YAP-transfected 293T cells after treatment with A$\beta_{25–35}$ comparing with control vector GFP. Furthermore, we found that the

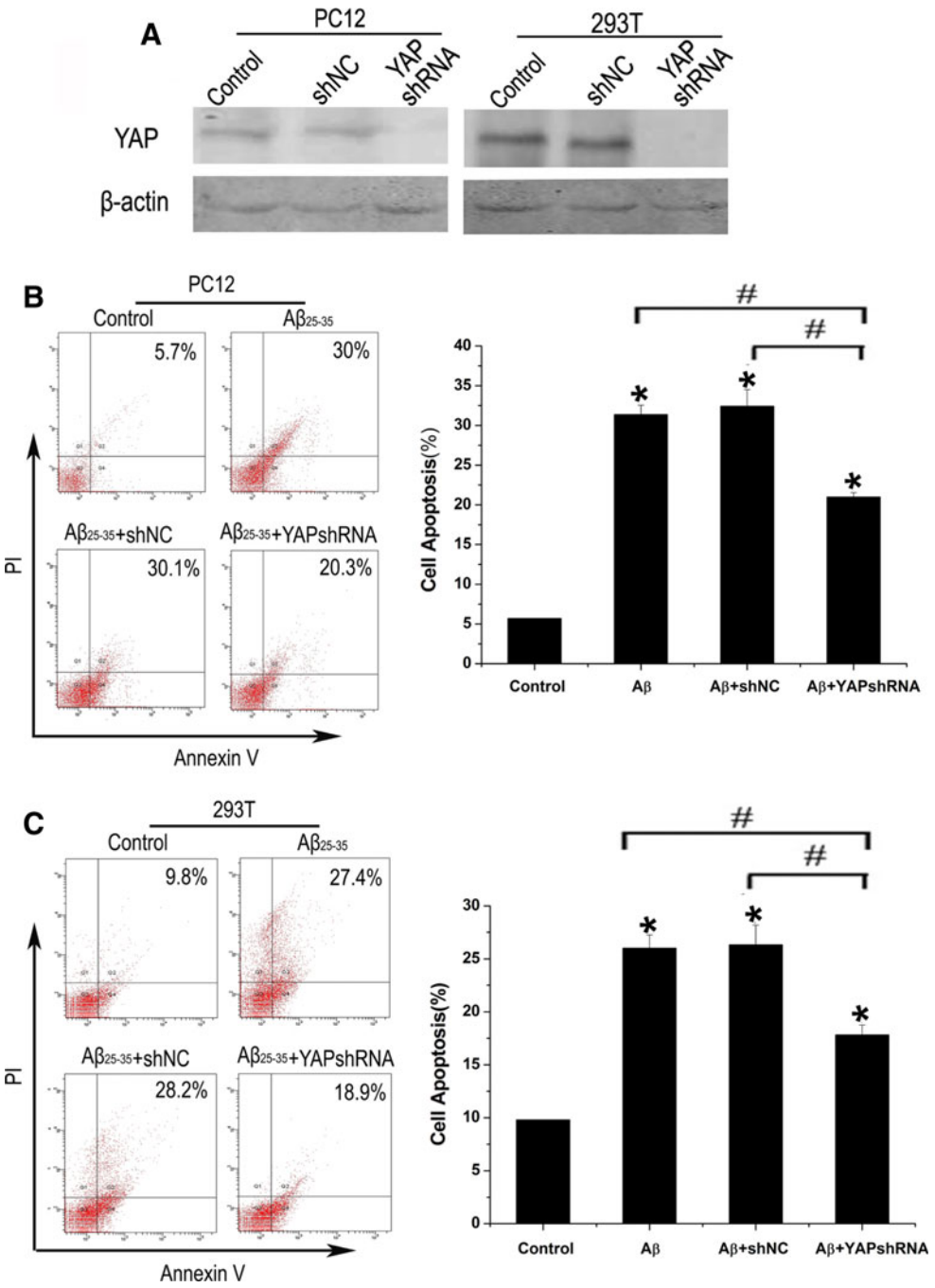


Fig. 2 Inhibition of Aβ_{25-35}-induced apoptosis in YAP–shRNA transfected cells. **a** Western blotting was performed to test the ability of YAP–shRNA to inhibit the expression of YAP. **b** Apoptosis induced by Aβ_{25-35} in PC12 cells decreased following transfection with YAP-directed shRNA. Unfixed cells were stained with Annexin V and propidium iodide (PI) 48 h following transfection with the indicated shRNA, then analyzed by flow cytometry. Numbers refer to the percent Annexin V- and/or PI-positive cells in this representative experiment. Quantitation of apoptotic cells (Annexin V- and/or PI-positive) treated and analyzed. **c** Apoptosis induced by Aβ_{25-35} in 293T cells decreased following transfection with YAP-directed shRNA. Quantitation of apoptotic cells (Annexin V- and/or PI-positive) treated and analyzed as in (**b**). $^{*}P < 0.005$, significant from the untreated cells, $^{\#}P < 0.005$, significant from the two indicated cells. Data represent the mean ± SEM of three independent experiments

cleaved fragments of caspase-3 were down-regulated by knocking down YAP expression (Fig. 5h). These results suggested that overexpression of YAP promoted caspase-3 activation during Aβ_{25-35}-induced apoptosis and caspase-3 activation was in a YAP-dependent manner.

Discussion

Apoptosis, the general neuronal death pathway, has been implicated in the pathogenesis of various neurodegenerative disorders including AD [7–9, 39]. Deposition of Aβ in the brain is considered as a pathological hallmark of AD [40]. Previous studies report that Aβ causes neurotoxicity and neuron cell apoptosis in vivo and in vitro [8–10]. However, the mechanisms of Aβ-induced apoptosis remain to be clarified. Here, we showed that YAP played an important role during Aβ_{25-35}-induced apoptosis. We found that YAP accelerated Aβ_{25-35}-induced apoptosis through translocating from cytoplasm to nucleus, and binding its target transcription factor p73 to stimulate the expression and activation of Bax. Our findings showed that YAP, p73, Bax

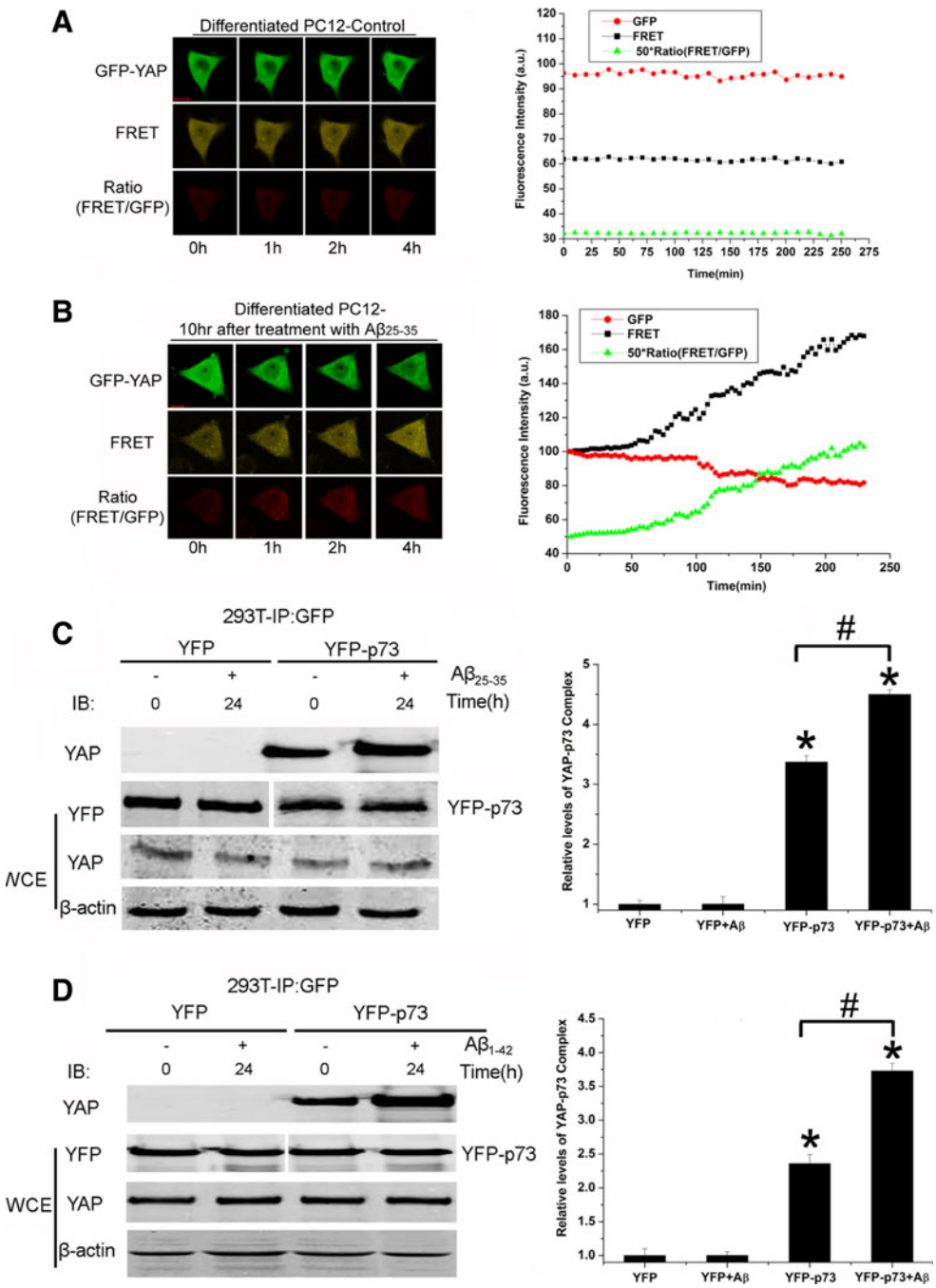


Fig. 3 Interaction between YAP and p73 increases during A$\beta_{25–35}$-induced apoptosis. PC12 cells were transiently cotransfected with YFP–p73 and GFP–YAP and then monitored under different conditions. **a** The time-course of one representative fluorescence image series of GFP, FRET, and FRET/GFP ratio in untreated cells. Quantitative analysis of GFP, FRET intensities, and FRET/GFP ratio corresponding to the images. **b** The time-course of one representative fluorescence image series of GFP, FRET, and FRET/GFP ratio in A$\beta_{25–35}$-treated cells. Quantitative analysis of GFP, FRET intensities, and FRET/GFP ratio corresponding to the images. **c**, **d** Co-IP was performed to detect the YAP–p73 complex at the indicated time after A$\beta_{25–35}$ (**c**) or A$\beta_{1–42}$ (**d**) treatment. After 293T cells transfected with YFP, YFP–p73, CO-IP with an anti-GFP/YFP antibody was used to pull down YFP, YFP–p73, respectively, western blotting was performed to detect YAP in the IP complexes. Similar results were obtained from three independent experiments. Quantitative analysis of YAP–p73 complex levels, $^{*}P < 0.005$, significant from the untreated cells, $^{\#}P < 0.005$, significant from the two indicated cells

and caspase-3 were involved in apoptosis triggered by A$\beta_{25–35}$. To our knowledge, this is the first evidence to demonstrate the proapoptotic role of the FL-YAP in A$\beta_{25–35}$-induced apoptosis, suggesting that YAP/p73/Bax signaling pathway was probably vital for mediating the process of AD.

YAP localizes in both nucleus and cytoplasm, and its activity is tightly regulated [13]. YAP was originally recognized by virtue of its ability to bind to the SH3 domain of Yes at the plasma membrane [14]. Akt phosphorylates YAP on serine 127 and enhances the interaction of YAP with the cytosolic 14-3-3, leading to relatively long-term inactivation of YAP [22]. As a transcriptional coactivator, YAP needs to bind nuclear transcription factors to stimulate gene expression [15]. In this study, we demonstrated for the first time that YAP translocated from cytoplasm to nucleus during A$\beta_{25–35}$-induced apoptosis. Furthermore, we found that knockdown of YAP attenuated the apoptosis

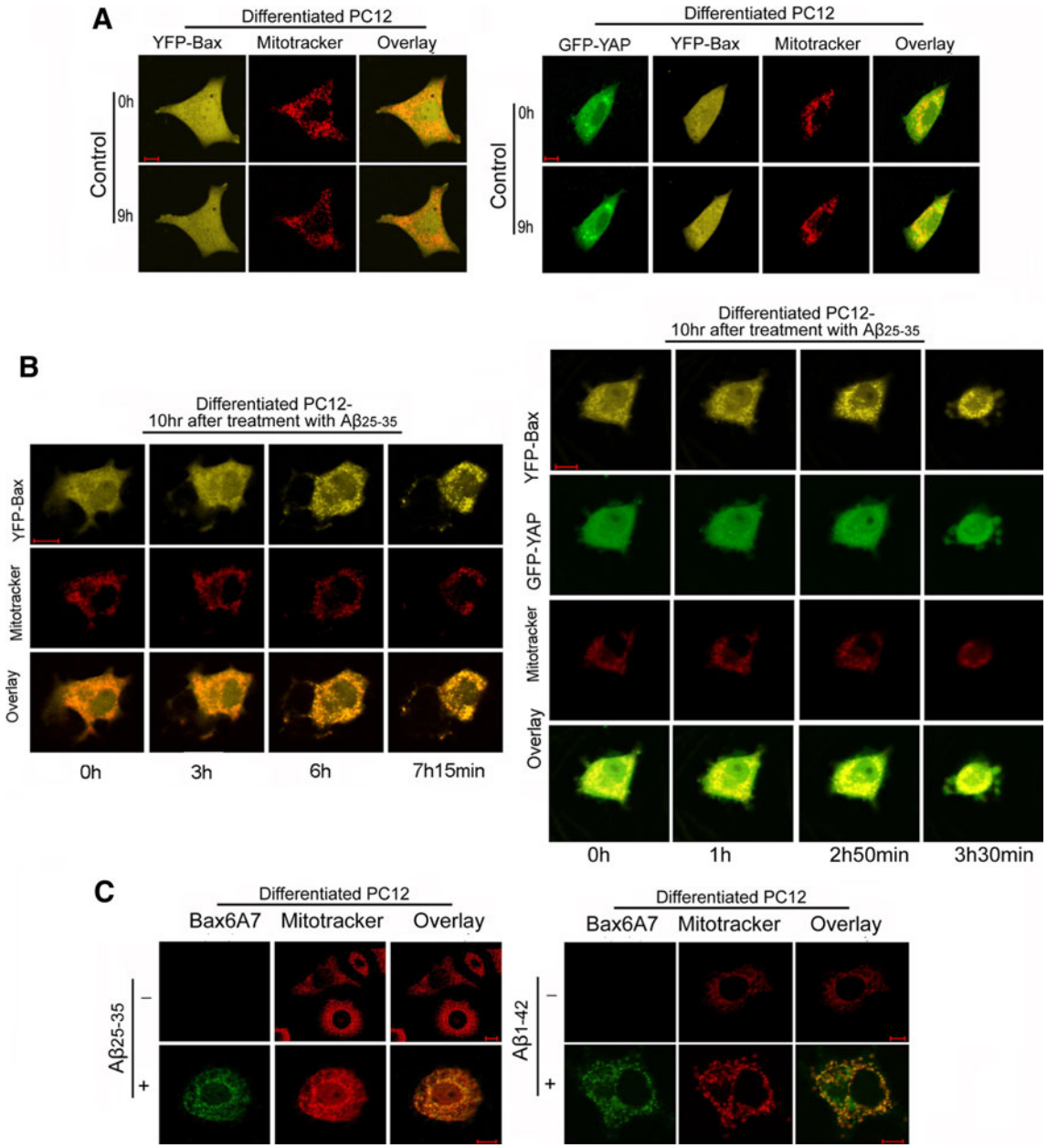


Fig. 4 Overexpression of YAP accelerates Bax translocation induced by A$\beta_{25–35}$. **a** YFP–Bax had a diffuse distribution in the whole cell in untreated cells. PC12 cells were transiently transfected with YFP–Bax. Similar results were obtained from three independent experiments. **b** GFP–YAP overexpression accelerated the Bax translocation from cytosol to mitochondria during A$\beta_{25–35}$-induced apoptosis. PC12 cells were transiently cotransfected with YFP–Bax and GFP–YAP, and then treated with A$\beta_{25–35}$. Similar results were obtained from three independent experiments. **c** Mitochondrial localization of conformationally activated Bax in cells treated by A$\beta_{25–35}$ and A$\beta_{1–42}$. Similar results were obtained from three independent experiments. The mitochondria were stained with MitoTracker Red

induced by Aβ. Thus, our results showed that YAP was required for Aβ-induced apoptosis and that apoptosis induced by Aβ was associated with a nuclear translocation of YAP.

Previous studies have shown that YAP translocates to the nucleus in a p73-dependent manner [27]. p73 activation is connected with the neurotoxicity of Aβ peptide [8, 9]. p73 is a reported YAP target transcription factors and YAP has been shown to positively regulate p73 in promoting apoptosis through mediating the expression of cell death-promoting genes [16, 22, 23, 41]. YAP works on two fronts to increase p73-mediated apoptosis: co-activation of p73 transcription and stabilization of the p73 protein [27]. However, whether YAP interacts with p73 during

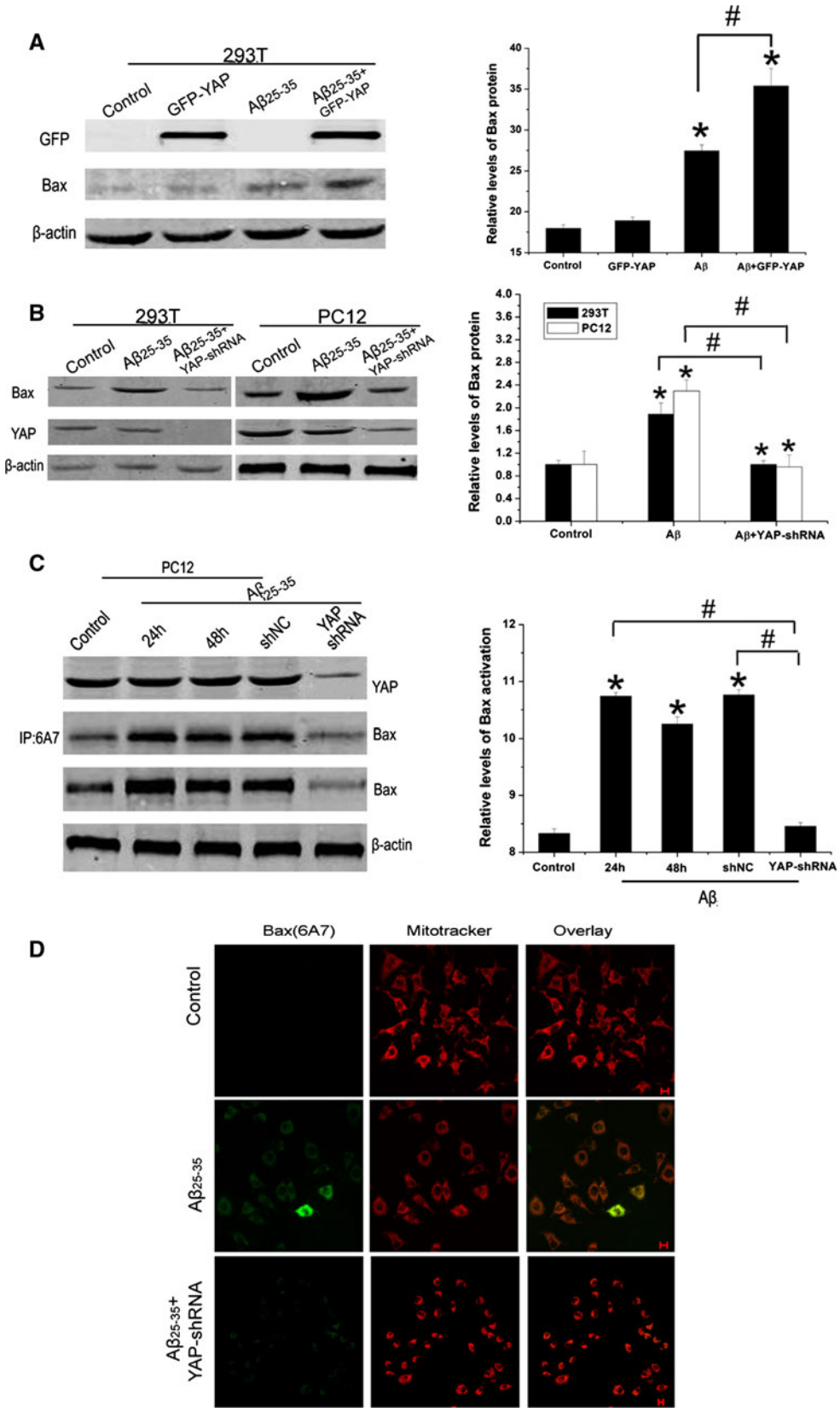
$A\beta_{25-35}$-induced cell apoptosis is unclear. Here, we showed that YAP mediated Aβ-induced apoptosis through interacting with p73 and the interaction increased after $A\beta_{25-35}$ and $A\beta_{1-42}$ treatment. We also found that $A\beta_{25-35}$ and $A\beta_{1-42}$ treatment promoted the phosphorylation of YAP on Y357. These results supported the idea that YAP may be involved in the apoptosis induced by Aβ and that the pro-apoptotic function of YAP was mediated by its functional interaction with p73. Our results were consistent with previous studies showing that tyrosine-phosphorylated YAP is more stable which displays higher affinity to p73 and selectively coactivates p73 proapoptotic target genes, *bax* or/and *puma* [22, 28]. Therefore, our results suggested that the increased interaction between YAP and p73 was crucial for mediating Aβ-induced apoptosis.

Bax is a transcriptional target of p73 [16, 22, 23]. Studies have proved that Aβ upregulates Bax expression [42]. $A\beta_{1-42}$ is selectively cytotoxic to neurons through the Bax-dependent cell death pathway [43]. Bax activates caspases by promoting the release of mitochondrial cytochrome *c*, which forms an apoptosome with a number of other factors including caspases [44]. In AD, apoptotic cell death is associated with the activation of caspases [11, 43, 45]. We showed that $A\beta_{25-35}$-treatment upregulated Bax expression and enhanced Bax activation. Overexpression of YAP further promoted Bax expression and accelerated Bax translocation. More importantly, YAP was essential for the activation of Bax and caspase-3 activation during $A\beta_{25-35}$-induced apoptosis. Hence, our findings suggested that Aβ-induced apoptosis was mediated by the YAP-dependent apoptotic pathway.

How YAP mediates Bax activation is still unknown. YAP has been shown to translocate to the nucleus and associate with p73, resulting in transcription of the proapoptotic target gene *puma* [24]. Melino et al. [46] have reported that the induction of apoptosis dependent on p73 appears to be mediated through the transcriptional activation of Puma, which facilitates mitochondrial translocation of Bax, rather than by direct transcription of Bax itself. Studies by Zhang et al. [35] have shown that Puma promotes Bax translocation by both directly interacting with Bax and by competitive binding to Bcl-X_L during apoptosis. We showed that Aβ-induced apoptosis was correlated with the expression of Bax and Puma. Taken together, our data suggested that YAP accelerated Aβ-induced apoptosis via interaction with p73, leading to directly upregulate Bax expression, and indirectly promote Bax translocation through the transcriptional activation of Puma. Further studies are needed to be conducted on the relationship among YAP, Puma and Bax activation during Aβ-induced apoptosis.

Fig. 5 YAP is essential for expression and activation of Bax, and caspase-3 activation during $A\beta_{25-35}$-induced cell apoptosis. **a–d** The upregulation of Bax expression and its activation during $A\beta_{25-35}$-induced cell apoptosis was dependent on YAP. **a** 293T cells were transiently transfected with GFP–YAP and then treated with $A\beta_{25-35}$. Western blotting was performed to detect the stimulation of endogenous Bax protein expression by $A\beta_{25-35}$-treatment. Similar results were obtained from three independent experiments. Quantitative analysis of Bax protein levels, $^{*}P < 0.005$, significant from the untreated cells, $^{\#}P < 0.005$, significant from the two indicated cells. **b** Knockdown of YAP decreased the level of endogenous Bax protein in $A\beta_{25-35}$-induced apoptosis. 293T cells and PC12 cells transfected with YAP–shRNA were treated with $A\beta_{25-35}$ for 24 h. Western blotting was performed to detect the level of endogenous Bax. Similar results were obtained from three independent experiments. Quantitative analysis of Bax protein levels, $^{*}P < 0.005$, significant from the untreated cells, $^{\#}P < 0.005$, significant from the two indicated cells. **c** IP was used to detect the activated Bax in PC12 cells. Bax6A7 was added to recognize the activated form of Bax and then immunoprecipitates were analyzed by western blotting with anti-Bax-antibody. Similar results were obtained from three independent experiments. Quantitative analysis of activated Bax levels, $^{*}P < 0.005$, significant from the untreated cells, $^{\#}P < 0.005$, significant from the two indicated cells. **d** Mitochondrial localization of conformationally activated Bax in YAP–shRNA transfected PC12 cells after $A\beta_{25-35}$ treatment. Similar results were obtained from three independent experiments. **e** Bax activation was in a YAP-dependent manner during $A\beta_{1-42}$-induced apoptosis. Bax6A7 was added to recognize the activated form of Bax and then immunoprecipitates were analyzed by western blotting with anti-Bax-antibody. Similar results were obtained from three independent experiments. Quantitative analysis of activated Bax levels, $^{*}P < 0.005$, significant from the untreated cells, $^{\#}P < 0.005$, significant from the two indicated cells. **f** Western blotting was performed to detect the Puma expression during $A\beta_{25-35}$-induced apoptosis. Similar results were obtained from three independent experiments. Quantitative analysis of Puma protein levels, $^{*}P < 0.005$, significant from the untreated cells, $^{\#}P < 0.005$, significant from the two indicated cells. **g**, **h** Caspase-3 activation was dependent on YAP during $A\beta_{25-35}$-induced apoptosis. **g** Caspase-3 activation increased significantly during $A\beta_{25-35}$-induced 293T cells apoptosis when GFP–YAP overexpression. Similar results were obtained from three independent experiments. **h** Caspase-3 activation was down-regulated during $A\beta_{25-35}$-induced apoptosis when YAP–shRNA transfected PC12 cells. Similar results were obtained from three independent experiments. Quantitative analysis of caspase-3 levels, $^{*}P < 0.005$, significant from the untreated cells, $^{\#}P < 0.005$, significant from the two indicated cells

In summary, we found that YAP accelerated $A\beta_{25-35}$-induced apoptosis through translocating from cytoplasm to nucleus to bind its target transcription factor p73 to stimulate *bax* gene expression and Bax activation. This raises the intriguing possibility that YAP/p73/Bax pathway plays a critical role in regulating neuronal cell apoptosis in AD. These results directly point to a potential therapeutic strategy for the treatment of AD through inhibiting YAP/p73/Bax pathway. Further studies are in progress to illustrate the mechanisms of AD and search for an effective therapeutic strategy.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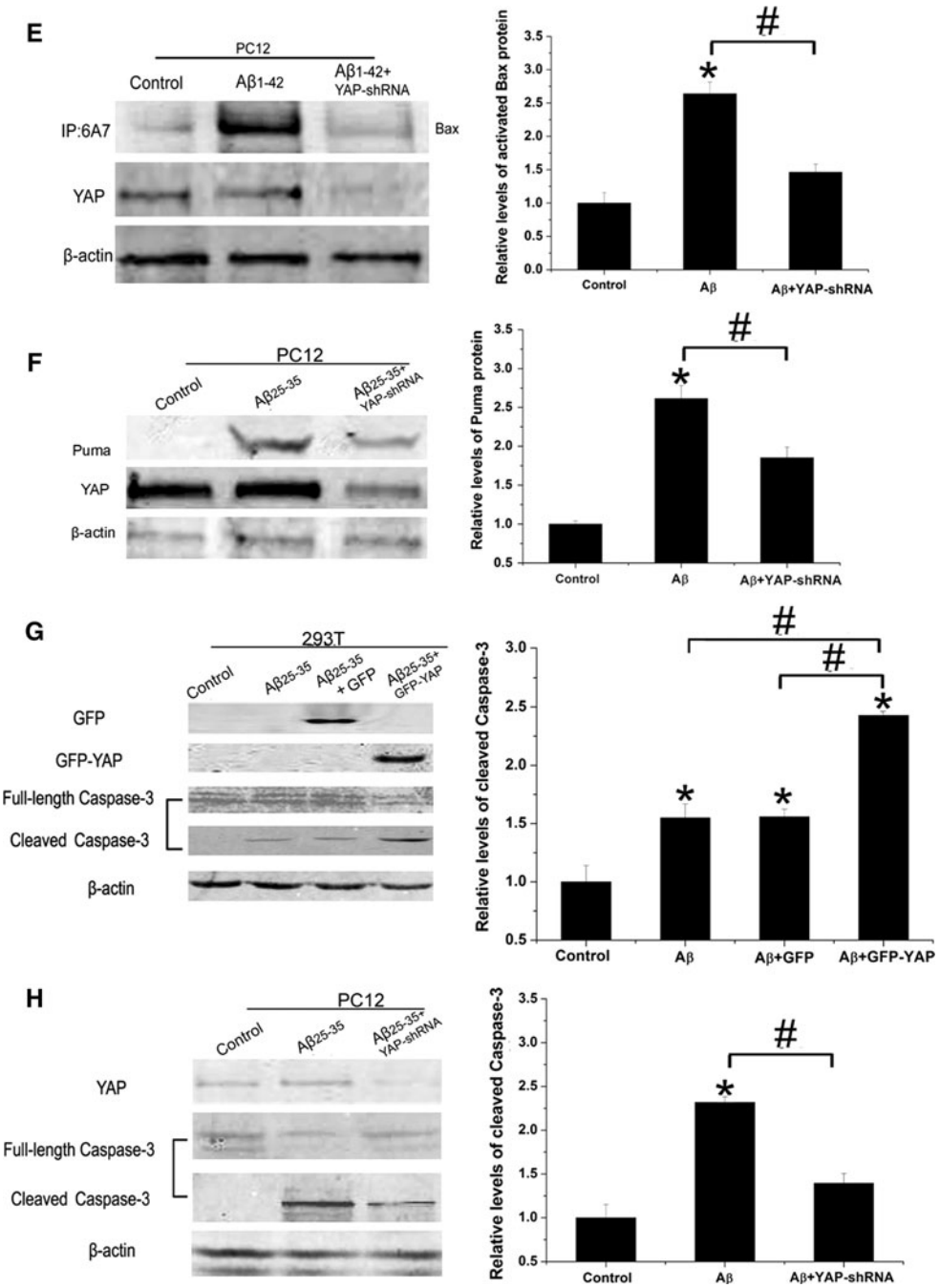

Fig. 5 continued

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