



## ARTICLE

# Cathepsin L induced PC-12 cell apoptosis via activation of B-Myb and regulation of cell cycle proteins

Xiao Shen<sup>1</sup>, Yi-fan Zhao<sup>1</sup>, Shu-qing Xu<sup>1,2</sup>, Long Wang<sup>1</sup>, Hui-min Cao<sup>1</sup>, Ying Cao<sup>1</sup>, Ying Zhu<sup>1</sup>, Yan Wang<sup>1</sup> and Zhong-qin Liang<sup>1</sup>

Cathepsin L (CTSL), a cysteine protease, is responsible for the degradation of a variety of proteins. It is known to participate in neuronal apoptosis associated with abnormal cell cycle. However, the mechanisms underlying CTSL-induced cell apoptosis remain largely unclear. We reported here that rotenone caused an activation of CTSL expression in PC-12 cells, while knockdown of CTSL by small interfering RNAs or its inhibitor reduced the rotenone-induced cell cycle arrest and apoptosis. Moreover, elevation of CTSL and increased-apoptosis were accompanied by induction of B-Myb, a crucial cell cycle regulator. We found that B-Myb was increased in rotenone-treated PC-12 cells and knockdown of B-Myb ameliorated rotenone-stimulated cell apoptosis. Further analysis demonstrated that CTSL influenced the expression of B-Myb as suppression of CTSL activity led to a decreased B-Myb expression, whereas overexpression of CTSL resulted in B-Myb induction. Reduction of B-Myb in CTSL-overexpressing cells revealed that regulation of cell cycle-related proteins, including cyclin A and cyclin B1, through CTSL was mediated by the transcription factor B-Myb. In addition, we demonstrated that the B-Myb target, Bim, and its regulator, Egr-1, which was also associated with CTSL closely, were both involved in rotenone-induced apoptosis in PC-12 cells. Our data not only revealed the role of CTSL in rotenone-induced neuronal apoptosis, but also indicated the involvement of B-Myb in CTSL-related cell cycle regulation.

**Keywords:** Cathepsin L; B-Myb; cyclin B1; cyclin A; cell cycle; apoptosis

*Acta Pharmacologica Sinica* (2019) 40:1394–1403; <https://doi.org/10.1038/s41401-019-0286-9>

## INTRODUCTION

Cathepsin L (CTSL), a member of the papain superfamily, was initially isolated from conditioned medium of apoptotic endothelial cells. It is widely expressed in many cell types. CTSL is one of the major lysosomal proteases responsible for antigen processing and lysosomal protein degradation [1, 2]. Previous studies found that CTSL was highly expressed in many diseased cells and supposedly associated with the disease occurrence. Moreover, the distribution of lysosomal proteases was altered in brains with Alzheimer's disease, suggesting the primary and/or secondary involvement of the lysosomal proteases in the pathological process of Alzheimer's disease [3]. Furthermore, our lab also indicated that CTSL may be involved in 6-OHDA-induced dopamine neuron death [4–6].

The main pathologic feature of neurodegenerative diseases is the loss of neurons through neuronal apoptosis [7–10]. The important molecular mechanism of neurodegenerative diseases is DNA damage-induced abnormal regulation of neuronal cell cycle checkpoints, followed by the denaturation and loss of dopaminergic neurons [11, 12]. Currently, the mechanisms involved in abnormal cell cycle regulation and degenerative process are complex and not fully understood. Cytotoxic and/or DNA-damaging agents were shown to induce cell cycle arrest resulting in apoptosis [13]. Our lab had previously found that the inhibition of CTSL nuclear translocation may contribute to neuroprotective

effects [14]. However, the CTSL-specific regulatory mechanisms through which it influences cell cycle progression and apoptosis remain unclear and require further investigation.

B-Myb, a eukaryotic transcription factor, is involved in the regulation of multiple physiological functions. It is acknowledged that B-Myb regulates cell cycle progression, cell differentiation, cell senescence, and apoptosis [15]. The level of B-Myb protein remains stable during the whole cell cycle and corresponds consistently with its mRNA level [16]. Skrzypczak M. et al. demonstrated a potential link between CTSL2 (also known as CTSV) and B-Myb [17]. CTSL, being a structurally homologous to CTSL2, shares 78% of its amino acid sequences with CTSL2 [18, 19]. However, there is at present no clear understanding of how CTSL interacts with B-Myb. Interestingly, several reports suggested that B-Myb regulated neuronal apoptosis through the expression of Bim in PC-12 cells [20, 21]. Bim, a protein activated upstream of the mitochondrial proapoptotic pathway, is a key regulator of the neuronal apoptosis. Overexpression of Bim induced neuronal apoptosis, whereas silencing of Bim protected neurons [22, 23], which suggested that it may be a potential pathway in neuronal apoptosis.

There was no conclusive evidence for a functional relationship between CTSL and B-Myb, or for the regulatory mechanisms by which they promote apoptosis. In this study, we found that both CTSL and B-Myb were involved in the regulation of cell cycle progression and apoptosis. Furthermore, CTSL upregulated cell

<sup>1</sup>Department of Pharmacology, College of Pharmaceutical Sciences, Soochow University, 215123 Suzhou, China and <sup>2</sup>Department of Pharmacy, The First People's Hospital of Wujiang, 215000 Suzhou, China

Correspondence: Yan Wang ([yanwang@suda.edu.cn](mailto:yanwang@suda.edu.cn)) or Zhong-qin Liang ([liangzhongqin@suda.edu.cn](mailto:liangzhongqin@suda.edu.cn))

These authors contributed equally: Xiao Shen, Yi-fan Zhao

Received: 16 February 2019 Accepted: 4 July 2019

Published online: 23 August 2019

cycle-related proteins and exerted its proapoptotic effect via B-Myb. Overall, this study has identified a proapoptotic CTSL/B-Myb signaling mechanism underlying rotenone-induced PC-12 cell apoptosis.

## MATERIALS AND METHODS

### Antibodies, reagents, and cell culture

The following antibodies were used: CTSL (Abcam, Massachusetts, US); Bim (Cell Signaling Technology, Massachusetts, US); B-Myb, GAPDH (Abcam, Massachusetts, US); Egr-1, cyclin A, cyclin B1,  $\beta$ -actin antibodies were bought from Santa Cruz Biotechnology; Alexa Fluor 594, Alexa Fluor 488 (Molecular probes, Australia).

PC-12 cells were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China), and were maintained in high-glucose DMEM (Hyclone, Los Angeles, USA) supplemented with 10 % (v/v) fetal bovine serum (Gibco, United States, California), at 37 °C in a humidified incubator with 5 % CO<sub>2</sub>. Cells in the mid-logarithmic phase were used for the experiments.

Rotenone (Sigma, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma, USA) and the final concentration was 1 mM. It was stored at -20 °C. Selected CTSL inhibitor, Z-Phe-Tyr (t-Bu)-diazomethylketone (Z-FY-DMK), was from Calbiochem company and dissolved in dimethylsulfoxide (DMSO, Sigma, USA) to 20 mM. It was stored at -30 °C. In each experiment, rotenone and Z-FY-DMK were diluted with the cell culture medium to obtain the desired final concentrations. Cells were treated with 0.5  $\mu$ M Rotenone and/or 10  $\mu$ M Z-FY-DMK in corresponding experiments.

### The sequence of siRNAs

CTSL siRNAs sequences and negative control siRNA sequence:

CTSL siRNA a: 5'-GGACAGAUGUCCUUAAGATT-3'

CTSL siRNA b: 5'-GGACUGUUCUCACGAUCAATT-3'

CTSL siRNA c: 5'-GGAGUCUUAUCCCUAUGAATT-3'

CTSL siRNA d: 5'-CAGCUAUCCUUAUCGUGAAUTT-3'

Negative control siRNA: 5'-UUCUCCGAACGUGUCACGUTT-3'

B-Myb siRNAs sequences and negative control siRNA sequence:

B-Myb siRNA 1: 5'-CUACUUCUAAGGAACAATT-3'

B-Myb siRNA 2: 5'-GACUGAGUAUCGCCUUGAUTT-3'

B-Myb siRNA 3: 5'-CACCAGAAGUAUCCAUCGUTT-3'

Negative control siRNA: 5'-UUCUCCGAACGUGUCACGUTT-3'

Egr-1 siRNAs sequences and negative control siRNA sequence:

Egr-1 siRNA: 5'-GGACUUAAGGCUCUUAUUTT-3'

Negative control siRNA: 5'-UUCUCCGAACGUGUCACGUTT-3'

Bim siRNAs sequences and negative control siRNA sequence:

Bim siRNA a: 5'-CAACCAUUAUCUCAGUGCATT-3'

Bim siRNA b: 5'-GACAGAGAAGGUGGACAAUUGTT-3'

Bim siRNA c: 5'-CCCAUGAGUUGUGACAAGUTT-3'

Bim siRNA d: 5'-CACCCGCAAUGGUUAUCUTT-3'

Negative control siRNA: 5'-UUCUCCGAACGUGUCACGUTT-3'

### Hoechst 33258 staining

Cells were seeded in 24-well plates and grown in high-glucose DMEM supplemented with 10% fetal bovine serum. Cells were transfected with corresponding small interfering RNAs (siRNAs) and treated with rotenone 48 h later. For another 24 h, treated cells were analyzed using a Hoechst 33258 staining kit (Beyotime, NanTong, Jiangsu). Staining was performed in accordance with the manufacturer's protocol. Cells were washed with PBS, and then treated with a fixing solution for 10 min at room temperature. Cells were then stained with 200  $\mu$ L Hoechst 33258 staining for 15 min at room temperature after washed with PBS. Nuclear morphological changes in the cells were observed and captured using an inverted fluorescence microscope.

### JC-1 assay

The depolarization of the mitochondrial membrane potential (MMP) was quantified using the Mitochondrial membrane

potential assay kit with JC-1 (Beyotime, NanTong, Jiangsu). Healthy polarized cell mitochondria form JC-1 aggregates (red fluorescence), whereas dead cells form JC-1 monomers (green fluorescence). Therefore the red/green ratio can be used as a sensitive measure of changes in MMP. A total of  $5 \times 10^5$  cells per well were seeded in a six-well culture plate, supplemented with serum medium, and incubated at 37 °C for 24 h. After the specified incubation period, the cells were exposed to different rotenone concentrations (0, 1, 2, 4  $\mu$ M). After another 24 h, the medium was removed, and cells were trypsinized, harvested, and directly resuspended with 500  $\mu$ L of JC-1 working solution. Cells were then incubated at 37 °C for 20 min. After incubation, the cells were washed twice and resuspended using the JC-1 assay buffer followed by centrifuge. The performance was repeated once and flow cytometry was used to detect red and green fluorescence.

### Western blot analysis

Cells were washed twice with PBS and then harvested with lysis buffer using a plastic scraper. Afterward, the cells were homogenized in lysis buffer. Proteins of the lysates were quantified using the BCATM Protein Assay Kit (Thermo Scientific, Massachusetts, US). The lysates were loaded and separated on SDS-PAGE gels, and the proteins were transferred onto nitrocellulose blotting membranes. The membranes were blocked with 5% BSA for 1 h and then incubated with primary antibodies overnight. After washing three times with TBST buffer, the membranes were incubated with rabbit or mouse secondary antibodies for 1 h in the dark. Immunoblots were detected by the Odyssey Infrared Imaging System (Li-COR Biosciences, NE, USA).

### Transfection

CTSL siRNA, B-Myb siRNA, Egr-1 siRNA, Bim siRNA, and control siRNA were purchased from GenePharma. Before transfection, PC-12 cells were seeded in six-well plates and grown in high-glucose DMEM supplemented with 10% fetal bovine serum for 24 h in a humidified 37 °C incubator with 5% CO<sub>2</sub>, then siRNA plasmid DNA was transfected into the cells by using an siRNA transfection reagent kit (Invitrogen, California, USA) in accordance with the manufacturer's protocol.

### Lentivirus infection

CTSL lentivirus was synthesized by Genechem (Shanghai, China). Cells were seeded in six-well plates and grown in high-glucose DMEM supplemented with 10% fetal bovine serum. After 24 h, cells were treated with  $2 \times 10^7$  titration units of lentivirus packaging. After 6–8 h, cells were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum.

### Immunofluorescence staining

Cells were seeded in 24-well plates firstly and the next day treated as required in the experiment. Cells were fixed by incubating in 4% paraformaldehyde solution for 10 min at room temperature and then incubated with primary antibodies against CTSL (1:200) and B-Myb (1:200) at 4 °C overnight. Primary antibodies were diluted in PBS-plus (0.01 M PBS + 0.1% Triton + 1% horse serum). The cells were then washed three times with PBS and incubated with appropriate biotinylated secondary antibodies for an hour and conjugated to Alexa Fluor 594 (Molecular Probes, 1:200) or 488 (Molecular Probes, 1:200) for an hour at room temperature. Cells were incubated with DAPI for 15 min. Fluorescence was observed under 630 $\times$  on an inverted microscopy (Carl ZEISS, LSM 710, Germany). Images were captured with a fluorescence microscopy.

### Cell viability assay

Cells were seeded in 96-well plates. Culture medium containing different concentrations of rotenone (0, 0.25, 0.5, and 1  $\mu$ M) and Z-FY-DMK was added to the medium 30 min before adding drug,

and cells were incubated at 37 °C. After 24 h, 10 µL CCK-8 reagent was added (Biosharp, Anhui, China) per well and incubated with cells at 37 °C with 5% CO<sub>2</sub> for 1–4 h. Optical density (OD) at 450 nm in each well was determined by using a microplate reader. The percentage of cell viability was calculated as follows: cell viability (%) = (OD of experimental well/OD of positive control well) × 100%.

#### Flow cytometry analysis of cell cycle distribution

The phase distribution of the cell cycle was determined using an Annexin V-fluorescein isothiocyanate (FITC)/PI Apoptosis Detection Kit (KeyGen, Nanjing, China). Staining was performed in accordance with the manufacturer's protocol. The cells (2 × 10<sup>5</sup> cells per well) were seeded in six-well plates and pretreated with rotenone and/or Z-FY-DMK for 24 h. Then, the cells were harvested and fixed in precooled 70% ethanol at 4 °C for at least 2 h. Then, the samples were centrifuged, resuspended in PBS with 20 µg/mL RNase A at 37 °C for an hour, and stained with 50 µg/mL of PI in the dark at 4 °C for 30 min. The percentage of apoptotic cells was measured using flow cytometer.

#### Flow cytometry analysis of cell apoptosis

The cell apoptosis was determined using an Annexin V-FITC/PI Apoptosis Detection Kit (KeyGen, Nanjing, China) and an Annexin V-PE/7-AAD Apoptosis Detection Kit (KeyGen, Nanjing, China). Staining was performed in accordance with the manufacturer's protocol. The cells were seeded in six-well plates and transfected with CTSL or B-Myb siRNAs for 48 h. Then, rotenone-treated cells for 24 h. Cells were harvested and stained as the corresponding instructions. The percentage of apoptotic cells was measured by flow cytometry.

#### Statistical analysis

At least three independent experiments were performed. All quantitative data were presented as mean ± SD. Multiple group comparisons were analyzed using a two-way ANOVA and two group comparisons were analyzed using Student's *t* test. Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software). Significance levels were interpreted at \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001.

## RESULTS

CTSL inhibition alleviated rotenone-induced apoptosis and cell cycle arrest in PC-12 cells

As an inhibitor of the mitochondrial respiratory chain, rotenone can induce apoptosis [24, 25]. Our lab previously found that CTSL was highly expressed in the rat model of Parkinson's disease (PD) abnormally and caused dopaminergic (DA) neuron loss [14]. To determine the role of CTSL in rotenone-induced cell apoptosis, we first examined the effects of rotenone on PC-12 cells. Hoechst 33258 staining and application of JC-1 proved that there was an increase in apoptotic cell numbers and the cytotoxicity of rotenone (Fig. 1a, b). Next result showed that rotenone treatment led to a time-dependent increase of CTSL expression (Fig. 1c). Immunofluorescence analysis confirmed the elevated level of CTSL (Fig. 1d). These results demonstrated that rotenone could enhance the expression of CTSL.

To verify involvement of CTSL in rotenone-induced apoptosis, cell viability was assessed in the presence of selected CTSL inhibitor Z-FY-DMK (10 µM). We found cell viability declined with rotenone, whereas it was slightly reversed by Z-FY-DMK (Fig. 1e). The data indicated that CTSL inhibition was associated with suppressed rotenone-induced cytotoxicity. CTSL siRNA was used to decrease CTSL expression in PC-12 cells for further study. CTSL siRNA b showed efficient interference (Fig. 2d). The transfected cells were treated with rotenone and examined for cell apoptosis. Fig. 2a demonstrated that silencing of CTSL expression partially

blocked the rotenone-induced apoptosis compared with the nontreated control (Fig. 2a). Hoechst 33258 staining analysis was consistent with the experiments demonstrating that the inactivation of CTSL either by siRNA silencing or treatment with selective CTSL inhibitor blocked apoptosis in PC-12 cells (Fig. 2b, c). Alternatively, CTSL-overexpressing cells displayed higher level of apoptosis (Fig. 5b, c).

It is well established that apoptosis is often initiated by the loss of cell cycle control. As CTSL was shown to be involved in rotenone-induced cell apoptosis, we suspected whether there was a potential link between the expression of CTSL and cell cycle regulation. Cells were treated with rotenone or Z-FY-DMK (10 µM) for specific periods of time and flow cytometry was used to determine cell cycle distribution. Our data showed that G2/M phase arrest was induced by rotenone, but was not reversed by CTSL inhibitor. The number of cells at S phase was gradually increased in a time-dependent manner. Z-FY-DMK obviously prevented S phase arrest, especially after 48 h of treatment (Fig. 2e). The results suggested involvement of CTSL in cell cycle regulation.

Above data indicated that decreased-CTSL partly reduced rotenone-induced cell apoptosis and cell cycle arrest in PC-12 cells.

B-Myb mediated rotenone-induced apoptosis and regulated expression of cell cycle-related proteins

To explore the role of B-Myb in rotenone-induced apoptosis, we reduced the expression of B-Myb through transfecting siRNA and then assessed apoptosis by flow cytometry and Hoechst 33258 staining. We chose siRNA 3 for transfections (Fig. 3b). Figure 3a, c showed that B-Myb interference reduced apoptosis compared with cells only treated with rotenone (Fig. 3a, c).

It has been discovered that B-Myb regulates expression of cell cycle proteins and influences cell cycle progression. We further tested the expression level of B-Myb and levels of associated cell cycle proteins in rotenone-treated PC-12 cells. Western blot analysis showed that proteins of interest were increased, including CTSL, B-Myb, cyclin A, and cyclin B1 (Fig. 3d). It proved that rotenone could induce the expression of these proteins and furthermore, they were increased at different time points for CTSL was earlier than others. Then we used B-Myb siRNA and the data indicated that both cyclin A and cyclin B1 were decreased, whereas CTSL level was not significantly altered (Fig. 3e).

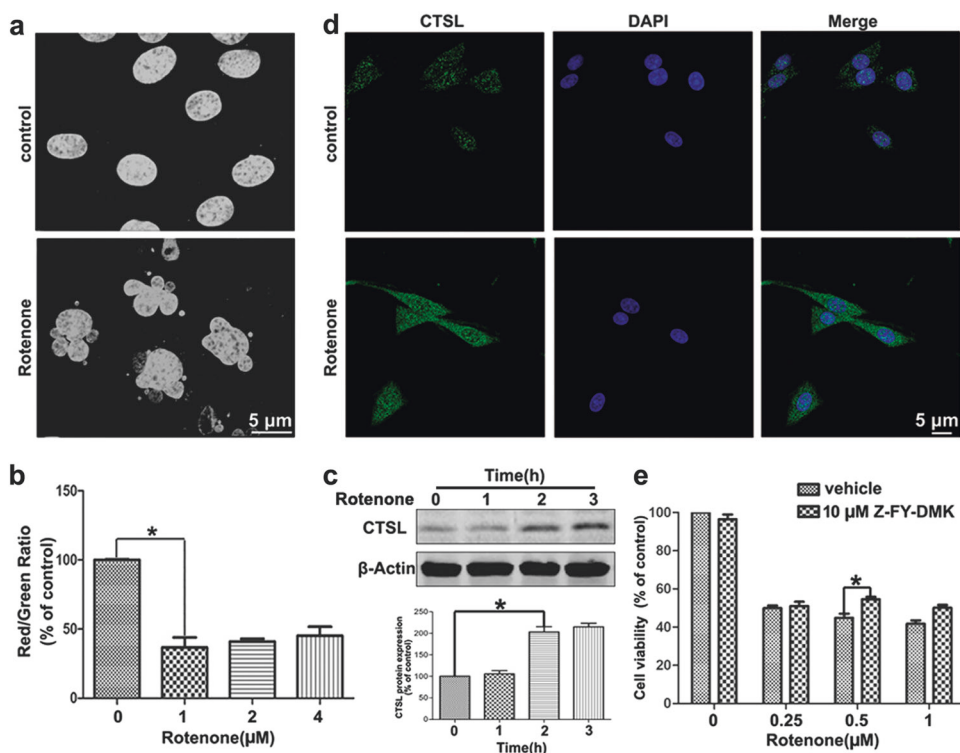
Our findings demonstrated that B-Myb was not only involved in rotenone-induced apoptosis, but also regulating cell cycle proteins.

CTSL had effect on B-Myb and further influenced cell cycle-related proteins via B-Myb

We found that both CTSL and B-Myb were involved in regulation of rotenone-induced apoptosis and cell cycle progression. Immunofluorescence experimental data revealed that CTSL and B-Myb levels were elevated in rotenone-treated cells (Fig. 4a). Moreover, we found that CTSL and B-Myb were co-expressed together in cell nuclei. However, CTSL level was not significantly altered when B-Myb was silenced (Fig. 3e). According to our observations, we aimed to explore whether CTSL can regulate B-Myb level in rotenone-treated PC-12 cells.

We performed experiments that overexpressed CTSL as well as decreased CTSL expression. The elevation of B-Myb level has been observed in PC-12 cells that overexpressed CTSL (Fig. 4b), whereas reduced level of CTSL decreased B-Myb expression (Fig. 4c). The effect of CTSL on the B-Myb level has been confirmed in experiments with the application of CTSL inhibitor (Fig. 4d).

Considering that CTSL affected B-Myb level and B-Myb regulated expression of cell cycle-related proteins, we questioned whether CTSL affected cell cycle proteins through B-Myb to promote cell apoptosis. The level of B-Myb was silenced in cells



**Fig. 1** CTSL was elevated in rotenone-treated PC-12 cells. **a** PC-12 cells were treated with 0.5 μM rotenone for 24 h and Hoechst 33258 staining was conducted. **b** PC-12 cells were treated with different concentrations (0, 1, 2, 4 μM) of rotenone and 24 h later analyzed by the Mitochondrial membrane potential assay kit with JC-1. Data were represented as red/green ratio (% of control). **c** PC-12 cells were treated with 0.5 μM rotenone and harvested at indicated time points. The expression level of CTSL was analyzed by Western blot, β-actin was used as an internal control. **d** PC-12 cells were treated with 0.5 μM rotenone for 3 h and stained for CTSL (Green) and DAPI (Blue), then analyzed by fluorescence microscopy. **e** PC-12 cells were treated with different concentrations (0, 0.25, 0.5, 1 μM) of rotenone with or without Z-FY-DMK (10 μM). Cell viability assay assessed cell viability 24 h later. At least three independent experiments were performed. \**P* < 0.05 compared with control

with overexpressed-CTSL. The collected results showed increased expression of cyclins, including cyclin A and cyclin B1, in cells with overexpressed-CTSL (Fig. 5a, panel 2). Alternatively, those proteins were decreased in cells with reduced level of B-Myb (Fig. 5a, panels 3 and 4). Hoechst 33258 staining and flow cytometry analysis of apoptosis confirmed the observed data (Fig. 5b, c). Apoptosis was increased when CTSL was overexpressed and it declined as B-Myb protein was interfered.

The findings suggested a key role of B-Myb in mediating CTSL effects on the regulation of cell cycle-related proteins and thus influenced cell apoptosis.

Egr-1 was associated with CTSL expression and Bim was involved in rotenone-induced apoptosis

We used Western blot to determine the expression level of Egr-1 in rotenone-treated PC-12 cells and found that Egr-1 was increased obviously after an hour (Fig. 6a). Consequently, we reduced CTSL expression using siRNA and measured Egr-1 level. Our data showed that CTSL reduction had no significant effect on the expression level of Egr-1 (Fig. 6b), whereas CTSL expression declined after Egr-1 was reduced (Fig. 6c). The observations suggested that CTSL expression was regulated by Egr-1.

It has been revealed in many apoptosis models that proapoptotic protein Bim is the downstream target of B-Myb. Reduced level of Bim suppressed apoptosis obviously in central and peripheral neurons. Moreover, Egr-1 affected cell apoptosis mainly via increased expression of Bim. To confirm the role of Bim in rotenone-induced cell apoptosis, we conducted the following experiments. PC-12 cells were treated with rotenone for different times and the data indicated increased expression of Bim (Fig. 6d).

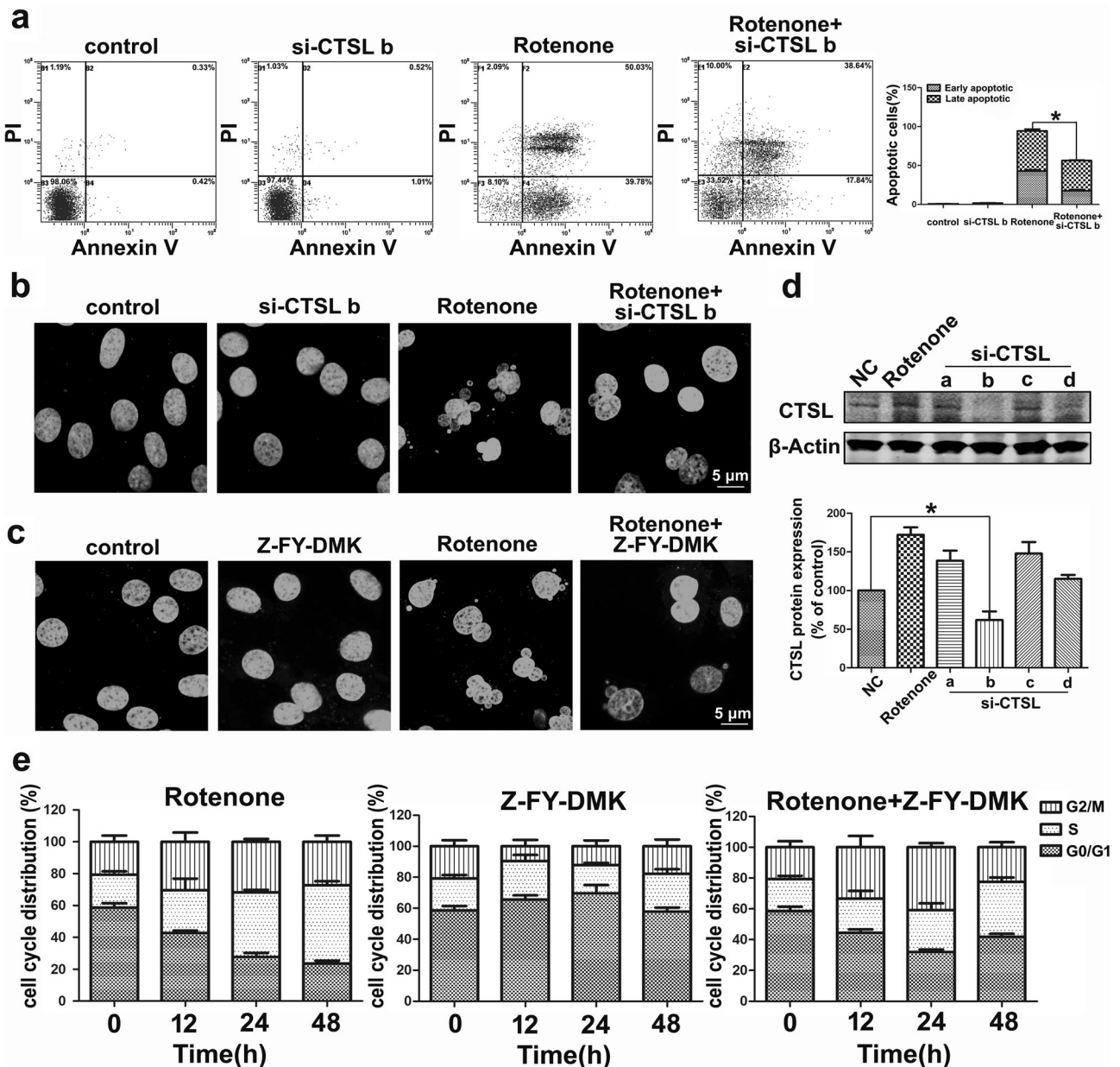
Efficient interference of Bim siRNA b was confirmed by Western blot (Fig. 6f). We then decreased the expression of Bim by transfecting cells with siRNA and Hoechst 33258 staining indicated that silencing of Bim was required for reduction of rotenone-induced apoptosis (Fig. 6e).

These results indicated Egr-1 could regulate CTSL expression and Bim also played a role in rotenone-induced apoptosis.

## DISCUSSION

This study mainly reported previously unrecognized roles of CTSL and B-Myb as well as provided a possible mechanism for how to regulate rotenone-induced PC-12 cell apoptosis. We identified B-Myb, an established cell cycle regulating transcription factor, as a mediator of CTSL signaling linked to cell cycle regulation and apoptosis.

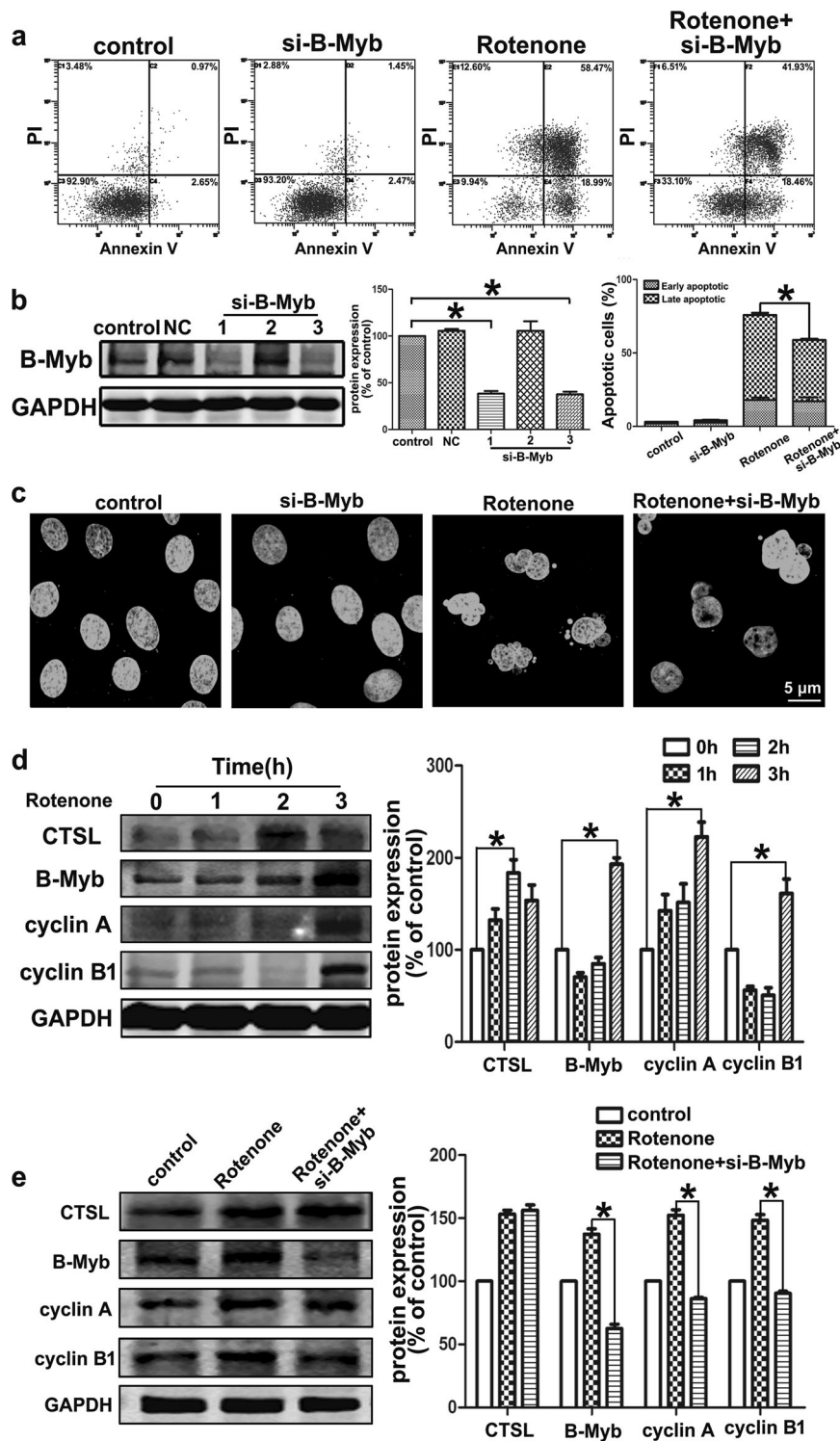
CTSL, an important cysteine protease that degrades large variety of proteins, widely existed in lysosomes. Recent discoveries suggested that CTSL could be involved in induction of apoptosis. Our lab had observed a markedly increased expression level of CTSL in the nigral dopaminergic neurons of 6-hydroxydopamine (6-OHDA)-treated Sprague Dawley (SD) rats, as well as in the brain of patients with PD [4, 6, 14]. However, the role of CTSL in apoptosis and the underlying mechanisms involved have not been completely elucidated. Consistently, here we found that CTSL was significantly upregulated in cultured PC-12 cells upon rotenone stimulation. In addition, we demonstrated that CTSL was indeed involved in regulation of apoptosis as rotenone-induced cell apoptosis was decreased either by addition of CTSL siRNA or its inhibitor.



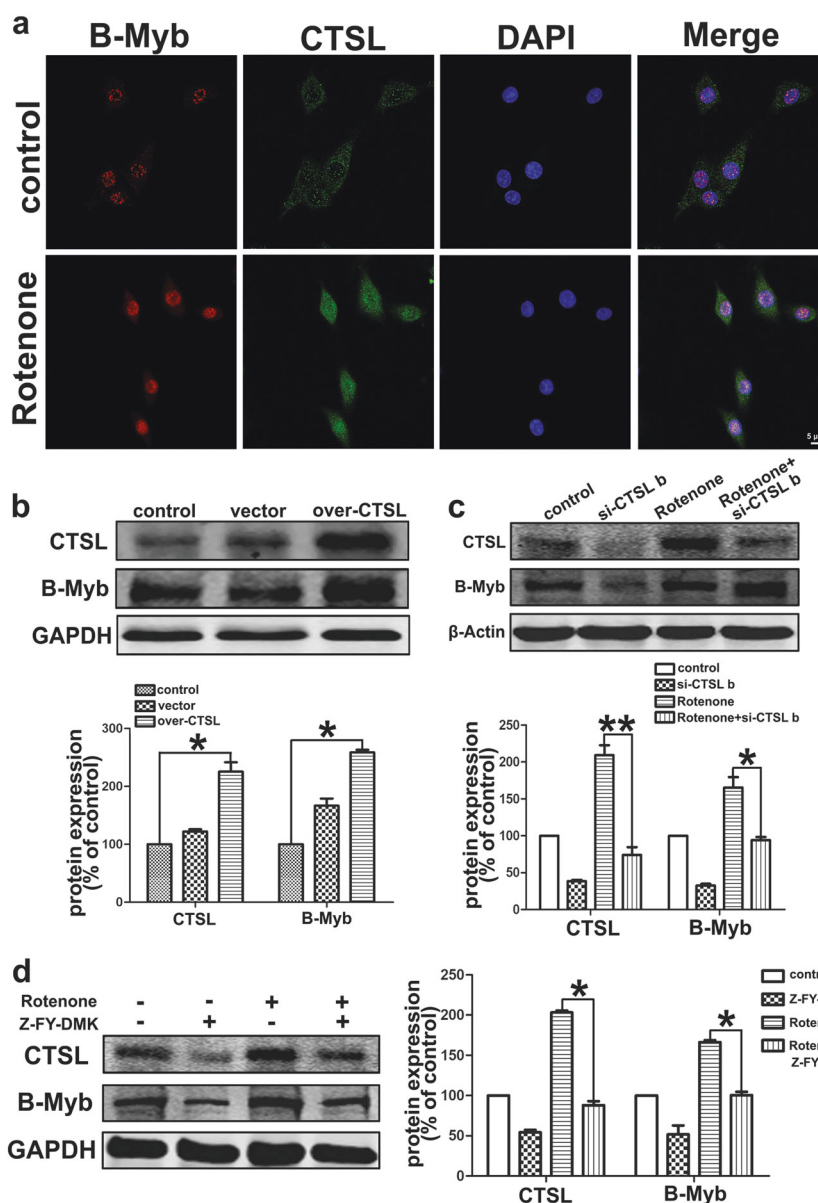
**Fig. 2** CTSL inhibition alleviated rotenone-induced apoptosis and cell cycle arrest in PC-12 cells. **a** PC-12 cells were transfected with CTSL siRNA b and 48 h later treated with 0.5 μM rotenone for 24 h. Annexin V-FITC/PI double dyes were used to stain the cells and the flow cytometry assay was performed. **b** PC-12 cells were transfected with CTSL siRNA b. Cells were treated with or without 0.5 μM rotenone and stained with Hoechst 33258 staining 24 h later. **c** PC-12 cells were treated with CTSL inhibitor, Z-FY-DMK, for 6 h firstly and then treated with 0.5 μM rotenone. Cells were stained with Hoechst 33258 staining to determine apoptotic cells 24 h later. **d** PC-12 cells were treated with 0.5 μM rotenone for 3 h or transfected with CTSL siRNAs or the negative control RNAs (NC) and 48 h later the expression level of CTSL was determined, β-actin was used as an internal control. Western blot showed efficient interference of CTSL siRNA b. **e** Cells were treated with 0.5 μM rotenone and/or 10 μM Z-FY-DMK for 0, 12, 24, and 48 h separately and cell cycle distribution was determined by flow cytometry after PI staining. At least three independent experiments were performed. \**P* < 0.05 compared with control

Proapoptotic stimulation results in the release of CTSL from lysosomes to cytoplasm. The secreted-CTSL leads to the cleavage of Bid to tBid [26], release of Cyt c and activation of caspase-3 [27]. Our previous investigations demonstrated that 6-OHDA stimulated CTSL secretion from lysosomes to cytoplasm as well as nuclear translocation of CTSL in DA neurons [4, 14]. The data indicated that CTSL was not only released to cytoplasm, but also translocated to nuclear space and thus resulted in the altered content of CTSL in cytoplasm. Nuclear translocated-CTSL can potentially affect the other cell signaling regulations during the cell cycle progression. Sanaregret L et al.

found that nuclear translocation of CTSL was followed by degradation of transcription factor CDP/Cux and production of CDP/Cux p110 isoform during the transitional period. The isoform is a transcription activator, which promotes the expression of many S phase-linked genes. The process accelerates cell cycle transition from G1 to S phase [28]. In this study we demonstrated that CTSL inhibitor reversed rotenone-induced cell cycle arrest at S phase. We suggested that rotenone stimulated the release of CTSL from lysosomes to cytoplasm, which was followed by its nuclear translocation. Nuclear translocated-CTSL could stimulate production of transcription



**Fig. 3** B-Myb mediated rotenone-induced apoptosis and regulated expression of cell cycle-related proteins. **a** Cells were transfected with B-Myb siRNA 3 and 48 h later treated with 0.5  $\mu$ M rotenone. Annexin V-FITC/PI double dyes were used to stain the cells and determine cell apoptosis 24 h later. **b** Cells were transfected with B-Myb siRNAs or the negative control RNAs (NC) and 48 h later the expression level of B-Myb was determined, GAPDH was used as an internal control. Western blot showed efficient interference of B-Myb siRNA 1 and 3. We chose siRNA 3 for the following experiments. **c** Cells were treated as described in **a** and Hoechst 33258 staining was conducted 24 h later. **d** PC-12 cells were treated with 0.5  $\mu$ M rotenone and harvested at indicated time points. The expression levels of CTSL, B-Myb, cyclin A, and cyclin B1 were analyzed by Western blot. GAPDH was used as an internal control. **e** Cells were transfected with B-Myb siRNA 3 and 48 h later treated with 0.5  $\mu$ M rotenone for 3 h. The expression levels of CTSL, B-Myb, cyclin A, and cyclin B1 were analyzed by Western blot. GAPDH was used as an internal control. At least three independent experiments were performed. \* $P < 0.05$  compared with control

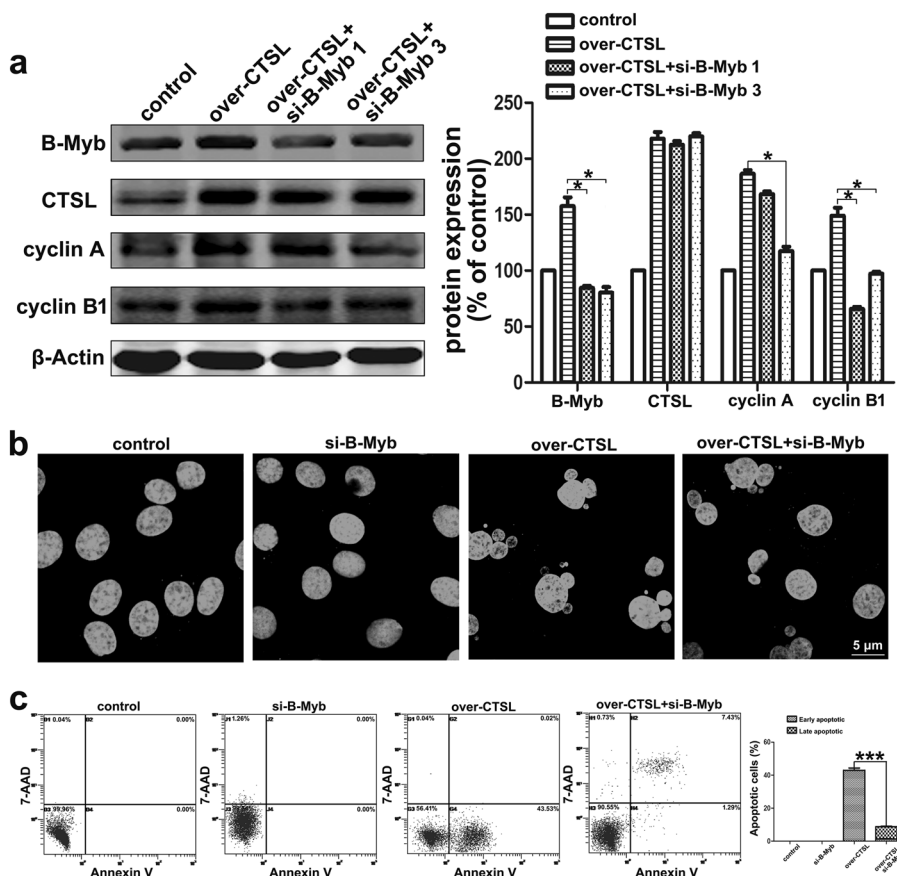


**Fig. 4** CTSL had effect on B-Myb expression. **a** PC-12 cells were treated with 0.5  $\mu$ M rotenone for 3 h and immunofluorescence staining was performed to determine the effect of rotenone on B-Myb (Red) and CTSL (Green). **b** Cells were infected of over-CTSL lentivirus or vector for 48 h and Western blot was used to analyze the expression of CTSL and B-Myb. GAPDH was used as an internal control. **c** PC-12 cells were transfected with CTSL siRNA b and then treated with 0.5  $\mu$ M rotenone. Western blot was used to determine the expression of CTSL and B-Myb,  $\beta$ -actin was used as an internal control. **d** PC-12 cells were treated with or without Z-FY-DMK for 6 h and then with 0.5  $\mu$ M rotenone for 3 h. The expression levels of CTSL and B-Myb were analyzed by Western blot. GAPDH was used as an internal control. At least three independent experiments were performed. \* $P < 0.05$ , \*\* $P < 0.01$  compared with control

activator CDP/Cux p110 isoform and cause elevated expression of S phase genes. The process could result in accelerated cell cycle arrest at S phase that we observed. Thus, CTSL translocation should be explored in future studies as it might be a plausible initiating mechanism for the detected cell cycle regulation in rotenone-treated PC-12 cells.

A positive correlation between expression levels of CTSL2 and B-Myb was previously observed [17]. Accordingly, we found CTSL as well as B-Myb expression levels were elevated. Immunofluorescence analysis showed that CTSL and B-Myb were present in nuclei of rotenone-treated PC-12 cells. The interaction between CTSL and B-Myb has not been exactly described yet. In our study, CTSL overexpression resulted in the increased expression of B-Myb. In contrast, siRNA- or inhibitor-induced lowered level of

CTSL resulted in the decreased B-Myb expression. However, the application of B-Myb siRNA failed to cause apparent changes in CTSL expression level. Furthermore, data showed decreased cell cycle protein levels and cell apoptosis when CTSL was overexpressed and, simultaneously, B-Myb was reduced. These results demonstrated that CTSL affected not only B-Myb but also cell cycle-related protein expressions via B-Myb. It has been reported that B-Myb regulates several cell signaling responses via binding proteins directly and activation of specific downstream targets when it is in the cytoplasm, whereas B-Myb acts as a transcription factor when exists in the nucleus [29]. Our data indicated the interaction of CTSL on B-Myb signaling, however, the exact regulatory mechanism between them has not been well understood yet and should be further clarified in future investigations.



**Fig. 5** CTSL influenced cell cycle-related proteins and apoptosis via B-Myb. **a** Cells were infected of over-CTSL lentivirus and then transfected with different B-Myb siRNAs for 48 h. Western blot analyzed the expression levels of B-Myb, CTSL, cyclin A, and cyclin B1,  $\beta$ -actin was used as an internal control. **b** Cells were infected of over-CTSL lentivirus and then transfected with B-Myb siRNA 3 for 48 h and another 24 h later Hoechst 33258 staining was used to determine cell apoptosis. **c** Cells were treated as described in **b** and cell apoptosis was determined by the Annexin V-PE/7-AAD Apoptosis Detection Kit 24 h later. At least three independent experiments were performed. \* $P < 0.05$ , \*\*\* $P < 0.001$  compared with control

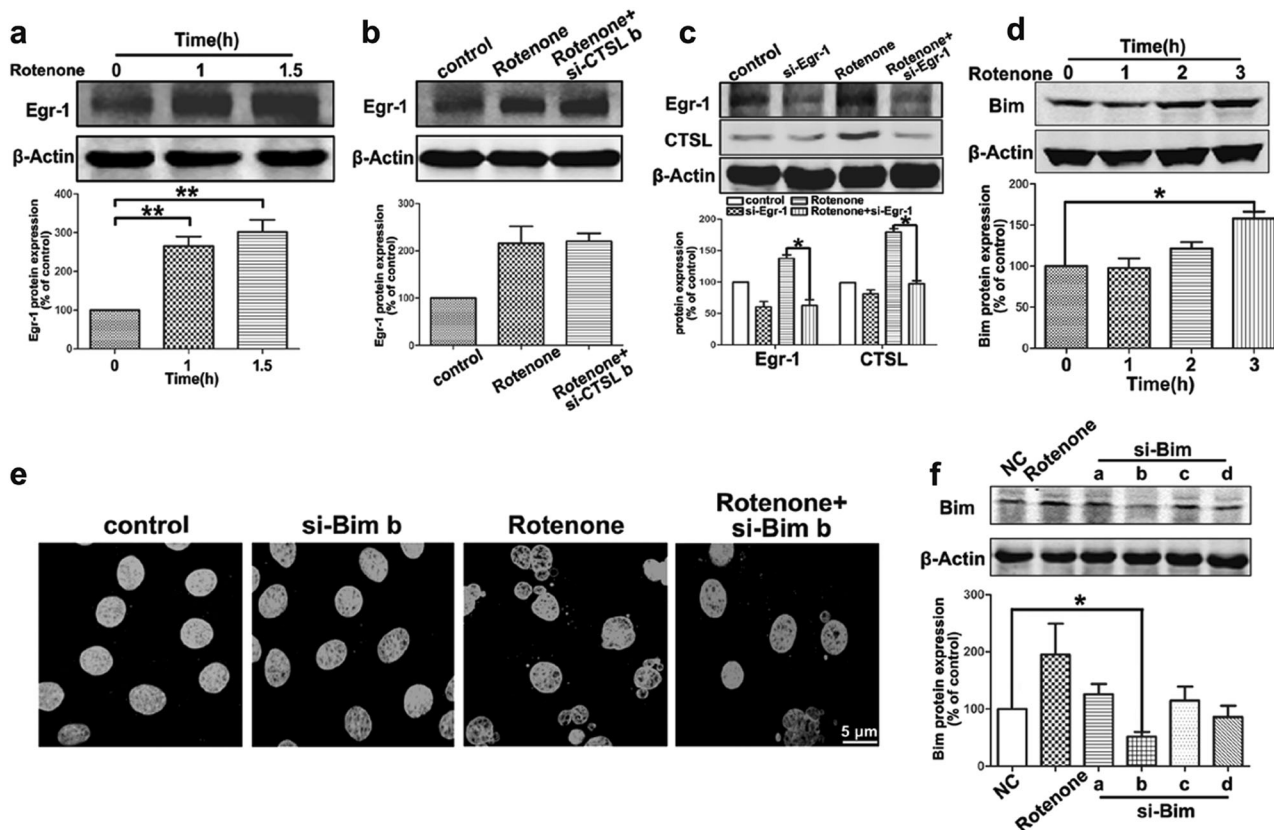
It is well established that B-Myb played a crucial role in regulation of cell cycle progression. As a transcription factor, B-Myb regulates cell cycle via direct interaction with other regulators of the cell cycle [30]. Cyclin A/CDK2 (cyclin-dependent kinase 2, CDK2) phosphorylates B-Myb and phosphorylation of it reaches the peaking level in early S phase [31–34]. Phosphorylated-B-Myb is the active form of B-Myb. It would be meaningful to test the relationship among phosphorylation of B-Myb, cell cycle progression, and induction of apoptosis. Notably, it was shown that cyclin D1 suppressed the activity of B-Myb [35, 36]. Cyclin F was also reported to inhibit the B-Myb/cyclin A pathway to ensure a DNA damage-induced checkpoint response in G2 phase [37]. B-Myb is required for recovery from the DNA damage-induced G2 checkpoint [38]. Furthermore, the interaction between B-Myb and cyclins is mutual. Bartusel et al. demonstrated that B-Myb activated cyclin A1 and cyclin D by binding to SP1 sites [39]. CTSL was also reported to participate in cell cycle regulation. Consistently, our data indicated that the CTSL inhibitor rescued cells from rotenone-induced cell cycle arrest. Furthermore, the lowered level of B-Myb was associated with decreased expression of some cell cycle-related proteins, such as cyclin A and B1. However, the specific interaction between cyclins and B-Myb remains unclear. Suggestively, cyclins might be able to alter B-Myb activity through modifying it and, thus, influence B-Myb impact on cell cycle progression. Further studies should address those questions.

Many reports presented interesting findings and demonstrated that B-Myb had proapoptotic and antiapoptotic effects in different

cells. B-Myb acts as an antiapoptotic factor in many cells, except nervous cells. B-Myb elevated expression of Bim in neuronal cells during stimulation of apoptosis [40]. We investigated the role of Bim, the downstream target of B-Myb, in rotenone-induced apoptosis. Accordingly, the expression level of Bim in rotenone-treated cells was increased as well as cell apoptosis. Xie et al. demonstrated that Egr-1 enhanced the expression of Bim and it was closely associated with activation of apoptosis [20, 41]. Furthermore, Egr-1 can bind CTSL promoter and, thus, regulate CTSL expression [42]. Whether Egr-1 is associated with rotenone-induced elevation of CTSL has not been reported currently. Our data preliminarily proved that reduced-Egr-1 caused the decrease of CTSL, while lowered-CTSL did not influence the level of Egr-1.

The present study has demonstrated that CTSL influenced cell cycle-related proteins and apoptosis via B-Myb in rotenone-treated PC-12 cells. First, we have shown that both CTSL and B-Myb were involved in regulation of cell cycle progression and apoptosis. Second, the suppression of CTSL activity or the knockdown of it blocked B-Myb induction, whereas the over-expression of CTSL elevated B-Myb expression, suggesting that CTSL had effects on B-Myb expression. Third, results showed that CTSL regulated cell cycle-related proteins, including cyclin A and cyclin B1, through B-Myb. Finally, the prevention of Egr-1 by its siRNAs decreased CTSL level and reduced level of proapoptotic protein Bim resulting in diminished apoptosis. Together, the findings indicated that CTSL-induced apoptosis through the





**Fig. 6** Egr-1 was associated with CTSL expression and Bim was involved in rotenone-induced apoptosis. **a** PC-12 cells were treated with 0.5  $\mu$ M rotenone and harvested at indicated time points. The expression level of Egr-1 was analyzed by Western blot,  $\beta$ -actin was used as an internal control. **b** PC-12 cells were transfected with CTSL siRNA b and 48 h later treated with 0.5  $\mu$ M rotenone for 3 h. Western blot determined the expression of Egr-1,  $\beta$ -actin was used as an internal control. **c** PC-12 cells were transfected with Egr-1 siRNA and 48 h later treated with 0.5  $\mu$ M rotenone for 3 h. Western blot was used to determine the expression levels of Egr-1 and CTSL,  $\beta$ -actin was used as an internal control. **d** PC-12 cells were treated with 0.5  $\mu$ M rotenone and harvested at indicated time points. The expression level of Bim was analyzed by Western blot,  $\beta$ -actin was used as an internal control. **e** PC-12 cells were transfected with Bim siRNA b and 48 h later treated with 0.5  $\mu$ M rotenone for another 24 h. Hoechst 33258 staining was conducted. **f** PC-12 cells were treated with 0.5  $\mu$ M rotenone for 3 h or transfected with Bim siRNAs or the negative control RNAs (NC) and 48 h later the expression level of Bim was determined,  $\beta$ -actin was used as an internal control. Western blot showed efficient interference of Bim siRNA b. At least three independent experiments were performed. \* $P < 0.05$ , \*\* $P < 0.01$  compared with control

activation of B-Myb and regulation of cell cycle-related proteins in rotenone-treated PC-12 cells.

#### ACKNOWLEDGEMENTS

This study was funded by the National Natural Science Foundation of China (Grant nos. 81773768 and 81671252) and the Priority Academic Program Development of the Jiangsu Higher Education Institutes (PAPD).

#### AUTHOR CONTRIBUTION

ZQL designed research; SQX, LW, HMC, YC, and YZ performed research; YW and ZQL contributed new reagents or analytic tools; XS and YFZ analyzed data; XS wrote the paper.

#### ADDITIONAL INFORMATION

**Competing interests:** The authors declare no competing interests.

#### REFERENCES

1. Cirman T, Oresic K, Mazovec GD, Turk V, Reed JC, Myers RM, et al. Selective disruption of lysosomes in HeLa cells triggers apoptosis mediated by cleavage of Bid by multiple papain-like lysosomal cathepsins. *J Biol Chem.* 2004;279:3578–87.

2. Nakamura Y, Takeda M, Suzuki H, Morita H, Tada K, Hariguchi S, et al. Lysosome instability in aged rat brain. *Neurosci Lett.* 1989;97:215–20.
3. Nakamura Y, Takeda M, Suzuki H, Hattori H, Tada K, Hariguchi S, et al. Abnormal distribution of cathepsins in the brain of patients with Alzheimer's disease. *Neurosci Lett.* 1991;130:195–8.
4. Xiang B, Fei X, Zhuang W, Fang Y, Qin Z, Liang Z. Cathepsin L is involved in 6-hydroxydopamine induced apoptosis of SH-SY5Y neuroblastoma cells. *Brain Res.* 2011;1387:29–38.
5. Li L, Gao L, Song Y, Qin ZH, Liang Z. Activated cathepsin L is associated with the switch from autophagy to apoptotic death of SH-SY5Y cells exposed to 6-hydroxydopamine. *Biochem Biophys Res Commun.* 2016;470:579–85.
6. Li L, Wang X, Fei X, Xia L, Qin Z, Liang Z. Parkinson's disease involves autophagy and abnormal distribution of cathepsin L. *Neurosci Lett.* 2011;489:62–7.
7. Mattson MP. Apoptosis in neurodegenerative disorders. *Nat Rev Mol Cell Biol.* 2000;1:120–9.
8. Bredesen DE, Rao RV, Mehlen P. Cell death in the nervous system. *Nature.* 2006;443:796–802.
9. Dickson DW. Apoptotic mechanisms in Alzheimer neurofibrillary degeneration: cause or effect? *J Clin Investig.* 2004;114:23–7.
10. Nicholson DW. Neuroscience: good and bad cell death. *Nature.* 2009;457:970–1.
11. West AB, Dawson VL, Dawson TM. To die or grow: Parkinson's disease and cancer. *Trends Neurosci.* 2005;28:348–52.
12. Smith PD, O'Hare MJ, Park DS. CDKs: taking on a role as mediators of dopaminergic loss in Parkinson's disease. *Trends Mol Med.* 2004;10:445–51.
13. Gamet-Payrastra L, Li P, Lumeau S, Cassar G, Dupont MA, Chevolleau S, et al. Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle

- arrest and apoptosis in HT29 human colon cancer cells. *Cancer Res.* 2000;60:1426–33.
14. Fei XF, Qin ZH, Xiang B, Li LY, Han F, Fukunaga K, et al. Olomoucine inhibits cathepsin L nuclear translocation, activates autophagy and attenuates toxicity of 6-hydroxydopamine. *Brain Res.* 2009;1264:85–97.
  15. Mowla SN, Lam EW, Jat PS. Cellular senescence and aging: the role of B-MYB. *Aging Cell.* 2014;13:773–9.
  16. Liu DX, Biswas SC, Greene LA. B-myb and C-myb play required roles in neuronal apoptosis evoked by nerve growth factor deprivation and DNA damage. *J Neurosci.* 2004;24:8720–5.
  17. Skrzypczak M, Springwald A, Lattrich C, Haring J, Schuler S, Ortmann O, et al. Expression of cysteine protease cathepsin L is increased in endometrial cancer and correlates with expression of growth regulatory genes. *Cancer Investig.* 2012;30:398–403.
  18. Du X, Chen NL, Wong A, Craik CS, Bromme D. Elastin degradation by cathepsin V requires two exosites. *J Biol Chem.* 2013;288:34871–81.
  19. Sevenich L, Hagemann S, Stoeckle C, Tolosa E, Peters C, Reinheckel T. Expression of human cathepsin L or human cathepsin V in mouse thymus mediates positive selection of T helper cells in cathepsin L knock-out mice. *Biochimie.* 2010;92:1674–80.
  20. Xie B, Wang C, Zheng Z, Song B, Ma C, Thiel G, et al. Egr-1 transactivates Bim gene expression to promote neuronal apoptosis. *J Neurosci.* 2011;31:5032–44.
  21. Biswas SC, Liu DX, Greene LA. Bim is a direct target of a neuronal E2F-dependent apoptotic pathway. *J Neurosci.* 2005;25:8349–58.
  22. Putcha GV, Le S, Frank S, Besirli CG, Clark K, Chu B, et al. JNK-mediated BIM phosphorylation potentiates BAX-dependent apoptosis. *Neuron.* 2003;38:899–914.
  23. Whitfield J, Neame SJ, Paquet L, Bernard O, Ham J. Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release. *Neuron.* 2001;29:629–43.
  24. Schober A. Classic toxin-induced animal models of Parkinson's disease: 6-OHDA and MPTP. *Cell Tissue Res.* 2004;318:215–24.
  25. Alam M, Schmidt WJ. Rotenone destroys dopaminergic neurons and induces parkinsonian symptoms in rats. *Behav Brain Res.* 2002;136:317–24.
  26. Blomgran R, Zheng L, Stendahl O. Cathepsin-cleaved Bid promotes apoptosis in human neutrophils via oxidative stress-induced lysosomal membrane permeabilization. *J Leukoc Biol.* 2007;81:1213–23.
  27. Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell.* 1996;86:147–57.
  28. Sansregret L, Goulet B, Harada R, Wilson B, Leduy L, Bertoglio J, et al. The p110 isoform of the CDP/Cux transcription factor accelerates entry into S phase. *Mol Cell Biol.* 2006;26:2441–55.
  29. Seong HA, Manoharan R, Ha H. B-MYB positively regulates serine-threonine kinase receptor-associated protein (STRAP) activity through direct interaction. *J Biol Chem.* 2011;286:7439–56.
  30. Joaquin M, Watson RJ. Cell cycle regulation by the B-Myb transcription factor. *Cell Mol Life Sci.* 2003;60:2389–401.
  31. Bessa M, Saville MK, Watson RJ. Inhibition of cyclin A/Cdk2 phosphorylation impairs B-Myb transactivation function without affecting interactions with DNA or the CBP coactivator. *Oncogene.* 2001;20:3376–86.
  32. Ziebold U, Bartsch O, Marais R, Ferrari S, Klempnauer KH. Phosphorylation and activation of B-Myb by cyclin A-Cdk2. *Curr Biol.* 1997;7:253–60.
  33. Bartsch O, Horstmann S, Toprak K, Klempnauer KH, Ferrari S. Identification of cyclin A/Cdk2 phosphorylation sites in B-Myb. *Eur J Biochem.* 1999;260:384–91.
  34. Werwein E, Cibis H, Hess D, Klempnauer KH. Activation of the oncogenic transcription factor B-Myb via multisite phosphorylation and prolyl *cis/trans* isomerization. *Nucleic Acids Res.* 2019;47:103–21.
  35. Schubert S, Horstmann S, Bartusel T, Klempnauer KH. The cooperation of B-Myb with the coactivator p300 is orchestrated by cyclins A and D1. *Oncogene.* 2004;23:1392–404.
  36. Horstmann S, Ferrari S, Klempnauer KH. Regulation of B-Myb activity by cyclin D1. *Oncogene.* 2000;19:298–306.
  37. Klein DK, Hoffmann S, Ahlskog JK, O'Hanlon K, Quaas M, Larsen BD, et al. Cyclin F suppresses B-Myb activity to promote cell cycle checkpoint control. *Nat Commun.* 2015;6:5800.
  38. Mannefeld M, Klassen E, Gaubatz S. B-MYB is required for recovery from the DNA damage-induced G2 checkpoint in p53 mutant cells. *Cancer Res.* 2009;69:4073–80.
  39. Bartusel T, Schubert S, Klempnauer KH. Regulation of the cyclin D1 and cyclin A1 promoters by B-Myb is mediated by Sp1 binding sites. *Gene.* 2005;351:171–80.
  40. Musa J, Aynaud MM, Mirabeau O, Delattre O, Grunewald TG. MYBL2 (B-Myb): a central regulator of cell proliferation, cell survival and differentiation involved in tumorigenesis. *Cell Death Dis.* 2017;8:e2895.
  41. Liu C, Rangnekar VM, Adamson E, Mercola D. Suppression of growth and transformation and induction of apoptosis by EGR-1. *Cancer Gene Ther.* 1998;5:3–28.
  42. Amuthan G, Biswas G, Zhang SY, Klein-Szanto A, Vijayarathay C, Avadhani NG. Mitochondria-to-nucleus stress signaling induces phenotypic changes, tumor progression and cell invasion. *EMBO J.* 2001;20:1910–20.