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ORIGINAL ARTICLE

GSK-3β inhibition by curcumin mitigates amyloidogenesis via TFEB activation and anti-oxidative activity in human neuroblastoma cells

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ABSTRACT

The translocation of transcription factor EB (TFEB) to the nucleus plays a pivotal role in the regulation of basic cellular processes, such as lysosome biogenesis and autophagy. Autophagy is an intracellular degradation system that delivers cytoplasmic constituents to the lysosome, which is important in maintaining cellular homeostasis during environmental stress. Furthermore, oxidative stress is a critical cause for the progression of neurodegenerative diseases. Curcumin has anti-oxidative and anti-inflammatory activities, and is expected to have potential therapeutic effects in various diseases. In this study, we demonstrated that curcumin regulated TFEB export signalling *via* inhibition of glycogen synthase kinase-3β (GSK-3β); GSK-3β was inactivated by curcumin, leading to reduced phosphorylation of TFEB. We further showed that H_2O_2-induced oxidative stress was reduced by curcumin *via* the Nrf2/HO-1 pathway in human neuroblastoma cells. In addition, we showed that curcumin induced the degradation of amyloidogenic proteins, including amyloid-β precursor protein and α-synuclein, through the TFEB-autophagy/lysosomal pathway. In conclusion, curcumin regulates autophagy by controlling TFEB through the inhibition of GSK-3β, and increases antioxidant gene expression in human neuroblastoma cells. These results contribute to the development of novel cellular therapies for neurodegenerative diseases.

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Autophagy; neurodegenerative diseases; heme oxygenase-1; Alzheimer's Disease; Parkinson's Disease

Introduction

Autophagy plays an important role in cellular homeostasis through the elimination of old or damaged organelles, as well as aggregated intracellular proteins [1,2]. It is associated with a variety of biological functions, such as development, cellular differentiation, defense against pathogens, and nutritional starvation. Defects in autophagic regulation play a central role in a number of diseases, including neurodegenerative diseases, cancer, pathogen infection, and metabolic diseases [3]. In addition, autophagy has been reported to be involved in the occurrence of pathological changes in many neurodegenerative disorders, including Alzheimer's disease (AD) and Parkinson's disease (PD) [4]. The most prevalent pathological features of many neurodegenerative diseases are the aggregation of misfolded proteins and the loss of certain neuronal populations [5]. Moreover, oxidative stress is a major factor in neurodegenerative disorders [6–8]. The induction of antioxidant enzymes, such as haem oxygenase-1 (HO-1), prevents a key early pathogenic process in AD [9].

Numerous studies have shown that curcumin has broad biological functions, including antioxidant and anti-inflammatory activities [10–12]. Curcumin exerts antioxidant activity in direct and indirect ways by scavenging reactive oxygen species (ROS) and inducing an antioxidant response, respectively [13,14]. Moreover, curcumin is known to promote transcription factor EB (TFEB) nuclear translocation [15]. TFEB, a master gene for lysosomal biogenesis, coordinates this programme by driving the expression of autophagy and lysosomal genes [16]. TFEB recognises and binds to a regulatory sequence, the coordinated lysosomal expression and

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Supplemental data for this article can be accessed here.

regulation (CLEAR) motif, which is present in the promoter region of several autophagy-related genes [17]. This TFEB activity depends on its phosphorylation status, which is mainly regulated by the mechanistic target of rapamycin complex 1 (mTORC1), calcineurin, which is a Ca^{2+}-dependent phosphatase, and mTOR-independent glycogen synthase kinase-3β (GSK-3β) [18,19].

GSK-3β is a serine/threonine kinase that is inhibited by a phosphorylated Ser9 residue, and its phosphorylation or dephosphorylation serves an important switch that regulates GSK-3β activity [20,21]. The binding of GSK-3β by curcumin potently inhibits its activity, and induces TFEB nuclear translocation by inhibiting TFEB phosphorylation [21,22]. Phosphorylated TFEB is retained in the cytoplasm, whereas dephosphorylated TFEB translocates to the nucleus to induce the transcription of target genes associated with autophagy [18]. The subcellular distribution of TFEB mainly depends on its phosphorylation status. A recent study has reported that phosphorylated TFEB by GSK-3β, at the serine 134 and 138 residues, can inhibit TFEB nuclear translocation. Phosphorylation of S134 and S138 appear to be critical for TFEB localisation to the surface of lysosome, where it interacts with mTORC1. Double mutations of S134A and S138A TFEB decreased lysosomal localisation, and leading to its nuclear localisation [23]. According to numerous reports, genetic or pharmacological activation of TFEB was shown to be beneficial in a variety of neurodegenerative diseases [24–26]. In neurodegenerative diseases, amyloidogenic proteins, such as amyloid-β (Aβ) precursor proteins or α-synuclein (α-syn), have an increased tendency to aggregate [27,28]. The hallmarks of AD are extracellular senile plaques of aggregated Aβ proteins, intracellular neurofibrillary tangles, and synaptic and neuronal loss [29–31]. In addition to the typical pathological changes of AD, oxidative stress is also reported to be an important pathological phenomenon that starts to manifest early in the course of AD [32–34]. Furthermore, accumulative evidence has shown that the aggregation of α-syn in neuronal cells is associated with PD [35,36]. It is well demonstrated that GSK-3β dysregulation can promote the pathology of neurodegenerative diseases such as PD [37]. Additionally, curcumin has been identified as a potential neuroprotective agent in PD [38].

The underlying mechanism of curcumin involved in the regulation of TFEB translocation in human neuroblastoma cells is largely unknown. Here, we hypothesised that the suppression of GSK-3β by curcumin could promote the TFEB-autophagy/lysosomal pathway, and protect cells from the deposition of aggregated proteins and H_2O_2-induced oxidative stress in human neuroblastoma cells.

Materials and methods

Cell culture and chemical treatment

A human neuroblastoma cell line (SH-SY5Y) was grown in Dulbecco's Modified Eagle medium, DMEM (Gibco, Grand Island, USA), supplemented with 4.5 g/L D-glucose, L-Glutamine, 110 mg/L Sodium pyruvate, 10% foetal bovine serum (Gibco, Melbourne, Australia) and 1% penicillin-streptomycin solution (Gibco, Grand Island, USA). The cells were maintained at 37 °C in a saturated humidity atmosphere containing 95% air and 5% CO2. SH-SY5Y cells were treated with 2.5, 5, 10, and 20 μM curcumin (Sigma Aldrich, St. Louis, MO, USA) for 12 and 24 h, respectively.

Immunofluorescence assay

SH-SY5Y cells inoculated on coverslips were cultured for 24 h. Then, cells were fixed with 4% paraformaldehyde for 15 min, and permeabilized with 0.1% Triton X − 100 for 5 min. Following blocking with 3% BSA in phosphate-buffered saline (PBS) for 30 min, cells were incubated with primary antibody: anti-α-syn (cell signalling, MA, USA, 1:500 dilution) and anti-amyloid-β precursor protein (Sigma, MO, USA, 1:500 dilution); overnight at 4 °C. After primary antibody incubation, cells were washed with PBS and incubated with Alexa-Fluor 594 goat anti-rabbit secondary antibody (Invitrogen, CA, USA, 1:500 dilution) for 1 h at room temperature, stained with DAPI (1 μg/mL) for 15 min and washed with PBS. Images were captured using an Olympus FV1200 confocal microscope (Olympus, Tokyo, Japan).

Nuclear/cytosol fraction

Cells were harvested *via* mechanical scraping and split into nuclear and cytoplasmic fractions utilising a nuclear/cytosol fractionation kit (BioVision, CA, USA), according to the manufacturer's protocol.

Cell viability assay

WST-8 (QuantiMax^{TM}-cell viability assay kit) was used to analyse cell viability according to the manufacturer's instructions. SH-SY5Y cells (1×10^5/well) were cultured in 96-well plates, with triplicate wells for each group. When 80–90% confluence was reached, cells were treated with curcumin (2.5, 5, 10, 20, 40 and 80 μM) at

different concentrations in 100 µl DMEM medium and incubated for 2 h. Then QuantiMax™ solution was added to each well followed by a further 4 h incubation under 5% CO_2 at 37 °C. Absorbance was measured at 450 nm with a microplate reader (SpectraMax iD3, TECAN Group Ltd.). The mean optical density (OD, absorbance) of three wells in the indicated groups was used to calculate the percentage of cell viability.

Transfection

SH-SY5Ycells were transfected with pEGFP-N1-TFEB using Lipofectamine™ 2000 (Invitrogen, CA, USA) in accordance with the manufacturer's protocol. To knock down the GSK-3β gene, SH-SY5Y cells were transfected with scramble siRNA (scRNA) and human GSK-3β siRNA (Santa Cruz, TX, USA) using Lipofectamine™ 2000 (Invitrogen, CA, USA). To assess the role of GSK-3β in TFEB nuclear translocation, SH-SY5Y cells were transfected with pcDNA3.1, constitutively active GSK3β (pcDNA3.1-GSK3β-S9A, CA), and kinase-dead GSK3β (pcDNA3.1-GSK3β-K85A, KD). To measure the effect of curcumin on the amyloid-β precursor protein (APP) and α-synuclein aggregation, SH-SY5Y cells were transfected with pCAX-APP695-WT (FL-APP WT), pCAX-APP-Swe/Ind (APP-Swe/Ind), p3x-flag-human α-synuclein-WT (α-syn WT), and p3x-flag-human α-synuclein-A53T (α-syn A53T) for 48 h and then processed for immunofluorescence microscopy. All plasmids were purchased from Addgene (MA, USA).

Western blot

Total proteins extracted from harvested cells were prepared in Mammalian Cell-PE LB™ Buffer (GBiosciences, MO, USA) containing phosphatase inhibitors (SIGMA-aldrich, MO, USA), and protease inhibitors (SIGMA-Aldrich, MO, USA). Protein concentration was determined using a BCA protein assay kit (Pierce Biotechnology, IL, USA). For western blot analysis, protein samples were separated using SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, MA, USA). The membrane was blocked with 5% skim milk (BD Difco™ Skim Milk, PA, USA) in PBS-Tween 20 and then incubated with a primary antibody against either TFEB (Cell signalling, MA, USA, 1:1000 dilution), GSK-3β (Cell signalling, MA, USA, 1:1000 dilution), p-GSK3β (Cell signalling, MA, USA, 1:1000 dilution), p-β-catenin (Cell signalling, MA, USA, 1:1000 dilution), β-catenin (Cell signalling, MA, USA, 1:1000 dilution), PARP1 (Cell signalling, MA, USA, 1:2000 dilution), α-tubulin (Cell signalling, MA, USA, 1:2000 dilution), LAMP1 (Abcam, MA, USA, 1:2000 dilution), LC3 (Novus Biologicals, CO, USA, 1:1000 dilution), FL-APP (Sigma Aldrich, MO, USA, 1:1000 dilution), α-synuclein (Cell signalling, MA, USA, 1:1000 dilution), or β-actin (Invitrogen, CA, USA, 1:2500 dilution) followed by incubation with a secondary antibody (Jackson ImmunoResearch Laboratories, PA, USA, 1:5000 dilution). All experiments were performed at least three times, and representative results are shown. Chemiluminescent signal was measured with an Azure Biosystems C300 analyser (Azure Biosystems, Dublin, CA) using an ECL detection system (GE Healthcare Bio-Sciences, Little Chalfont, UK). The band density was analysed by using Image J2x software (US National Institutes of Health, Bethesda, MD).

Real-time quantitative-PCR (qRT-PCR)

Total RNA was extracted from cellular lysates using Trizol (Life Technologies, CA, USA) according to the manufacturer's instructions. cDNA was synthesised by using oligo (dT) primers (Bioneer, Daejeon, Korea) and M-MLV reverse transcriptase (Promega, WI, USA) according to the manufacturer's protocol. The cDNA product was amplified using SYBR Green PCR Master Mix (Applied Biosystems, CA, USA) on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, CA, USA). mRNA expression data were normalised to GAPDH gene expression. The forward and reverse primers for human MCOLN1 were 5′- GAGTGGGTGCGACAAGTTTC-3′ (forward) and 5′-TGTTCTCTTCCCGGAATGTC-3′ (reverse), and for human LAMP1 were 5′-CGTACCTTTCC AACAGCAGC-3′ (forward) and 5′-CGCTCACGTTGTACTT GTCC-3′(reverse), and for human GAPDH were 5′-CAA TGACCCCTTCATCCTC-3′ (forward) and 5′-AGCATCGCC CCACTTGATT −3′ (reverse).

Autophagy flux assay using tandem fluorescent-tagged LC3

SH-SY5Y cells were transfected with pBABE-puro-mCherry-EGFP-LC3B (Addgene, Cambridge, MA, USA, #22418). After incubation with curcumin or other treatments, the cells were fixed with 4% parpformadehyde and then observed under Olympus FV1200 confocal microscope (Olympus, Tokyo, Japan).

Luciferase assay

For the dual luciferase assay, SH-SY5Y cells were grown in a 96-well plate and co-transduced with a pCignal Lenti-ARE reporter (Qiagen, Hilden, Germany) and pCignal Lenti-TK-Renilla (Qiagen, Hilden, Germany).

After 72 h, cells were pre-treated with 20 μM curcumin and 10 mM LiCl for 30 min followed by the administration of H_2O_2 (200 μM) for 6 h. Then, treated cells were lysed with passive lysis buffer (Promega, Fitchburg, WI, USA) and mixed with luciferase assay reagents (Promega). The chemiluminescent signal was detected using a SpectraMax L Microplate reader (Molecular Devices, Sunnyvale, CA). Firefly luciferase was normalised to Renilla luciferase in each sample.

ROS measurement

To measure intracellular ROS by flow cytometry, SH-SY5Y cells were first transfected with scRNA and siRNA against GSK-3β for 36 h. Then cells were pre-treated with curcumin (20 μM) and LiCl (10 mM) for 3 h followed by the stimulation of 200 μM H_2O_2 for 6 h. Then cells were placed in fluorescence-activated cell sorting tubes, and washed with 1xPBS. Cells were incubated in 1xPBS containing 3 μM CM-H_2DCFDA (C6827, Invitrogen, CA) for 40 min at 37 °C. DCF-DA fluorescence was detected using a fluorescence-activated cell sorter (FACSCanto II) and data was analysed with FlowJo V10 software (Tree Star Inc., San Carlos, CA). The fold of change is presented by mean fluorescence intensity.

Statistical analysis

For statistical comparisons, all values were expressed as mean ± SD. Statistical differences between samples were assessed by ANOVA with *post hoc* Turkey's honestly significant difference (HSD) test. Data were analysed and presented using GraphPad Prism software version 5.03 (San Diego, CA). Probability values of $p \leq 0.05$ were considered to represent a statistically significant change.

Results

Curcumin promotes TFEB nuclear translocation through the inhibition of GSK-3β activity in SH-SY5Y cells

A cell viability assay was initially employed to investigate any potential cytotoxic effects of curcumin in the neuroblastoma cell line (SH-SY5Y cells); the results showed that treatment with various concentrations of curcumin had no significant impact on cell viability (Supplementary Figure S1(A)). Then, to determine the effect of curcumin on GSK-3β activity, we examined GSK-3β phosphorylated at S9 (p-GSK-3β (S9)), which is considered an inactive form of GSK-3β, under different concentrations of curcumin (0, 2.5, 5, 10, and 20 μM). Here, we treated cells with LiCl, a GSK-3β inhibitor, to stimulate p-GSK-3β (S9), as a positive control to assess the effect of curcumin on GSK-3β activity. Immunoblotting results clearly supported that curcumin enhanced p-GSK-3β (S9) in a dose-dependent manner (Figure 1(A)). To further assess the inhibitory effect of curcumin on the activity of GSK-3β, we measured the levels of phosphorylation of a well-characterized substrate of GSK-3β, β-catenin. Our results showed that the administration of curcumin dramatically decreased the phosphorylation of β-catenin due to the inactivation of GSK-3β (Figure 1(A)). A previous study has shown that a novel curcumin analog can induce the translocation of TFEB from the cytosol to the nucleus [39]. Next, to examine the role of curcumin in TFEB nuclear translocation in a human neuroblastoma cell line, we transfected SH-SY5Y cells with GFP-tagged TFEB for 48 h, and observed that the curcumin treatment could dramatically increase the nuclear translocation of TFEB (Figure 1(B,C)). Torin1, an mTOR inhibitor, (Figure 1(B)) or LiCl (Supplementary Figure S1(B)) was used as positive controls. A previous study showed that cells transfected with siRNA against GSK-3β (siGSK-3β) and pharmacological inhibition of GSK-3β could both promote TFEB nuclear translocation and subsequently elevate the autophagy-lysosomal pathway [40]. To evaluate the critical role of GSK-3β in TFEB nuclear translocation followed by curcumin treatment, we transfected SH-SY5Y neuroblastoma cells with two kinds of mutant forms of GSK-3β, GSK-3β CA and GSK-3β KD. We first isolated the nuclear and cytoplasmic fraction, and examined the relative expression of TFEB in these fractions. As we expected, curcumin treatment could significantly increase the nuclear translocation in cells transfected with pcDNA, while GSK-3β CA transfection compromised the beneficial effect of curcumin on TFEB nuclear translocation. Interestingly, the nuclear fraction isolated from cells transfected with GSK-3β KD showed a remarkable increase of TFEB expression in the presence or absence of curcumin treatment (Figure 1(D)). SH-SY5Y cells were treated with Torin1 (Figure 1(D)) and LiCl (Supplementary Figure S1(C)) as positive controls. These results clearly demonstrated that curcumin could promote TFEB nuclear translocation *via* the phosphorylation of GSK-3β at the serine 9 residue in SH-SY5Y cells.

Inhibition of GSK-3β by curcumin facilitates autophagy progression

Our results demonstrated that curcumin could promote TFEB nuclear localisation, which was found to be associated with the inactivation of GSK-3β (Figure 1). TFEB

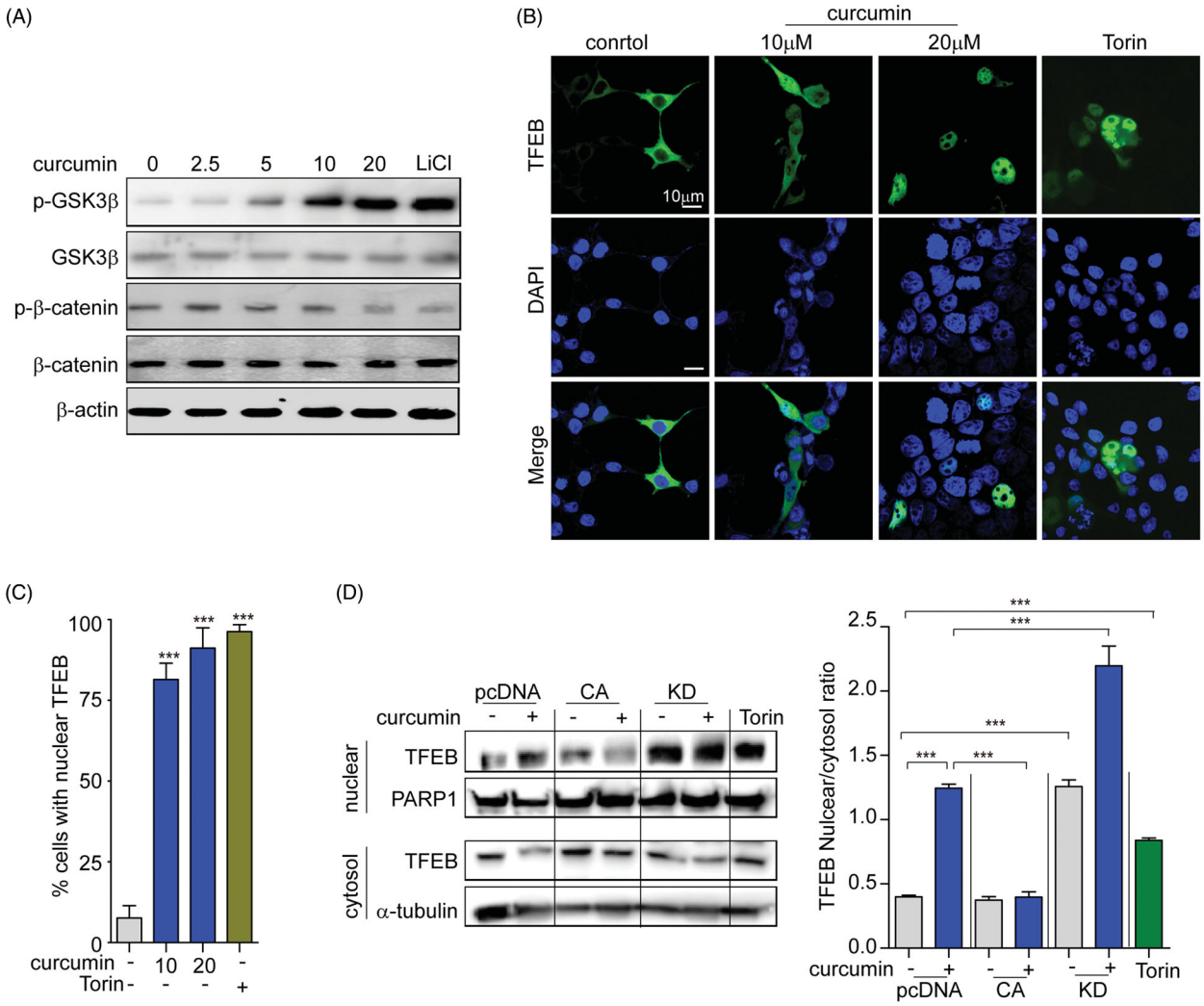

Figure 1. Curcumin promotes TFEB nuclear translocation through the inhibition of GSK-3β activity in SH-SY5Y cells. (A) SH-SY5Y cells were treated with curcumin in various concentrations (0, 2.5, 5, 10 and 20 μM) for 2 h. The protein levels of p-GSK-3β, GSK-3β, p-β-catenin, β-catenin, and β-actin were evaluated by western blot analysis. LiCl is used as positive control for the phosphorylation of GSK-3β. (B) An immunofluorescence assay was performed to observe the nuclear translocation of TFEB. SH-SY5Y cells were transfected with pEGFP-N1-TFEB for 48 h, and then cells were treated with curcumin (10 μM and 20 μM) and Torin1 (5 μM) for 6 h. Confocal microscopy was utilised to detect localisation of TFEB (green) and nuclei (blue). Scale bar = 10 μm. (C) The percentage of cells with nuclear TFEB. (D) SH-SY5Y cells were transfected with pcDNA3.1, GSK3β CA, and GSK3β KD for 48 h, followed by the administration of 20 μM curcumin for 2 h, and the protein level of TFEB was assessed in the nuclear and cytoplasmic fractions by western blot analysis (*left panel*). The quantification of TFEB nucleus-cytoplasm ratios from these experiments is represented in the histogram (*right panel*). Data presented are the means ± SD ($n = 3$); *** $p < 0.001$.

nuclear translocation plays a fundamental role in the progression of autophagy by upregulating master genes involved in lysosomal biogenesis [16]. Therefore, we next investigated whether curcumin could induce autophagy through the inactivation of GSK-3β. We first examined autophagy- related genes, including lysosomal-associated membrane protein 1 (LAMP-1), which is one of the target genes of TFEB, and lipid bound LC3-II, in various concentrations of curcumin. As shown in Figure 2(A), we found that curcumin treatment could significantly increase the protein levels of LAMP1 and LC3-II, and decrease the levels of p62. In addition, SH-SY5Y cells were treated with Torin1, as a positive control to assess the beneficial effect of curcumin on autophagy. Then, in order to investigate whether the inhibition of GSK-3β mediated by curcumin contributes to the induction of autophagy, we transfected SH-SY5Y cells with scRNA or siGSK-3β, after 48 h transfection, cells were treated with either curcumin or Torin1 (Figure 2(B)). Cells transfected with scRNA showed a dramatic increase in LAMP1 mRNA and LC3-II protein levels in the treatment of curcumin and torin1. Interestingly, we observed siGSK-3β-transfected cells without treatment could significantly increase the

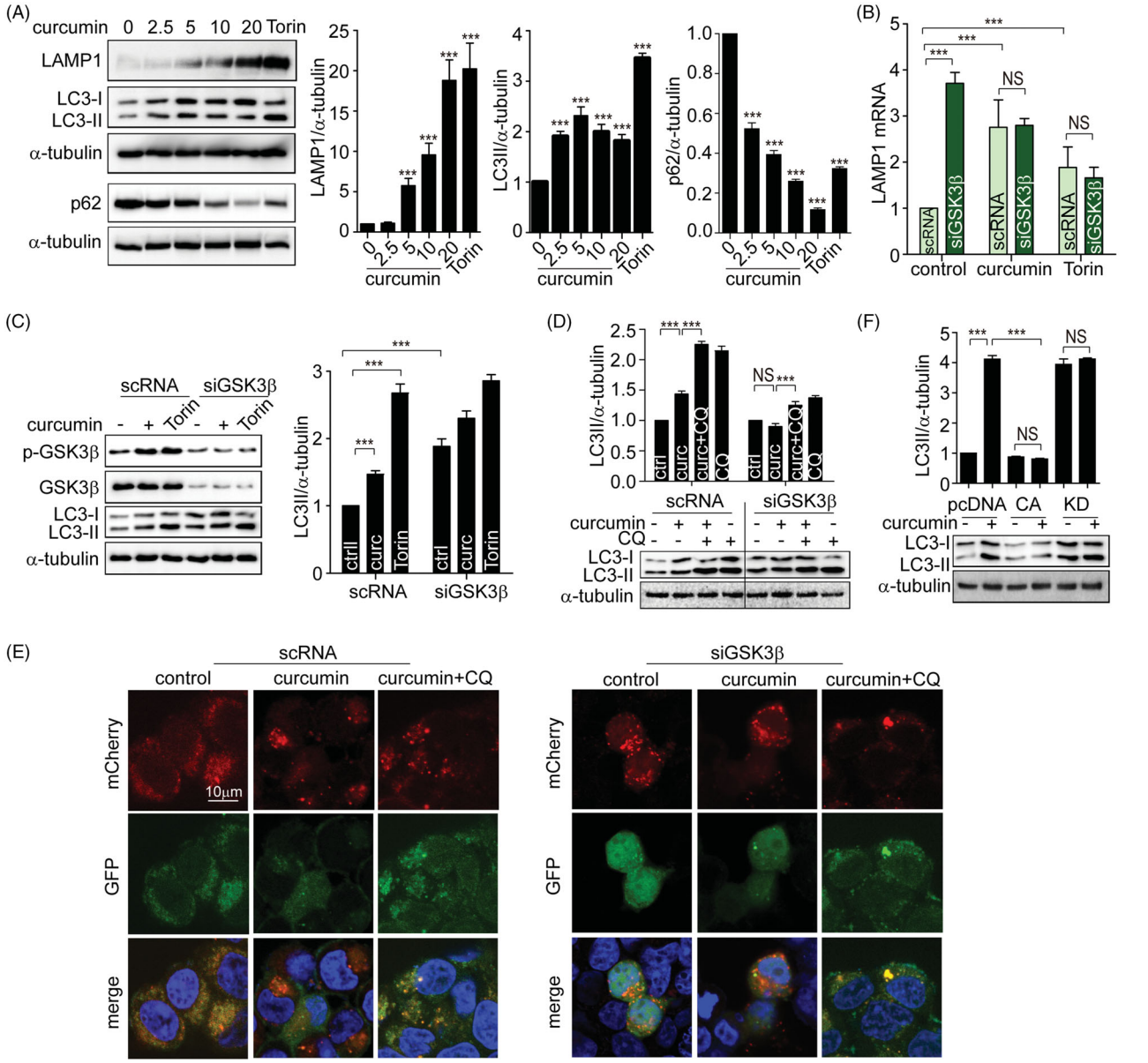

Figure 2. Inhibition of GSK-3β by curcumin facilitates autophagy progression in SH-SY5Y cells. (A) SH-SY5Y cells were treated with curcumin in a dose-dependent manner (0, 2.5, 5, 10, and 20 μM), and with Torin 1 (5 μM, 3 h) as a positive control. The protein levels of LAMP1, LC3, p62, and α-tubulin were detected by western blot analysis (*left panel*). The quantification of LAMP1/α-tubulin, LC3-II/α-tubulin, and p62/α-tubulin (*right panel*) from these experiments is represented. (B) SH-SY5Y cells transfected with scramble siRNA (scRNA) and siRNA against GSK-3β (siGSK-3β) for 36 h, then cells were treated with curcumin and torin 1. scRNA and siGSK-3β transfected cells were treated with curcumin (20 μM) and torin 1 (5 μM) for 24 h, and the mRNA expression of LAMP1 was analysed by quantitative real time (qRT)-PCR. (C, D) SH-SY5Y cells were transfected with scRNA or siGSK-3β for 36 h, and then cells were treated with curcumin (20 μM) for 24 h. (C) The transfected cells were treated with Torin 1 (5 μM) for 3 h. The protein levels of p-GSK-3β, GSK-3β, LC3 and α-tubulin were detected by western blot analysis (*left panel*). The quantification of LC3-II to α-tubulin ratio from these experiments is represented in *right panel*. (D) The transfected cells were pre-treated with CQ (5 μM) for 1 h. The protein levels of LC3 and α-tubulin were detected by western blot analysis (*bottom panel*). The quantification of LC3-II to α-tubulin ratio from these experiments is represented in *upper panel*. (E) SH-SY5Y cells were co-transfected with either scRNA or siGSK3β and mCherry-EGFP-LC3 and then treated with 20 μM curcumin for 24 h in presence or absence of 10 μM chloroquine (CQ). The confocal images of SH-SY5Y cells showed yellow (autophagosomes) and red (autolysosomes) puncta. Scale bar represents 10 μm in all images. (F) SH-SY5Y cells transfected with pcDNA3.1, GSK-3β CA, and GSK-3β KD of GSK-3β for 36 h. Then, cells were treated in the presence or absence of curcumin (20 μM) for 24 h. The protein level of LC3 was determined by western blot. Data presented are mean ± SD; *** $p < 0.001$ and NS: not significant.

expression of LAMP1 and LC3-II compared to cells transfected with scRNA, while curcumin and torin1 treatment showed no significant changes in LAMP1 and LC3-II in both cells transfected with scRNA or siGSK-3β (Figure 2(B,C)). To investigate the effect of curcumin on the autophagy flux, we challenged the cells with chloroquine (CQ), an inhibitor of autophagy flux. As shown in Figure 2(D), CQ induced an increase in LC3-II as it inhibits the autophagy flux. Although the LC3-II levels were increased with curcumin treatment, the combined treatment of curcumin and CQ showed a much greater increase, indicating that curcumin did not inhibit the autophagolysosomal maturation. The deficiency of GSK-3β caused the increase of LC3-II levels, compared with the control of scRNA-transfected cells, but curcumin did not induce an increase in the levels of LC3-II relative to the control in siGSK3β-transfected SH-SY5Y cells. However, co-treatment with curcumin and CQ or the treatment with CQ alone induced a higher accumulation of LC3-II, compared with curcumin-treated SH-SY5Y cells in the deficiency of GSK-3β. To further monitor autophagy flux induced by curcumin, a tandem fluorescent-tagged LC3 (GFP-mCherry-LC3) expression plasmid was used. Consistent with Figure 2(D), curcumin increased autolysosomes (red puncta) and co-treatment with curcumin and CQ showed the increase of autophagosomes (yellow puncta) by inhibiting autophagy flux in presence of GSK3β (Figure 2(E)). Taken together, we could conclude that curcumin increases autophagy *via* GSK-3β inhibition. Next, to confirm the activity of GSK-3β on autophagy, we transfected cells with GSK-3β CA or KD mutants, and found that the increase of LC3-II by curcumin treatment was significantly decreased in GSK-3β CA-transfected SH-SY5Y cells. However, in cells transfected with GSK-3β KD, the level of LC3-II was dramatically elevated in both the presence and absence of curcumin administration (Figure 2(F)). All of these results demonstrated that the induction of autophagy by curcumin is mainly associated with the inhibition of GSK-3β.

Oxidative stress induced by H_2O_2 is mitigated by inhibition of GSK-3β

Curcumin has received increased interest due to its unique interaction with inflammatory and antioxidant pathways, as well as amyloid aggregation [41]. In addition, the phosphorylation of GSK-3β (S9) has been shown to exert anti-oxidant effects through promoting nuclear accumulation of Nrf2 and the expression of anti-oxidant genes [42]. To determine whether the anti-oxidant effect of curcumin is mediated by the inhibition of GSK-3β in SH-SY5Y cells, we first explored the phosphorylation of GSK-3β (S9) by curcumin in the presence of H_2O_2. According to our western blot results, cells co-treated with H_2O_2 and curcumin showed a significant increase in p-GSK-3β (S9) (Figure 3(A)), suggesting that curcumin could inhibit GSK-3β activation under oxidative stress conditions. Several studies have indicated that curcumin could increase the antioxidant gene, HO-1, expression through the promotion of Nrf2 nuclear translocation. Activation of the mouse *Hmox-1* gene promoter by curcumin is mediated by antioxidant-responsive elements (AREs) [43]. To investigate whether curcumin could enhance ARE promoter activity through the inhibition of GSK-3β, we next performed a luciferase assay, and found that cells co-treated with H_2O_2 and curcumin showed a robust increase in ARE luciferase activity compared to the cells treated with H_2O_2 alone (Figure 3(B)). Interestingly, we also observed that LiCl could significantly increase ARE promoter activity in the presence or absence of H_2O_2 (Figure 3(B)), suggesting that the inhibition of GSK-3β can enhance ARE promoter activity. Next, to detect whether curcumin could elevate the expression of anti-oxidant genes, we analysed HO-1 mRNA expression. Curcumin dramatically increased the mRNA level of HO-1 in the presence or absence of H_2O_2 (Figure 3(C)). ARE promoter activity has been reported to increase the anti-oxidant response and reduce ROS levels [29]. Therefore, we performed flow cytometry analysis to detect the production of ROS in SH-SY5Y cells transfected with scRNA or siGSK-3β. Treatment with curcumin or LiCl significantly decreased ROS levels in the presence of H_2O_2. Additionally, we also observed that cells transfected with siGSK-3β could significantly diminish the production of ROS under H_2O_2 treatment compared to cells transfected with scRNA (Figure 3(D)). Taken together, these results demonstrated that the anti-oxidant effect of curcumin requires the activation of the Nrf2/ARE pathway and induction of HO-1 through the inhibition of GSK-3β.

GSK-3β inhibition by curcumin degrades aggregated APP through autophagy in SH-SY5Y cells

APP is a key protein in the pathogenesis of AD. Autophagy is reported as a critical feature necessary for the clearance of aggregated amyloid-β protein [44]. According to our results (Figures 2 and 3), we showed that curcumin promoted autophagy and exerted anti-oxidant effects on H_2O_2-mediated oxidative stress. Therefore, to investigate whether curcumin could

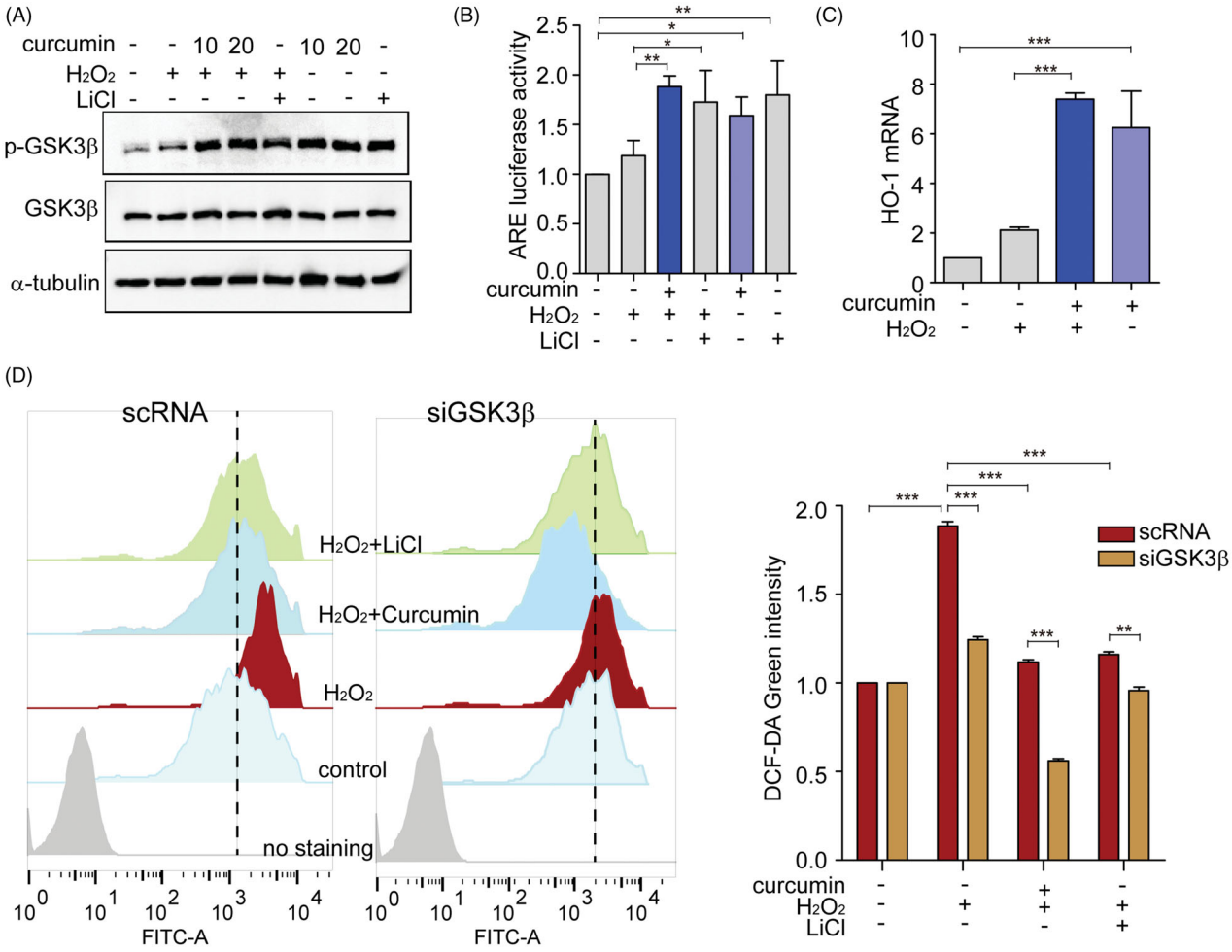

Figure 3. Oxidative stress induced by H_2O_2 is mitigated by inhibition of GSK-3β in SH-SY5Y cells. (A) SH-SY5Y cells were pre-treated with curcumin (10 μM and 20 μM) and LiCl (20 mM) for 2 h, and then cells were changed into fresh medium followed by the administration of H_2O_2 (200 μM) for 48 h. The expression of p-GSK-3β and GSK-3β was evaluated by western blot analysis. (B) SH-SY5Y cells were pre-treated with curcumin (20 μM) and LiCl (20 mM) for 30 min followed by the treatment of H_2O_2 (200 μM) for 6 h, before being co-transfected with a pCignal Lenti-ARE reporter and pCignal Lenti-TK-Renilla. After treatment with curcumin, luciferase activity was analysed. The expression levels obtained from pCignal Lenti-ARE reporter-transduced cells without 4-PG treatment were normalised to 1. (C) SH-SY5Y cells were pre-treated with curcumin (20 μM) for 30min followed by stimulation with H_2O_2 (200 μM) for 6 h; HO-1 mRNA levels were detected by qRT-PCR. (D) SH-SY5Y cells were transfected with scRNA and human GSK-3β siRNA. After 20 h incubation, cells were treated with curcumin (20 μM) and LiCl (20 mM) before being incubated in, fresh medium treated with H_2O_2 (200 μM) for 48 h; after incubation the production of intracellular ROS was detected by staining with DCF-DA and examining with a fluorescence-activated cell sorter (FACS Canto II). The fold change of fluorescence mean intensity is presented as mean ± SD ($n = 3$). Data presented are the mean ± SD ($n = 3$); $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

inhibit the aggregation of APP through the induction of autophagy, SH-SY5Y cells were transfected with human FL-APP-WT and a mutant form of APP (APP-Swe/Ind) for 48 h, prior to treatment with curcumin for 24 h. First of all, we evaluated the effect of curcumin on cell viability in WT or mutant of APP-transfected cells. As shown in Supplementary Figure S2(A), curcumin did not affect cell viability. Immunofluorescence results showed that the accumulation of APP was dramatically decreased in the curcumin-treated SH-SY5Y cells (Figure 4(A)). Moreover, curcumin treatment decreased the levels of FL-APP, while the levels of LC3-II significantly increased (Figure 4(B)). These data supported that curcumin could inhibit the aggregation of APP in neuronal cells. In order to explore whether the inhibition of APP aggregation by curcumin was linked to GSK-3β inactivation, SH-SY5Y cells were transfected with CA and KD mutant forms of GSK-3β for 48 h, prior to treatment with 20 μM curcumin for 24 h. Our results revealed that curcumin significantly increased the mRNA expression of several lysosomal markers, including that of LAMP1 and MCOLN1. However, in cells transfected with the CA-

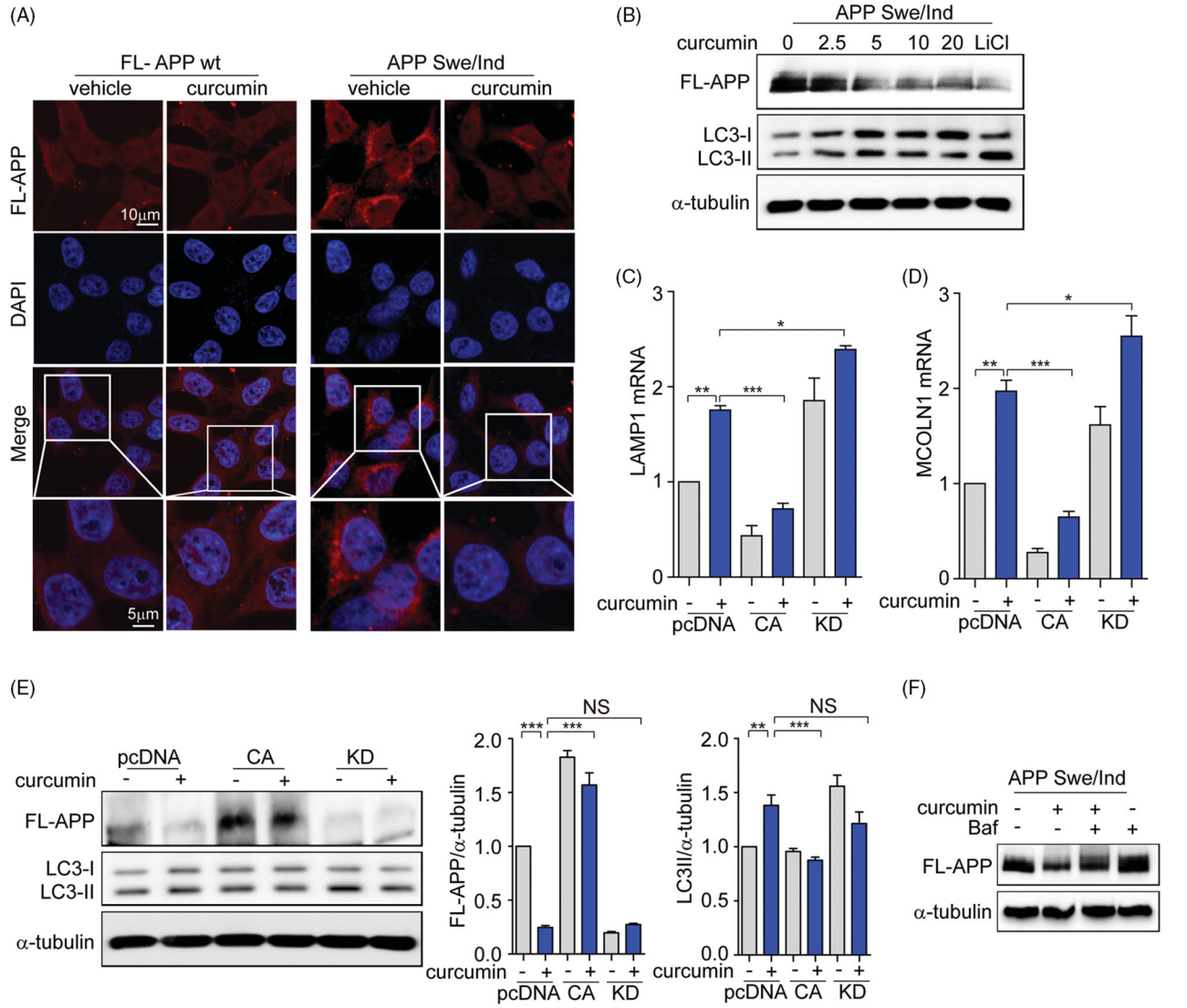

Figure 4. Curcumin-induced GSK-3β inhibition degrades aggregated APP through autophagy in SH-SY5Y cells. (A) SH-SY5Y cells transfected with a wild type full length-APP (FL-APP WT) and a mutant form of APP (APP-Swe/Ind) for 36h. Then, cells were treated with curcumin (20 μM) for 24 h. Immunofluorescence was performed to detect the FL-APP (red). Scale bars represent 10 μm in all images. (B) SH-SY5Y cells were transfected with a APP-Swe/Ind for 36 h, and then cells were treated with curcumin at different concentrations (0, 2.5, 5, 10, and 20 μM) for 24 h and LiCl (20 mM) for 12 h. The protein levels of FL-APP and LC3 were determined by western blot analysis. (C-E) SH-SY5Y cells were transfected with pcDNA3.1, GSK3β CA, and GSK3β KD for 36 h, and then treated with 20 μM curcumin for 24 h. The mRNA levels of LAMP1 (C) and MCOLN1 (D) were measured by qRT-PCR. (E) The protein level of FL-APP and LC3 were analysed by western blot (*left panel*). The quantification of FL-APP/α-tubulin (*middle panel*) and LC3-II/α-tubulin (*right panel*) from these experiments is represented. (F) SH-SY5Y cells were transfected with a APP-Swe/Ind for 36 h, and then cells were treated with curcumin (20 μM) for 24 h and Bafilomycin (100 nM) for 3 h. The protein levels of FL-APP and α-tubulin were determined by western blot analysis. Data represented are the means ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and *** $p < 0.001$. NS: not significant.

GSK-3β mutant, the beneficial effect of curcumin on LAMP1 and MCOLN1 expression was abolished, and was dramatically decreased compared to cells transfected with pcDNA. These lysosomal biomarkers were significantly increased in KD-GSK-3β transfected cells (Figure 4(C,D)). These data suggested that GSK-3β inhibition plays a critical role in curcumin-mediated autophagy. Furthermore, we investigated whether curcumin could decrease the expression of APP due to the induction of autophagy; the levels of APP were remarkably decreased by curcumin treatment in cells transfected with pcDNA (Figure 4(E)), and significantly increased in CA-GSK-3β-transfected cells treated with or without curcumin. Conversely, we found that the levels of LC3-II were decreased compared to the cells transfected with pcDNA. In KD-GSK-3β transfected cells, APP expression was significantly decreased, while LC3-II levels were enhanced (Figure 4(E)). To confirm the

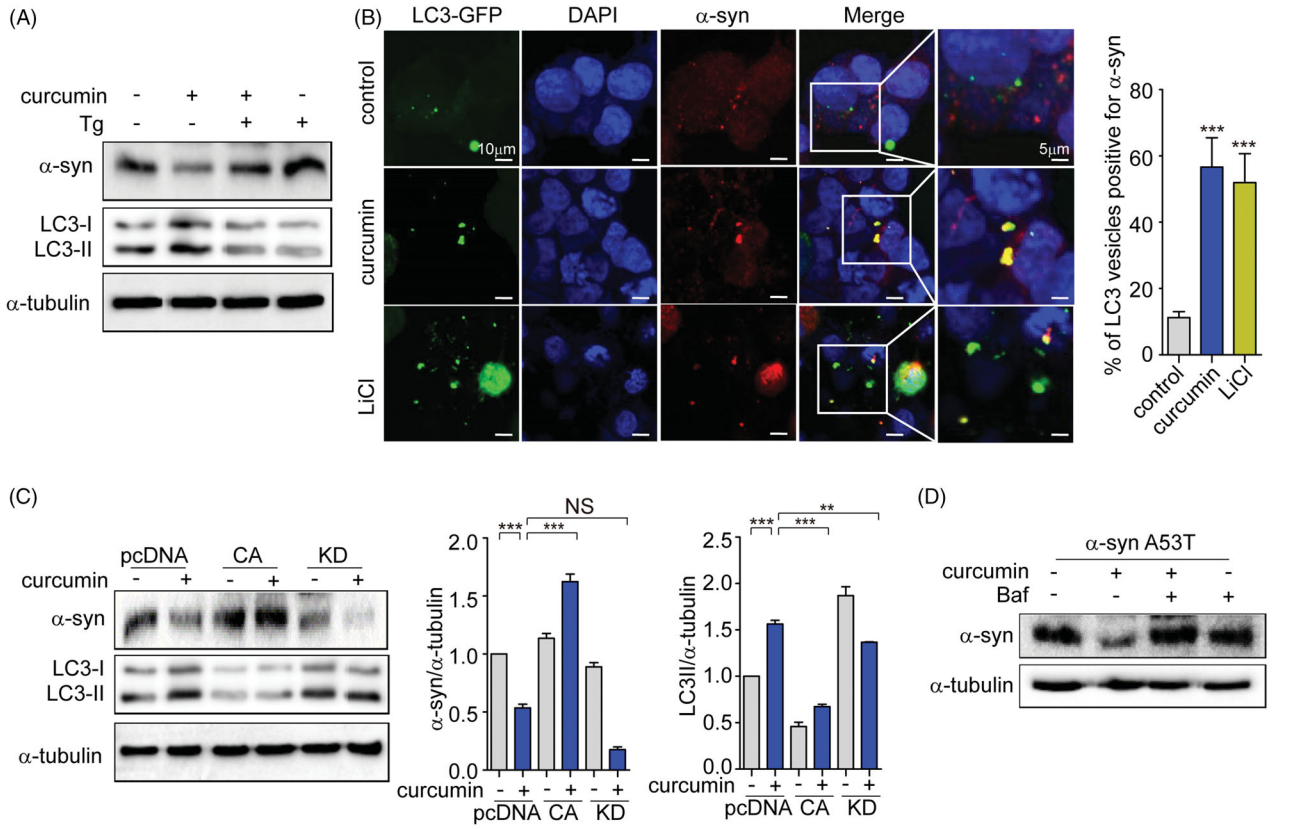

Figure 5. Activation of autophagy by curcumin reduces α-syn accumulation through inhibition of GSK-3β in SH-SY5Y cells. (A) SH-SY5Y cells were transfected with a human α-syn mutant (A53T) for 48 h, and then cells were treated with or without 200nM thapsigargin for 1h. The protein levels of α-syn and LC3 were assessed by western blot analysis. (B) SH-SY5Y cells were transfected with pEGFP-LC3 and a human α-syn mutant for 36 h. Then, cells were treated with curcumin (20 μM) or LiCl (20 mM) for 24 h. Immunofluorescence was performed to detect the co-localisation of LC3 (green) vesicles and α-syn (red) (*right panel*). The percentage of cells with co-localisation of LC3 and α-syn (*left panel*). (C) SH-SY5Y cells were transfected with pcDNA3.1, GSK3β CA, and GSK3β KD for 36 h, and then cells were treated with or without 20 μM curcumin for 24 h. The protein levels of α-syn, LC3 and α-tubulin were detected by western blot analysis (*left panel*). The quantification of α-syn/α-tubulin (*middle panel*) and LC3-II/α-tubulin (*right panel*) from these experiments is represented. (D) SH-SY5Y cells were transfected with a human α-syn mutant (A53T) for 48 h, and then cells were treated with curcumin (20 μM) for 24 h and Bafilomycin (100 nM) for 3 h. The protein levels of α-syn and α-tubulin were assessed by western blot analysis. Data represented are the means ± SD of three independent experiments. $^{**}p < 0.01$, and $^{***}\ p < 0.001$. NS: not significant.

clearance of APP through lysosome activation, APP-Swe/Ind-transfected SH-SY5Y cells were treated with Bafilomycin A, a lysosome inhibitor. The decrease of APP levels with curcumin was reversed by Bafilomycin A treatment (Figure 4(F)). All these data suggested that autophagy induced by curcumin could promote the clearance of APP, which is determined by the inactivation of GSK-3β.

Activation of autophagy by curcumin reduces α-syn accumulation through inhibition of GSK-3β in SH-SY5Y cells

Here, to determine whether curcumin could decrease other aggregative proteins, namely α-syn, through promotion of autophagy and inhibition of GSK-3β, SH-SY5Y cells were first transfected with mutant form of α-syn (A53T) to construct an *in vitro* PD model, and were then treated with thapsigargin (Tg, 200 nM) for 1 h, which is a GSK-3β activator [45,46]. Our results showed that curcumin has no effect on cell viability (Supplementary Figure S2(B)), increases the levels of LC3-II and reduces the accumulation of α-syn, while Tg treatment significantly attenuates the levels of LC3-II and reversibly increases the levels of α-syn (Figure 5(A)). Tg has different effects depending on incubation times. At short incubation time (30 min), Tg inhibited Ca^{2+}-ATPase [19]. Up to 3 h treatment with Tg did not affect mTOR activity [19], but it suppressed mTOR activity for 24 h incubation [47]. Previous studies reported Tg treatment for 1 h activated GSK-3β [48]. Thus, in this study, we postulated that Tg treatment for 1 h may be responsible for GSK-3β activation without the effects of Tg on Ca^{2+}-ATPases inhibitor and mTOR activity. To further confirm the

mechanism of curcumin-mediated down-regulation of α-syn, SH-SY5Y cells were co-transfected with LC3-GFP and α-syn and treated with curcumin or LiCl. The treatment of curcumin or LiCl significantly increased the percentage of LC3 vesicles containing α-syn (Figure 5(B)). In this study, our results demonstrated that curcumin facilitates autophagy progression, which is associated with the inactivation of GSK-3β (Figure 2). Next, to confirm whether the activity of GSK-3β is linked to the degradation of α-syn through the induction of autophagy, we transfected cells with CA or KD mutants. Cells transfected with GSK-3β CA or KD showed an opposite induction of the expression of α-syn and the level of LC3-II, indicating that GSK-3β exhibits a critical role in the α-syn degradation through the enhancement of autophagy (Figure 5(C)). We confirmed whether curcumin promotes the clearance of α-syn through the activation of the lysosome. As shown in Figure 5(D), Bafilomycin A reversed the levels of α-syn decreased by curcumin. To Thus, these results demonstrated that curcumin-induced GSK-3β inhibition is required for the removal of aggregated protein by autophagy.

Discussion

Our data demonstrated, for the first time, that the regulation of curcumin on autophagy in human neuroblastoma cells is dependent on the inactivation of GSK-3β and the nuclear translocation of TFEB. As curcumin activity leads to an increase in autophagy through GSK-3β inhibition, we suggest that curcumin attenuates amyloidogenesis in human neuroblastoma cells. Curcumin by the binding pocket of GSK-3β potently inhibits its activity [49]. GSK-3β activity is regulated by two different sites of phosphorylation, Tyr216 and Ser9 [37]; upon phosphorylation of Ser9, the activity of GSK-3β is inhibited, which will subsequently relieve autophagy inhibition. Inactivation of GSK-3β has been reported to be involved in the protective effect of curcumin against oxidative stress [50,51]. The main targets of autophagy are intracellular damaged or aging organelles, misfolded proteins, and excessive biological macromolecules [52]. Consistent with other studies, we observed that the inhibition of GSK-3β by both LiCl and curcumin could elevate the process of autophagy and protect cells from H_2O_2-induced oxidative stress. A previous study showed that the phosphorylation of different serine residues in TFEB could control its nuclear-cytoplasmic shuttling [53]. As shown in Figure 1(D), continuous nuclear accumulation of TFEB can be observed in cells transfected with the GSK-3β KD mutant plasmid, even without the treatment of curcumin. Soluble LC3-I can be converted into LC3-II, which is involved in the formation of autophagosomes [54]. The autophagy-related involvement of LC3 and LAMP1 are the main reason why these proteins were selected as markers to explore the effect of GSK-3β inhibition on autophagy. In addition, curcumin inhibited mTORC1 activity [55]. In human neuroblastoma cells, we suggested that curcumin has the possibility to enhance the TFEB activity by decreasing mTORC1 activity (Supplementary Figure 1S (D,E)). Oxidative stress plays a critical role in various neurodegenerative diseases, thus alleviation of oxidative stress is a potential strategy for therapeutic intervention and/or prevention of these diseases [56]. It has been found that curcumin confers protection against oxidative stress induced by H_2O_2 [12]. The Nrf2/HO-1 pathway plays a crucial role in protecting against oxidative stress. GSK-3β may be linked to regulating Nrf2 in a Kelch-like ECH-associated protein 1 (Keap1)-in dependent manner [57–59]. Multiple studies have shown that curcumin exhibits numerous biological activities, including the ability to induce the expression of Nrf2/HO-1, anti-oxidant enzymes, and ARE pathways [11,60,61]. Additionally, inhibition of GSK-3β by LiCl upregulates the Nrf2/HO-1 pathway by enhancing Nrf2-ARE binding activity, and increasing HO-1 expression [62]. Consistent with these reports, our results suggested that ARE promoter activity, assessed by luciferase assay, was significantly enhanced by the administration of curcumin. Thus, we propose that the protective effect of curcumin against oxidative stress might be due to the upregulation of the Nrf2/HO-1 pathway through the phosphorylation of S9-GSK-3β. Next, to evaluate the relationship between GSK-3β activity and autophagy induced by curcumin in the neuron, SH-SY5Y cells were transfected with mutant FL-APP and α-syn, which are considered important risk factors in AD and PD, respectively. Curcumin-induced autophagy can effectively increase the clearance of these aggregated proteins in SH-SY5Y cells. To confirm these cellular mechanisms of curcumin on the neuron, we need to further study using primary neuronal cells because SH-SY5Y cells have cancerous properties that differ greatly from normal neuronal features [63].

In summary, our data revealed that curcumin can increase the nuclear translocation of TFEB to promote the process of autophagy, and exert an anti-oxidant effect through the activation of ARE promoter activity and HO-1 expression. All of these beneficial effects of curcumin are associated with the inhibition of GSK-3β through the phosphorylation of the serine 9 residue. These finding may unveil new strategies for pharmacological intervention in neurodegenerative disorders.

Disclosure statement

All authors have disclosed that they have no conflicts of interest.

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Data availability statement

All relevant data are presented within the paper and its Supporting Information files.

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