Overexpression of BAT3 Alleviates Prion Protein Fragment PrP106-126-Induced Neuronal Apoptosis

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Keywords

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SUMMARY

Backgrounds and aims: Prion diseases are a group of infectious neurodegenerative diseases characterized by neuronal death and degeneration. Human leukocyte antigen-B-associated transcript 3 (BAT3) is an important apoptosis regulator. We therefore investigated the interactions between BAT3 and prion protein and the potential role of BAT3 in PrP106-126-induced apoptosis. **Methods:** BAT3 and prion protein were overexpressed in HeLa, Neuro2A, or primary neuronal cells by transfection with BAT3-HA or PRNP-EGFP expression plasmids and their relationship studied by immunofluorescence and Western blotting. The effect of BAT3 on PrP106-126-induced cytotoxicity and apoptosis was detected by the CCK-8 assay and terminal-deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay. The expression of cytochrome *c* and Bcl-2 was examined by Western blotting. **Results:** BAT3 interacted with prion protein and enhanced PrP expression. After PrP106-126 peptide treated, BAT3 was transported from the nucleus to cytoplasm, increased cell viability, and protected neurons from PrP106-126-induced apoptosis through stabilizing the level of Bcl-2 protein and inhibiting the release of cytochrome *c* to cytoplasm. **Conclusions:** Our present data showed a novel molecular mechanism of PrP106-126-induced apoptotic process regulation through the overexpression of BAT3, which may be important for the basic regulatory mechanism of neuron survival in prion diseases and associated neurodegenerative diseases *in vivo*.

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Introduction

Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), are fatal neurodegenerative disorders affecting human and other mammalian species. The pathology is characterized by brain vacuolation, neuronal cell death and microgliosis [1,2]. Prion diseases arise from the conformational conversion of cellular prion protein (PrP^C) to the misfolded form, termed PrP^{Sc}, which has a higher proportion of β -sheet structure in place of the normal α -helix structure [3] and is protease-resistant [4]. The physiological PrP^C performs a variety of functions including involvement in cell death and survival, oxidative stress, metal ion trafficking, cell adhesion and transmembrane signaling [5], while the misfolded PrP^{Sc} is pathologic. The neurotoxic prion protein fragment 106-126 (PrP106-126) possesses similar physicochemical and pathogenic properties to PrP^{Sc}, in that it forms amyloid fibrils with a high β -sheet content, shows partial proteinase K resistance

and is neurotoxic *in vitro* including the ability to cause apoptosis in hippocampal neurons and induce proliferation of astrocytes. Therefore, PrP106-126 is commonly used as a model for the investigation of PrP^{Sc} neurotoxicity [6–8].

Hessa and his colleagues showed that human leukocyte antigen-B-associated transcript 3 (BAT3, also called BAG6 and Scythe) interacts with PrP and mediates the ubiquitylation of mislocalized PrP [9]. BAT3 was initially identified within the class III region of the major histocompatibility complex (MHC) on human chromosome 6 [10]. One of cellular functions of BAT3 was regulating apoptosis [10–12]. Genetic ablation of BAT3 in mice caused embryonic lethality [13] as a result of developmental defects associated with increased apoptosis and aberrant cell proliferation, confirming the involvement of BAT3 in regulated apoptotic events during embryofetal development. It has also been shown that BAT3 regulates apoptosis through the modulation of apoptosis-inducing factor (AIF) [14] and stabilizing antiapoptotic protein

Bcl-2 (B cell lymphoma-2) [15]. BAT3 may also protect cells from apoptosis by sequestering proapoptotic factors and promoting the degradation of certain apoptotic factors [16].

Given that both PrP and BAT3 have been implicated in cell apoptosis, their interaction may represent an important regulatory mechanism of the misfolded PrP-induced apoptotic process. We therefore undertook the present study to investigate the interactions between BAT3 and PrP106-126 and the potential role of BAT3 in PrP106-126-induced neuronal cell death.

Materials and methods

Ethics Statement

All of the animal experiments were conducted in accordance with the guidelines of Beijing Municipality on the Review of Welfare and Ethics of Laboratory Animals approved by the Beijing Municipality Administration Office of Laboratory Animals (BAOLA).

Reagents

Monoclonal antibody (mAb) to BAG-6(D-1) cytochrome c and PrP (AH6) raised in mice were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse HA-tag mAb, rabbit anti-mouse Bcl-2 (P65) polyclonal antibody (pAb), rabbit anti-mouse β -actin pAb, mouse GAPDH mAb, mouse Lamin B1 mAb, and goat anti-rabbit IgG (H&L)-HRP secondary antibody were purchased from Bioworld Technology (Bioworld Technology, Inc., Nanjing, China). Goat anti-mouse IgG (H&L)-HRP secondary antibody and Alexa Fluor 594-conjugated AffiniPure goat anti-mouse IgG (H+L) were obtained from Beijing ZSGB Biotechnology. Goat anti-rabbit IgG (H+L) fluorescein isothiocyanate (FITC)-conjugated secondary antibodies, goat anti-rabbit IgG (H+L) Cy5-conjugated secondary antibodies, and rabbit anti-TUBB3/ β III tubulin (Neuronal Marker) were purchased from Bioss (Beijing Boisynthesis Biotechnology, Haidian District, China). Cell Counting Kit-8, Neuronal Class III β -Tubulin (Tuj1) antibody, DAPI dihydrochloride, and propidium iodide (PI) were purchased from Beyotime Biotechnology (Wuhan, Hubei, China). Reagents and apparatus used in immunoblotting assays were obtained from Bio-Rad (Richmond, CA, USA).

Prion Protein Peptide

The PrP peptide and PrP106-126 (sequences KTNMKHMAGAAAA GAVVGGLG) were synthesized by Sangon Bio-Tech (Beijing, China). The purity of prion peptides was >95% according to the data from the synthesizer. FITC-labeled PrP106-126 peptides were purchased from FANBO BIOCHEMICALS (Beijing, China). The peptides were dissolved in 0.1 M PBS to a concentration of 1 mM and allowed to aggregate at 37°C for 12 h. Experiments were conducted with a final peptide concentration of 200 μ M [17].

Plasmids

The prion protein plasmid was synthesized the full-length human prion protein (PRNP) cDNAs originally cloned from Hela cells by PCR. The primers were designed according to the gene sequence

of Homo sapiens prion protein (PRNP) mRNA in GenBank (AY569456). Oligonucleotides ATA CTC GAG ATG GCG AAC CTT GGC TGC T (forward) and ACT AAG CTT TCC CAC TAT CAG GAA GAT GAG (reverse) were used to introduce XhoI and HindIII sites flanking the cDNA. The PRNP-EGFP expression plasmid was constructed in the pEGFP-N1 vector (Clontech, Kyoto, Japan) by standard molecular biology techniques and confirmed by sequencing. All the primers were synthesized by Sangon Company (Shanghai, China). The BAT3-HA expression plasmid was kindly provided by Prof. Tang (State Key Laboratory of Agrobiotechnology, China Agricultural University, Beijing, China, Department of Basic Veterinary Medicine, College of Veterinary Medicine, China Agricultural University, Beijing, China).

Cell Culture

Isolation and culture of primary neuronal cells

Cerebral cortex neuronal cells were prepared from a 1-day-old Sprague–Dawley rat. The cells were gently dissociated with a plastic pipette after digestion with papain (Invitrogen, Carlsbad, CA, USA) at 37°C. The dissociated cells were plated at a final density of 2×10^6 cells/mL on polyethyleneimine (Sigma, St. Louis, MO, USA) 25 μ g/mL-coated 6-well or 12-well plates overnight at 37°C before seeding. The culture medium contained 2% B27 (Invitrogen), 0.5% penicillin–streptomycin solution (Gibco, Grand Island, NY, USA), 98% DMEM and F12 medium (Hyclone, Logan, UT, USA). Two days later, 10 μ M cytarabine (Sigma) was added to repress the growth of glial cells. Then, the medium was changed every 2 days. Experimental treatments were begun after 7 days in culture.

The neuroblastoma Neuro2A cells and human epitheloid carcinoma Hela cells were obtained from (Xiehe Medical University, Cell Culture Center, Beijing, China). Hela and Neuro2A cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone, Waltham, MA, USA) and 100 μ g/mL streptomycin and 100 U/mL penicillin (Gibco). The cells were maintained in a humidified incubator at 5% CO₂ and 37°C.

Transfection

Hela cells or Neuro2A cells were transfected with PRNP-EGFP or BAT3-HA expression plasmids by lipofection. The cells were grown in a 12-well dish to 80–90% confluency, then washed with Opti-MEM (Invitrogen), transfected with 2 μ g of the PRNP-EGFP or BAT3-HA plasmids, empty pcDNA3.1 vector control, or empty pGFP-N1 vector control using 3 μ L Lipofectamine 2000 reagent (Invitrogen) in Opti-MEM (Invitrogen) without serum according to the manufacturer's instructions. The same volume of DMEM containing 10% FBS or primary cell culture medium was added to the culture medium 5 h after transfection. The cells were observed 24 and 48 h after transfection by fluorescence confocal microscopy (Olympus, Tokyo, Japan) and subjected to immunoblot analyses.

Immunofluorescence

For plasmids localization analysis, Hela cells and cultured primary neuronal cells grown on cover slips were washed twice with PBS,

fixed by Immunol Staining Fix Solution, blocked 1 h at room temperature by Immunol Staining Blocking Buffer (P0102) and then incubated overnight at 4°C with anti-BAT3 antibody (1:100) or anti-HA-tag mAb (1:1000) followed by incubation with Alexa Fluor 594-conjugated AffiniPure goat anti-mouse IgG (H+L) secondary antibodies (1:100). The nuclei were stained with DAPI.

For cultured primary neuronal cells, we used rabbit anti-TUBB3/beta III tubulin (Neuronal Marker) (1:100) and anti-Tuj1 (neuronal class III -tubulin) antibody (Beyotime, Wuhan, China) (1:250) as the primary antibody and Alexa Fluor 594-conjugated AffiniPure goat anti-mouse IgG (H+L) (1:100) or fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit (Bioss, Beijing, China) (1:100) antibodies as the secondary antibody to confirm the identification of neurons.

To visualize the intracellular localization of PrP106-126 in Neuro2A cells or primary neuronal cells, the cells were treated with the FITC-labeled PrP106-126 in culture medium for 12 h before observation [18], then treated as above. All the treated cells were observed using an upright fluorescence confocal microscopy. All reagents for fixation, washing, and blocking were purchased from Beyotime Biotechnology (Beyotime Institute of Biotechnology, China).

Extraction of Nuclear, Cytoplasmic and Total Cellular Proteins

Total cellular proteins: Hela or Neuro2A cells were washed in PBS after overexpression or peptide treatment and were homogenized in pre-chilled RIPA lysis buffer (Beyotime Biotechnology) supplemented with a proteinase inhibitor cocktail (Novasyn, Beijing, China). The homogenate was centrifuged at 4000 *g* for 10 min at 4°C. The supernatant was frozen in aliquots at -80°C for Western blot analysis to protein analysis.

Nuclear and cytoplasmic proteins: Nuclear and cytoplasmic proteins were extracted using a cytoplasmic and nuclear protein extraction kit (Wuhan Boster Biotech, Wuhan, Hubei, China). BAT3, cytochrome *c* and Bcl-2 protein levels were determined by Western blotting. The blot was stripped and reprobed with anti- β -actin (for cytoplasmic extracts) or anti-Lamin B (for nuclear extracts) to estimate the total amount of protein loaded on the gel.

Western Blotting

The extracted proteins (exactly 40 μ g in each lane) were separated by SDS-PAGE on 10–15% gels, and the separated proteins were transferred onto a nitrocellulose membrane. Nonspecific binding sites were blocked by incubating the membrane with 5% fat-free dried milk in Tris-buffered saline (TBS-T: 10 mM Tris, 0.15 M NaCl, 0.05% Tween-20, pH of the solution adjusted to 7.5). The membrane was incubated with the following primary antibodies at 4°C overnight: murine anti-BAT3 (1:100), anti-HA (1:3000), anti-LaminB (1:1000), anti-PrP (AH6) (1:1000), rabbit anticytochrome *c* (1:500), anti-Bcl-2 (1:500), and anti- β -actin (1:3000) antibodies. The membrane was washed with TBS-T and then incubated with the secondary antibody, goat anti-mouse IgG, or anti-rabbit IgG-conjugated to horseradish peroxidase (1:5000). Bands of immunoreactive proteins were visualized on an image system (Versadoc; Bio-Rad) after incubation with enhanced chemifluorescence (ECF) reagent for 5 min.

TUNEL Assay

Neuro2A cells were grown on cover slips in poly-D-lysine-coated 12-well Lab-Tek® culture dishes at a density of 5×10^5 cells/well. TUNEL analysis was performed to measure the degree of cellular apoptosis using an *in situ* cell death detection kit, POD (Roche, Nutley, NJ, USA) following the manufacturer's instructions. The cells were counterstained with propidium iodide (PI) for nuclei. The TUNEL-positive control kit was purchased from Beyotime Biotechnology. All the slides were visualized using a fluorescence microscope.

Cell Viability Assays

Cell viability in response to the different treatments was evaluated using the CCK-8 assay. Briefly, after each treatment, 10 μ M of WST-8 reagent was added, and the primary cultures of rat cortical neurons were incubated for 2 h at 37°C and 5% CO₂. The absorbance of the samples was measured at 450 nm using a microplate reader with a background control as the blank. The cell survival ratio was expressed as the percentage of the control.

Statistical Analysis

All assays were performed on three separate occasions. Data were expressed as means $\pm$ SD of three experiments. All comparisons for parametric data were made using Student's *t*-test or one-way ANOVA followed by *post hoc* Tukey's test using the SPSS software (version 13.0: SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5 software (La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

Results

BAT3 Interacted with Full-length Prion Protein

Hessa and colleagues showed that BAT3 is involved in the ubiquitylation of mislocalized full-length PrP through interactions with the hydrophobic domains of the protein [9]. To validate their interactions, we overexpressed wild-type PrP tagged with the GFP marker to localize PrP in Hela cells and examined endogenous BAT3 and PrP localization in the cytosol by immunofluorescence microscopy. Confocal microscopy revealed expression of BAT3 in the plasma and partially colocalized with PrP in Hela cells (Figure 1A). To characterize this interaction further in neurons, we isolated and cultured primary cortical neurons. First, we identified the primary cultured neurons with neuronal specific antibodies (Figure S1), then we used the neurons to examine the subcellular localization of EGFP-tagged PrP and endogenous BAT3. In line with the results in Hela cells, the two proteins colocalized in the cytoplasm of primary neuronal cells (Figure 1B). Overexpressed PrP was mainly cytoplasmic in both cell types. We observed that the morphology of the full-length PrP-EGFP aggregates displayed cell-to-cell variations and differences between cell types. In Hela cells, the aggregates were larger and fewer than in primary neurons, whereas the aggregates in primary neuronal cultures were numerous and dispersed. Our data demonstrated that despite the primary localization of BAT3 in nucleus and

Figure 1 BAT3 interacts with GFP-tagged full-length prion protein in cytoplasm. HeLa cells and primary cultures of rat cortical neurons (PCCN) (7 days *in vitro*) were plated on coverslips and were transfected with green fluorescent protein (GFP)-tagged full-length prion protein (GFP-PrP), then stained with a monoclonal BAT3 antibody for evaluation by fluorescence confocal microscopy. The left panel displays BAT3 (red), the middle panel displays GFP-PrP (green), and the right panel is a merged image of the other two images. Scale bars, 5 μm (A) or 10 μm (B). Crosstalk of fluorescence emission was minimized by serial acquisition of the fluorescence color channels.

variations in distribution in different cell lines, BAT3 colocalized with PrP in the cytoplasm of HeLa cells and primary cultures of rat cortical neurons. Interestingly, BAT3 was also detected in the nuclei of PCCN cells, but not HeLa cells. This difference in subcellular localization in different cell types is consistent with the findings by Kamper *et al.* [19], who reported cell type specific expression of BAT3.

BAT3 Expression Enhanced Cellular Levels of Endogenous PrP

As BAT3 has been proposed to interact with PrP *in vitro* [9], and it acts as an essential factor in the protein quality control of aggregation-prone nascent chain polypeptides and mislocalized protein [20,21], we speculated that BAT3 might modulate PrP expression or turnover. Under normal circumstances, the synthesis of endogenous PrP closely parallels PrP degeneration [9]. It is difficult to detect the alteration of endogenous PrP expression. To test our assumption, we expressed different amounts of HA-tagged full-length BAT3 in HeLa cells and determined the expression of endogenous PrP by Western blotting. As shown in Figure 2A, endogenous PrP levels rose with increasing exogenous BAT3. We used three different PrP antibodies to confirm the results [data with the other two anti-PrP antibodies, (PrP(6D11) sc-58581, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and AH6 (PrP, RC 058, TSE Research Center, Compton, UK) are not shown], and all these experiments gave similar results. As one of the characteristics of brain damage in prion diseases is widespread neuronal loss, we further investigated whether BAT3 could influence the expression of PrP in mouse neuroblastoma cells. Neuro2A cells were transfected with different amounts of HA-tagged full-length BAT3 plasmids, and the total cell lysates were subjected to Western blotting. To our surprise, the findings were very encouraging and consistent with the previous results in HeLa cells (Figure 2B). With the increasing expression of exogenous BAT3, the endogenous PrP levels enhanced in Neuro2A cells. Taken together, these data indicate that BAT3 can markedly increase the endogenous

expression of PrP in HeLa and Neuro2A cells. It is possible that BAT3 might stabilize PrP and thereby promote its potential maturation and subsequent cellular function(s).

BAT3 was Translocated to Cytoplasm in Response to PrP106-126 Stimulation

The synthetic peptide PrP106-126 has previously been shown to be neurotoxic and induce apoptosis in the human neuroblastoma cell line SH-SY5Y [22]. In the meantime, several studies have demonstrated that BAT3 could be exported to the cytoplasm and bind to its interacting proteins in cells exposed to specific apoptotic stimuli [15,23]. We therefore wondered whether BAT3 is engaged in PrP106-126-induced apoptosis in neurons, or BAT3 merely serves as a buffer for dislocated substrates. To understand the influence of PrP106-126 fragment to the BAT3 protein, we used PrP106-126-FITC to realize the change by fluorescence microscopy. First, we characterized that PrP106-126-FITC has similar properties with PrP106-126 fragment by the CCK8 assay and Western blot (Figure 3).

We treated primary cultures of rat cortical neurons with the PrP106-126-FITC peptide and then examined endogenous BAT3 levels by immunofluorescence microscopy. To our surprise, BAT3 not only colocalized with PrP106-126-FITC in the cytosol, but also had a tendency to translocate from the nucleus to cytoplasm of cells exposed to PrP106-126-FITC compared with the control group (Figure 4A). Studies have proved that the expression and subcellular distribution of BAT3 are adjusted to cellular function dependent on the cell types [19]. After the stimulation of PrP (106-126)-FITC, BAT3 translocated from the nucleus to the cytoplasm, in line with assigned roles for BAT3 in the cytosol [15,20,23].

To further confirm nuclear export of BAT3 in response to PrP106-126, Neuro2A cells were treated with 200 μM PrP106-126 and harvested at different time points. The expression levels of BAT3 in the cytoplasmic and nuclear fractions at each time point were analyzed by Western blotting. The quality of BAT3 in the cytoplasmic and nuclear fractions was determined using β -actin

Figure 2 BAT3 expression enhances cellular levels of endogenous PrP. HeLa cells **(A)** or Neuro2A cells **(B)** were transfected with empty vectors or BAT3-HA-expressing vectors (0.1, 0.3, 1 or 3 µg). The transfected cells were immunoblotted with anti-PrP (AH6), anti-HA, and anti-BAT3 antibodies as described in Materials and Methods. β -actin was used as a loading control. Three histogram plots show the densitometric quantification of PrP protein levels, total BAT3 protein levels (probed with anti-BAT3 antibody), and exogenous BAT3 levels (detected with anti-HA antibody) normalized to β -actin. Three different anti-PrP antibodies were used to confirm the results (data with the other two anti-PrP antibodies, PrP (6D11) (sc-58581, Santa Cruz, CA, USA) and AH6(RC 058, Compton, UK) are not shown). Data are presented as mean $\pm$ SD of triplicate experiments; * P < 0.05, ** P < 0.01 versus cells transfected with empty vectors only group.

and lamin B as the cytoplasmic and nuclear markers, respectively. As expected, upon stimulation with PrP106-126, the amounts of BAT3 in cytoplasm were enhanced and the increase was derived from nuclear export (Figure 4B and C). At 6 h, BAT3 was distributed prominently in the nuclear fractions. With the increasing incubation time, PrP106-126 treatment promoted the export of BAT3 to the cytoplasm in Neuro2A cells with a corresponding decrease in its level in the nucleus fraction. Taken together, these results in concert with the previous reports suggest that BAT3 is a nucleus-cytoplasm shuttling protein. This is the first report describing the nuclear export of BAT3 in neuronal cells in response to PrP106-126 stimulation.

BAT3 Modulated PrP106-126-induced Cell Death

As one of the cellular functions of BAT3 is the regulation of apoptosis [11,12] and the synthetic peptide PrP106-126 induces apoptosis *in vitro* [22,24,25], we further explored the role of BAT3 in PrP106-126-induced cell death.

First, we overexpressed the full-length recombinant BAT3 protein or HA vectors in primary cultures of rat cortical neurons and then stimulated the cells with 200 µM PrP106-126 to observe the cell viability by the CCK-8 assay in different experiment groups. Compared with the group treated with PrP106-126 only or the group transfected with empty vectors and then stimulated with

Figure 3 PrP106-126-FITC has similar cytotoxicity to PrP106-126. **(A)** Subcellular localization of PrP106-126-FITC in the cytoplasm around the nucleus. **(B)** PrP106-126-FITC has similar cytotoxicity to PrP106-126 measured by the CCK-8 assay. $**P < 0.01$ versus the control. **(C)** Both PrP106-126-FITC and PrP106-126 induced Cytochrome c release. Primary cultures of rat cortical neurons (PCCN) were treated with PrP106-126-FITC or PrP106-126 (200 μ M) for 24 h, and then, cytoplasmic fractions were collected and processed for immunoblotting with anti-cytochrome c antibody. The β -actin immunoblot was indicated as an internal control.

Figure 4 PrP106-126 stimulation induces BAT3 export from nucleus to cytoplasm. **(A)** Primary cultured cortical neurons were incubated with FITC-labeled PrP106-126 or PBS for 24 h as described in Material and Methods. The left panel displays FITC-labeled PrP106-126 (green), the second left panel displays BAT3 staining (red), the second right panel displays DAPI staining (blue), and the right panel is a merged image of the other images. Scale bars = 5 μ m. **(B, C)** Neuro2A cells were treated with PrP106-126 (200 μ M) for the indicated time periods. Nuclear and cytoplasmic fractions were collected, and the fractions were immunoblotted for BAT3. The nucleus-localized protein Lamin B and β -actin or GAPDH confirmed the separation of the nuclear and cytoplasmic fractions, respectively. The right histogram plots show the densitometric quantification of BAT3 in the indicated groups. The BAT3 expression was normalized to the expression of Lamin B and β -actin or GAPDH. Data are represented as mean $\pm$ SD of triplicate experiments. In **(B)**, $**P < 0.01$ versus cells treated with PrP106-126 for 3 h group; In **(C)**, $**P < 0.01$ versus cells treated with PrP106-126 for 6 h group, $##P < 0.01$ versus cells treated with PrP106-126 for 12 h.

PrP106-126, the cell viability percentage of the group transfected with BAT3 and stimulated with PrP106-126 has a significant difference between them, at the same time their cell viability percentage similar to the control group and group transfected with BAT3 vector.

Then, to further confirm the function of BAT3 in PrP106-126-induced cell death, we overexpressed the full-length recombinant BAT3 protein in Neuro2A cells, stimulated the cells with 200 μ M PrP106-126, and measured apoptosis by TUNEL assay using confocal microscopy. BAT3 protein levels were determined by Western

blot analysis (Figure 4B). In Neuro2A cells transfected with BAT3-HA vectors for 48 h before exposure to 200 μ M PrP106-126, there was significantly lower apoptosis as shown by weak fluorescence intensity in the TUNEL staining. Whereas significant apoptosis occurred in the control vector-transfected cells and untransfected cells incubated with PrP106-126 as shown by intense TUNEL-positive staining (Figure 5A). These data indicated that BAT3 downregulated PrP106-126-induced apoptotic cell death.

Overexpression of Full-length BAT3 Inhibited PrP106-126-induced Cytochrome *c* Release and Stabilized the Bcl-2 Protein

Next, we investigated possible protective mechanisms of BAT3 against PrP106-126-induced neuronal apoptosis. Previously, there had been studies showing that the apoptotic cell death induced by proteins such as ricin [26] and the immunodominant *M. tuberculosis* antigen ESAT-6 (early secreted antigenic target-6) [15] is downregulated by BAT3 via direct interaction of BAT3 with the protein itself or anti-apoptotic proteins, such as amyloid beta (A4) precursor-like protein 2 (APLP2) [23] and antiapoptotic protein

Bcl-2 (B cell lymphoma-2) [15]. Several researches showed that PrP106-126 peptide induced mitochondrial cytochrome *c* release, caspase 3 activation and the reduction in Bcl-2 [2,22]. We reasoned that BAT3 might inhibit PrP106-126-induced apoptosis by modulating mitochondrial cytochrome *c* release and Bcl-2 [2,22].

First, we stimulated Neuro2A cells with 200 μ M PrP106-126 and harvested at different time points, confirming enhanced cytochrome *c* release to the cytoplasm and decreased Bcl-2 protein levels by Western blotting (Figure 6A). Next, we determined the intracellular localization of exogenous full-length BAT3 and whether it interacted with PrP106-126. We transfected primary neurons with the full-length HA-tagged BAT3 vector and stained with a monoclonal antibody against the HA-tag. Expression of BAT3 was visualized with Alexa Fluor 594-conjugated goat anti-mouse IgG and inspected by fluorescence microscopy. Figure 6B displays that full-length exogenous BAT3 was prominently expressed in the cytoplasm of primary neurons with negligible distribution in the nucleus. After exposure to PrP106-126-FITC, primary neurons displayed enhanced cytosolic labeling of BAT3 in comparison to cells treated with PBS. Exogenous full-length BAT3 colocalized with PrP106-126 in the cytoplasm (Figure 6B).

Figure 5 BAT3 overexpression increases cell viability and suppresses PrP106-126-induced neuronal apoptosis. **(A)** Protective effects of BAT3 in cell viability measured by the CCK-8 assay. Primary cultures of rat cortical neurons transfected with the HA control vector or full-length BAT3-HA vector for 48 h before incubation with PrP-106-126 (200 μ M) for 24 h. The values are percent viable cells relative to the control (no treatment) (mean $\pm$ SD, $n = 5$). The data were analyzed using a one-way ANOVA followed by *post hoc* Tukey's multiple comparison tests. ** $P < 0.01$ versus the PrP106-126+BAT3 Vector group. **(B)** Apoptotic cells were labeled using the *in situ* cell death detection kit, POD (TUNEL). Representative fluorescent images of TUNEL-positive (green) Neuro2A cells transfected with the HA control vector or full-length BAT3-HA vector for 48 h before incubation with PrP-106-126 (200 μ M) for 24 h. The cells were counterstained with propidium iodide (PI) (red) to show nuclei. Scale bar = 5 μ m. **(C)** Western blot analysis of BAT3 from Neuro2A cells described in **(A)**. β -actin was used as a loading control. Total cell lysate was loaded in 10% SDS-PAGE gel for Western blotting.

Figure 6 (A) PrP-(106-126) induced Cytochrome *c* release and BCL-2 protein decrease in the cytoplasm. Neuro2A cells were treated with PrP-(106-126) (200 μ M) for 24 h, and then, cytoplasmic fractions were collected and processed for immunoblotting with anticytochrome *c* antibody and Bcl-2 antibody. The values are normalized to the expression of β -actin. The under histogram plots show the densitometric quantification of cytochrome *c* and Bcl-2 in the indicated groups. The values are normalized to the expression of β -actin. Data are represented as mean $\pm$ SD of triplicate experiments; * P < 0.05, ** P < 0.01 versus cells treated with PrP106-126 for 6 h.

(B) The exogenous BAT3 colocalized with PrP-(106-126) in the cytoplasm. Primary cultured cortical neurons were transfected with BAT3-HA vector for 48 h; then, they were incubated in FITC-labeled PrP106-126 or PBS for 24 h, stained with anti-HA antibody for observing the exogenous BAT3 by an upright fluorescence confocal microscopy. Left panel displays the control or FITC-labeled PrP106-126 groups (green), second panel displays exogenous BAT3-HA staining (red), third panel displays DAPI staining (blue), right panel merging of images. Scale bars = 5 μ m.

We further examined the role of BAT3 in PrP106-126-induced neuronal apoptosis and its relationship with Bcl-2 and cytochrome *c* in the apoptotic process. We transfected the BAT3-HA vector in Neuro2A cells and exposed the cells to PrP106-126. Compared with empty vector-transfected cells, overexpression of BAT3 suppressed PrP106-126-induced cytochrome *c* release and caused an elevation of the endogenous Bcl-2 protein level in the cytoplasm (Figure 7A). To further confirm the inhibitory activity of BAT3 overexpression on the release of cytochrome *c*, Neuro2A cells transfected with various amounts of plasmids encoding full-length BAT3 were treated with PrP106-126. The release of cytochrome *c* was decreased in a dose (BAT3 plasmid dose)-dependent manner (Figure 7B). The optimal BAT3 protein expression not only depended on the BAT3 plasmid dose but also related to cell number and the amount of Lipofectamine reagent. These findings suggest that BAT3 interacts with PrP106-126 and inhibits apoptotic activity of the prion peptide through the modulation of cytochrome *c* and Bcl-2. We describe a schematic model of signaling pathways for BAT3 regulates PrP106-126-induced neurons apoptosis (Figure 8).

Discussion

In this study, we showed a positive correlation of BAT3 and PrP expression in the cytoplasm of HeLa and neuronal cells. BAT3 translocated from the nucleus to cytoplasm in response to exposures to PrP106-126, and overexpression of BAT3 alleviated PrP106-126-induced apoptosis, downregulated cytochrome *c* release from mitochondria, and stabilized Bcl-2. We propose that shuttling of BAT3 between the nucleus and the cytoplasm may contribute to its function of regulating apoptotic cell death induced by PrP106-126, and BAT3 modulates PrP106-126-induced cell death by downregulation of cytochrome *c* release and stabilization of Bcl-2.

In prion diseases, only brain appears to be predominantly affected despite widespread expression of PrP in multiple cell types both in and out of the nervous system [27,28]. Thus, we used Neuro2A cells and primary neurons, in addition to HeLa cells, in our experiments. Using GFP-tagged full-length PrP protein, we showed the colocalization of PrP with endogenous BAT3 in cytoplasm. Endogenous PrP protein level was upregulated by

Figure 7 Overexpression of full-length BAT3 inhibits PrP-(106-126)-induced cytochrome c release and stabilizes the BCL-2 protein. **(A)** Cytosolic fractions of BAT3-HA vector or empty vector-transfected Neuro2A cells were treated with PrP106-126 for 24 h and then processed as Fig. 6 **(A)**. Cytosolic fractions were analyzed by immunoblotting with anti-HA, anti-cytochrome c, anti-Bcl-2, or anti- β -actin antibody. The β -actin immunoblot was indicated as an internal control. The histogram plots analysis methods were the same as before. **(B)** BAT3 inhibits PrP106-126-induced cytochrome c release in a dose-response manner. Neuro2A cells transfected with various amounts of plasmids encoding BAT3 were treated with PrP106-126, and the cytosolic extracts were analyzed by immunoblotting with anti-BAT3 or anticytochrome c antibody. The β -actin immunoblotting was indicated as a control of protein loading. The histogram plots analyzed as above and showed the dose-response manner of cytochrome c release after BAT3 overexpression. Data are represented as mean $\pm$ SD of triplicate experiments; ** P < 0.01 versus cells transfected with control vectors only group.

Figure 8 Schematic model of signaling pathways for the regulation of PrP106-126-induced neuronal apoptosis by BAT3. Upon the stimulation of neuronal cells by PrP106-126, endogenous BAT3 translocates from nucleus to cytoplasm. Endogenous and exogenous BAT3 in cytoplasm function as prosurvival proteins by stabilizing the level of BCL-2 protein and inhibiting the release of cytochrome c from mitochondria to cytoplasm induced by PrP106-126. EX-BAT3, exogenous BAT3; EN-BAT3, endogenous BAT3; Mito. stress, mitochondrial stress; Cyt. c, cytochrome c. — or — inhibition.

overexpression of the full-length BAT3 in HeLa and Neuro2A cells, a mouse neuroblastoma cell line, confirming the ability of BAT3 to stabilize PrP. For mislocalized proteins, deubiquitination may enable productive interactions of proteins with specific delivery factors and/or molecular chaperones that facilitate the acquisition of a native structure and thereby counteract premature degradation. It is possible that if deubiquitinated precursors are unable to initiate productive interactions, the proteins might be reubiquitinated via BAT3 to facilitate their degradation.

It has been reported that BAT3 enhances the stability of interacting proteins by reducing their ubiquitylation and degradation [23,29]. The ubiquitin-like domains of BAT3 are required for apoptotic control during the normal development of the *Xenopus* embryo through interactions with the Xrpn10c subunit of the proteasome via the N-terminal 436 residues [16,30]. PrP as a BAT3-interacting protein [9] was also reported to exert cytoprotection of neuronal cells, particularly against internal or environmental stresses that initiate apoptosis [31,32]. The mechanisms of the induction of PrP expression by BAT3 and the involvement of BAT3 in prion diseases and associated cell death are open for further research.

The phenomenon of proteins shuttling between the nucleus and cytoplasm is a mechanism of regulating apoptosis and maintaining basal activities of transcription factors by signaling molecules [33–37]. BAT3 was reported to act as a nucleus-cytoplasm

shuttling protein regulating apoptotic cell death induced by M. tuberculosis antigen ESAT-6 (early secreted antigenic target-6) [15], camptothecin (CPT)-a topoisomerase inhibitor known to induce apoptosis [23], thapsigargin-a stimulus for endoplasmic reticulum stress-induced apoptosis [14], and papillomavirus binding factor (PBF) [38]. BAT3 is a nuclear protein with a nuclear localization signal (NLS) and a nuclear export signal (NES) [14,39], and thus, it is subject to nucleus export. Consistent with the already reported findings, our present study also demonstrated a time-dependent export of BAT3 from nucleus to cytoplasm in Neuro2A cells exposed to PrP106-126. The translocation of BAT3 is thought to be at least in part due to the interaction of BAT3 with a cofactor named TRC35 (transmembrane domain recognition complex 35), which masks the NLS [20,40]. We propose that the transiently elevated protein levels of BAT3 induced by PrP106-126 in the cytoplasm initially resist apoptotic changes in the cell, but BAT3 then may degrade due to caspase-3 cleavage and proteasomal degradation *in vitro* in a dynamic equilibrium. The pathway of its degradation may be similar to another member of the BAG family of protein, BAG3, which is transiently increased before degradation by caspases and proteasomal degradation during ER stress-induced apoptosis [41]. The nucleus to cytoplasm export mechanism and association of BAT3 with specific cellular partners in response to PrP106-126 are yet to be elucidated.

Considering the regulatory role of BAT3 in apoptosis [13,42], we reasoned that BAT3 translocation may be a protective mechanism against PrP106-126-induced apoptosis. We explored whether the BAT3 protein affects Neuro2A cell apoptosis. The most interesting result of the present study is the finding that overexpression of BAT3 protected Neuro2A cells from PrP106-126-induced cell death. We further demonstrated that increased expression of BAT3 inhibited the release of cytochrome *c* and stabilized the antiapoptotic protein Bcl-2 level in cytoplasm of Neuro2A cells stimulated with PrP106-126. The release of cytochrome *c* was inhibited by BAT3 in a dose (BAT3 plasmids dose)-dependent manner in response to PrP106-126 stimulation, indicating that BAT3 either directly regulates cytochrome *c* release from mitochondria or indirectly through the stabilization of Bcl-2. It has been shown that overexpression of Bcl-2 inhibits programmed cell death induced by various stimuli [43,44] and especially Bcl-2 was shown to block apoptosis by inhibiting the release of

cytochrome *c* from mitochondria [45,46]. On the other hand, BAT3 (aka BAG6) belongs to the BAG family of proteins that have an evolutionarily conserved BAG domain. Other members of the BAG family, such as BAG1 [47], BAG3 [48] and BAG4 [49], associate with Bcl-2 through the BAG domain. BAT3 may behave similarly and interact with Bcl-2.

In brief, our study demonstrated that overexpression of BAT3 inhibits PrP106-126 induced apoptosis by reducing the release of cytochrome *c*, and this pathway may at least partly through the stabilization of Bcl-2. In addition to the diverse regulatory functions in protein biogenesis and degradation [50,51], BAT3 may also protect cells from apoptosis by influencing the degradation of certain apoptotic factors [16] or unknown chaperones. Further evaluation of the signal pathway of BAT3 as a prosurvival protein in PrP106-126 induced apoptosis is the next challenge to help understand the exact role of this enigmatic but multitasking protein in prion diseases.

Conclusions

Our present data describes a novel molecular mechanism regulating PrP106-126 induced apoptotic process through the overexpression of BAT3, which may be important for the basic regulatory mechanism of neuron survival in prion diseases and associated neurodegenerative diseases *in vivo*.

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Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

The following supplementary material is available for this article:

Figure S1. Primary cultures of rat cortical neurons were plated on coverslips, 7 days later stained with two kinds of neuronal specific antibodies, Rabbit Anti-TUBB3/beta III Tubulin (Neuronal

Marker) (bs-4512R) (β III-tub) (left, red) and Neuronal Class III β -Tubulin (Tuj1) antibody (AT809) (Beyotime, China) (right, green), to identified the primary cultures of rat cortical neurons. The cells were counterstained with DAPI (blue) to show all cell nuclei. Scale bars = 20 μ m.