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·Original Article·

Regulation of autophagic flux by CHIP

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ABSTRACT

Autophagy is a major degradation system which processes substrates through the steps of autophagosome formation, autophagosome-lysosome fusion, and substrate degradation. Aberrant autophagic flux is present in many pathological conditions including neurodegeneration and tumors. CHIP/STUB1, an E3 ligase, plays an important role in neurodegeneration. In this study, we identified the regulation of autophagic flux by CHIP (carboxy-terminus of Hsc70-interacting protein). Knockdown of CHIP induced autophagosome formation through increasing the PTEN protein level and decreasing the AKT/mTOR activity as well as decreasing phosphorylation of ULK1 on Ser757. However, degradation of the autophagic substrate p62 was disturbed by knockdown of CHIP, suggesting an abnormality of autophagic flux. Furthermore, knockdown of CHIP increased the susceptibility of cells to autophagic cell death induced by bafilomycin A1. Thus, our data suggest that CHIP plays roles in the regulation of autophagic flux.

Keywords: CHIP/STUB1; autophagic flux; neurodegeneration; mTOR; AKT

INTRODUCTION

The autophagy-lysosome pathway (ALP) and the ubiquitin proteasome system (UPS) are major intracellular protein-degradation systems. Autophagy mediates the lysosomal degradation of cytoplasmic components including proteins with longer half-lives, aggregates, and damaged organelles[1]. The process of autophagy includes autophagosome formation, a step in which the cytoplasmic components are sequestered in a double membrane; autolysosome formation by fusion of mature autophagosomes with lysosomes; and substrate degradation in which the cargo-containing substrates are degraded by proteases in the lysosome[2]. Aberrant autophagy contributes to the pathogenesis in many neurodegenerative diseases[3-5].

Many neurodegenerative disease-related proteins act either as key regulators of autophagy or as autophagic substrates[3,4]. For example, amyloid β (Aβ) can be generated in autophagic vacuoles and degraded by the ALP[6,7]. Wild-type and mutant α-synuclein, expanded polyglutamine Huntingtin, and mutants of SOD1 are degraded through the ALP[8]. Moreover, neurodegenerative disease-related proteins such as presenilin 1, α-synuclein, DJ-1, parkin, PINK1, LRRK2, and SOD1 are involved in the regulation of autophagy either at the initiation, maturation, or substrate-recognition steps[4, 9,10].

The carboxy-terminus of Hsc70-interacting protein (CHIP), the product of the *STUB1* gene, has been identified as a co-chaperone and U-box type E3 ubiquitin ligase that has multiple functions in many cellular processes such as protein degradation, trafficking, transcription, signaling, and apoptosis[11]. CHIP is involved in many pathological processes, especially those of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS) and several types of ataxia[12,13]. For example, CHIP exists in Lewy bodies, promotes the degradation of phosphorylated α-synuclein[14,15], binds to Parkin and enhances its E3 ligase activity[16], enhances

the degradation of phosphorylated tau, and reduces Aβ toxicity[17,18]. CHIP also represses the toxicity induced by LRRK2[19,20], expanded polyglutamine[21,22], and mutant SOD1[23]. Thus, CHIP has protective effects against the toxicity of neurodegenerative disease proteins. Recently, loss-of-function mutations in CHIP were identified as a causative genetic factor for a group of autosomal recessive cerebellar ataxias[24-28]. However, the precise mechanism of the involvement of CHIP in neurodegeneration remains unclear. In this study, we set out to address the role of CHIP in the regulation of autophagy, which may be associated with CHIP deficiency-related neurodegeneration.

MATERIALS AND METHODS

Plasmid Constructs and siRNAs

The EGFP-LC3 and FLAG-p62 expression plasmids were described previously[29,30]. mCherry-EGFP-LC3B was kindly provided by Jayanta Debnath (University of California at San Francisco, USA) (Addgene plasmid #22418)[31]. The fidelity of all constructs was confirmed by sequencing. siRNAs against human or mouse CHIP were synthesized with the following sequences: human CHIP, sense: 5'-UGCCGCCACUAUCUGUGUAAUTT-3', anti-sense: 5'-AUUACACAGAUAGUGGCGGCATT-3'; mouse CHIP, sense: 5'-AUACAUGGCAGAUAUGGAUTT-3', anti-sense: 5'-AUCCAUAUCUGCCAUGUAUTT-3'; negative control siRNA (si-NC), sense: 5'-UUCUCCGAACGUGUCACGUTT-3', anti-sense: 5'ACGUGACACGUUCGGAGAATT-3'.

Cell Culture, Transfection, and Drug Treatment

Human embryonic kidney 293 (HEK293) cells and mouse neuroblastoma Neuro 2a (N2a) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco), penicillin (100 U/mL), and streptomycin (100 μg/mL) (Gibco). Cells were transfected with siRNAs using lipofectamine RNAiMAX transfection reagent (Invitrogen, La Jolla, CA), or transfected with expression plasmids using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were treated with DMSO or 100 nmol/L bafilomycin A1 (Sigma, St. Louis, MO).

Immunoblot Analysis and Antibodies

Cells were lysed in lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl pH 7.5, 0.5% deoxycholate, 1% NP40, and protease inhibitor cocktail (Roche, Indianapolis, IN)]. Proteins were separated by 10% or 15% SDS-PAGE and transferred onto a PVDF (polyvinylidene difluoride) membrane (Millipore, Bedford, MA). The PVDF membrane was incubated with the following primary antibodies overnight at 4°C: anti-FLAG, anti-ATG1/ULK1 (Sigma), anti-GAPDH (Chemicon, Temecula, CA), anti-CHIP/STUB1, anti-p70S6K, anti-PTEN (Epitomics, Burlingame, CA), anti-phospho-p70S6K (S371), anti-phospho-AKT (S473), anti-phospho-mTOR (S2448), anti-phospho-ULK1 (S757), anti-mTOR, anti-cleaved caspase-3 (Cell Signaling Technology, Beverly, MA), anti-LC3 (Novus Biologicals, Littleton, CO), anti-AKT, and anti-p62/SQSTM1 (Santa Cruz, Santa Cruz, CA). Then the following secondary antibodies were used: horseradish peroxidase-conjugated sheep anti-mouse, anti-rabbit, or anti-goat IgG (Amersham Pharmacia Biotech, Peapack, NJ). Proteins were visualized with an ECL detection kit (Amersham Biosciences, Piscataway, NJ) using a chemiluminescence imaging system (Bioshine ChemiQ 4800) (Shanghai, China).

Fluorescence and Immunofluorescence

Transfected HEK293 cells were washed with PBS (Gibco, pH 7.4) and fixed in 4% paraformaldehyde for 5 min, then the fixed cells were visualized under an inverted microscope (IX71, Olympus, Tokyo, Japan) or a confocal microscope (LSM710, Zeiss, Oberkochen, Germany). For immunofluorescence assays, after permeabilization with 0.25% Triton X-100 in PBS, the cells were washed 3 times in PBS and pre-blocked with 4% fetal bovine serum for 1 h in PBST (0.1% Tween 20 in PBS). Then the cells were incubated with primary antibodies overnight, followed by incubation with fluorescent secondary antibodies (Alexa Fluor 594-conjugated Affinipure donkey anti-rabbit or anti-mouse IgG, Alexa Fluor 488-conjugated Affinipure donkey anti-mouse IgG, Invitrogen) for 2 h. The labeled cells were visualized under the confocal microscope.

MTT Cell Viability Assay

Cells were washed with PBS and incubated with 0.5 mg/mL MTT (Sigma) dissolved in DMEM without phenol red. After 3 h, the medium was removed and the formazan crystals were dissolved in DMSO by incubation at 37°C for 30 min. The absorbance was measured by a photometer at 570

nm by subtracting background at 630 nm. The data were normalized to the control and the ratios are presented as mean ± SEM from three independent experiments.

Statistical Analysis

Immunoblot densitometric analysis of three independent experiments was performed using Photoshop 7.0 software (Adobe, San Jose, CA). The data were analyzed by one-way analysis of variance (ANOVA) using Origin 6.0 software (OriginLab, Northampton, MA).

RESULTS

Knockdown of CHIP Induces Autophagosome Formation

To investigate whether CHIP is associated with autophagy, CHIP was knocked down in two cell lines, N2a and HEK293, and LC3 protein levels were measured. LC3 is cleaved at its carboxyl termini to form LC3-I, which is conjugated with phosphatidylethanolamine to generate LC3-II during the initiation of autophagy[29, 32-33]. LC3-II binds tightly to the autophagosomal membrane and its level or the ratio of LC3-II to LC3-I is a useful marker of autophagosome numbers[33]. Knockdown of CHIP increased the LC-II level in both N2a and HEK293 cells in three independent experiments (Fig. 1A and B), suggesting that CHIP affects autophagosome formation across diverse types of cells. Consistently, the fluorescence of EGFP-LC3 showed significantly more puncta in CHIP knockdown cells than in control cells (Fig. 1C). The ubiquitin-binding autophagic adaptor p62/SQSTM1 (p62) binds to LC3 and mediates the engulfment of autophagic cargoes into autophagosomes, so the co-localization of p62 and LC3 puncta also serves as a marker of autophagosome formation[34-36]. Here, we found dramatically increased co-localization of FLAG-p62 with endogenous LC3 or EGFP-LC3 puncta in CHIP-knockdown cells (Fig. 1D and E). These data indicate that CHIP deficiency induces autophagosome formation.

AKT/mTOR/p70S6K Is Involved in CHIP-Mediated Autophagosome Formation

PI3K/AKT/mTOR is the major pathway for the regulation of autophagosome formation[37,38]. Considering that CHIP is an E3 ligase for PTEN degradation[39,40] and that PTEN is a key negative regulator of the PI3K/AKT/mTOR pathway[37], we investigated whether CHIP has effects on the PI3K/AKT/mTOR pathway. With CHIP-knockdown, the phosphorylation of AKT, mTOR, and p70S6K decreased in both N2a and HEK293 cells (Fig. 2A and B). We also assessed the protein level of PTEN, as well as the phosphorylation of ULK1 on Ser757, which is phosphorylated by activation of mTOR[41,42]. Increased PTEN protein levels and decreased phosphorylation of ULK1 on Ser757 were detected in both N2a and HEK293 cells (Fig. 2C and D). These results indicated that loss of CHIP affects the PTEN/AKT/mTOR pathway, leading to the induction of autophagosome formation.

CHIP Influences Autophagic Flux and p62 Degradation

Increased LC3-II levels and puncta with a co-localization of LC3 and p62 may reflect increased autophagic flux towards lysosomes or accumulation of autophagosomes by impaired autophagosome-lysosome fusion[33]. To further confirm the effect of CHIP on autophagic flux, we performed CHIP knockdown experiments in cells expressing mCherry-GFP-LC3B. Tandem mCherry-GFP-LC3B is a useful tool for monitoring autophagic flux. Both mCherry (red) and GFP (green) emit fluorescence resulting in yellow fluorescence in autophagosomes; however, when autolysosomes are formed, pH-sensitive GFP fluorescence is lost while red fluorescence is preserved[43,44]. Interestingly, an increased autophagosome (yellow) ratio and a decreased red fluorescence ratio were present in CHIP knockdown cells compared with controls (Fig. 3A and B), giving an appearance similar to treatment with bafilomycin (Baf) A1, a vacuolar-type H^+-ATPase inhibitor that decreases lysosome acidification and affects the fusion of autophagosomes with lysosomse (Fig. 3A and B). In addition, the yellow fluorescence further increased in CHIP-depleted cells compared with controls under Baf A1 treatment (Fig. 3A and B). Next, we examined the effect of CHIP on autophagic substrate degradation, and found that the autophagic substrate p62 protein was increased in CHIP-depleted cells (Fig. 3C). Together, these results suggested that knockdown of CHIP leads to abnormal autophagosome accumulation and substrate degradation with increased autophagosome formation but inhibition of autophagosome-lysosome fusion and substrate degradation.

Knockdown of CHIP Increases Cell Death in Response to Inhibition of Autophagic Flux

Inhibition of autophagic flux leads to cell death in response

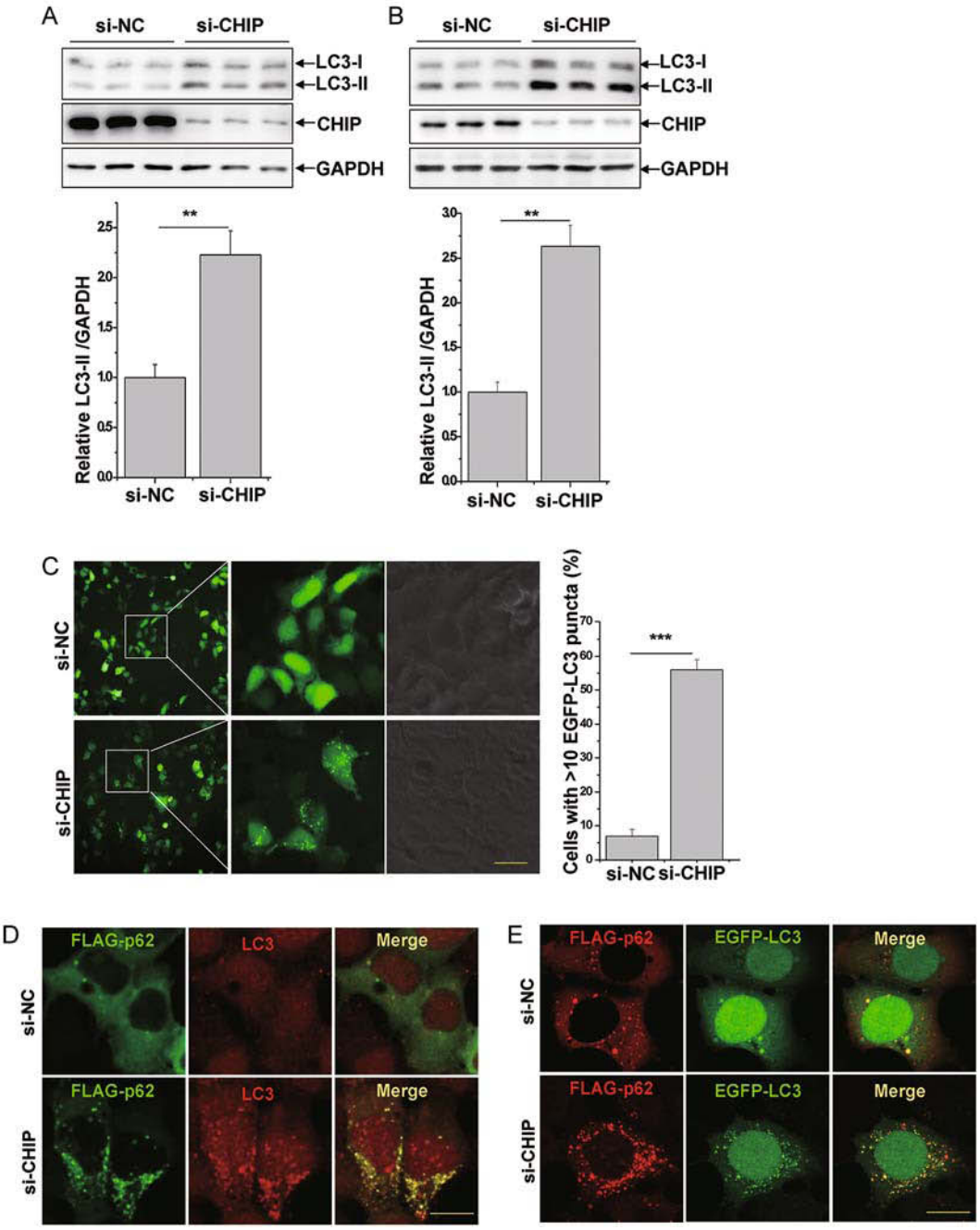

Fig. 1. Autophagosome formation induced by knockdown of CHIP. (A-B) Upper panels, HEK293 cells (A) and N2a cells (B) were transfected with indicated siRNAs. Seventy-two hours later, cells were lysed and the lysates subjected to immunoblot analysis using anti-LC3, anti-CHIP, and anti-GAPDH antibodies. Lower panels, relative ratios of LC3-II to GAPDH (mean ± SEM; *****P* **<0.01, one-way ANOVA). (C) HEK293 cells expressing EGFP-LC3 were transfected with indicated siRNAs. Seventy-two hours later, the green fluorescence was visualized under an inverted fluorescent microscope. Scale bar, 10 μm. The quantitative data presented the percentages of cells with >10 EGFP-LC3 puncta (mean ± SEM; ******P*** **<0.001, one-way ANOVA). (D) HEK293 cells expressing FLAG-p62 were transfected with indicated siRNAs. Seventy-two hours later, cells were immuno-stained with anti-FLAG (green) and anti-LC3 (red) and were visualized under a confocal microscope. Scale bar, 10 μm. (E) HEK293 cells expressing FLAG-p62 and EGFP-LC3 were transfected with indicated siRNAs. Seventy-two hours later, cells were immuno-stained with anti-FLAG (red), then visualized with a confocal microscope. Scale bar, 10 μm.**

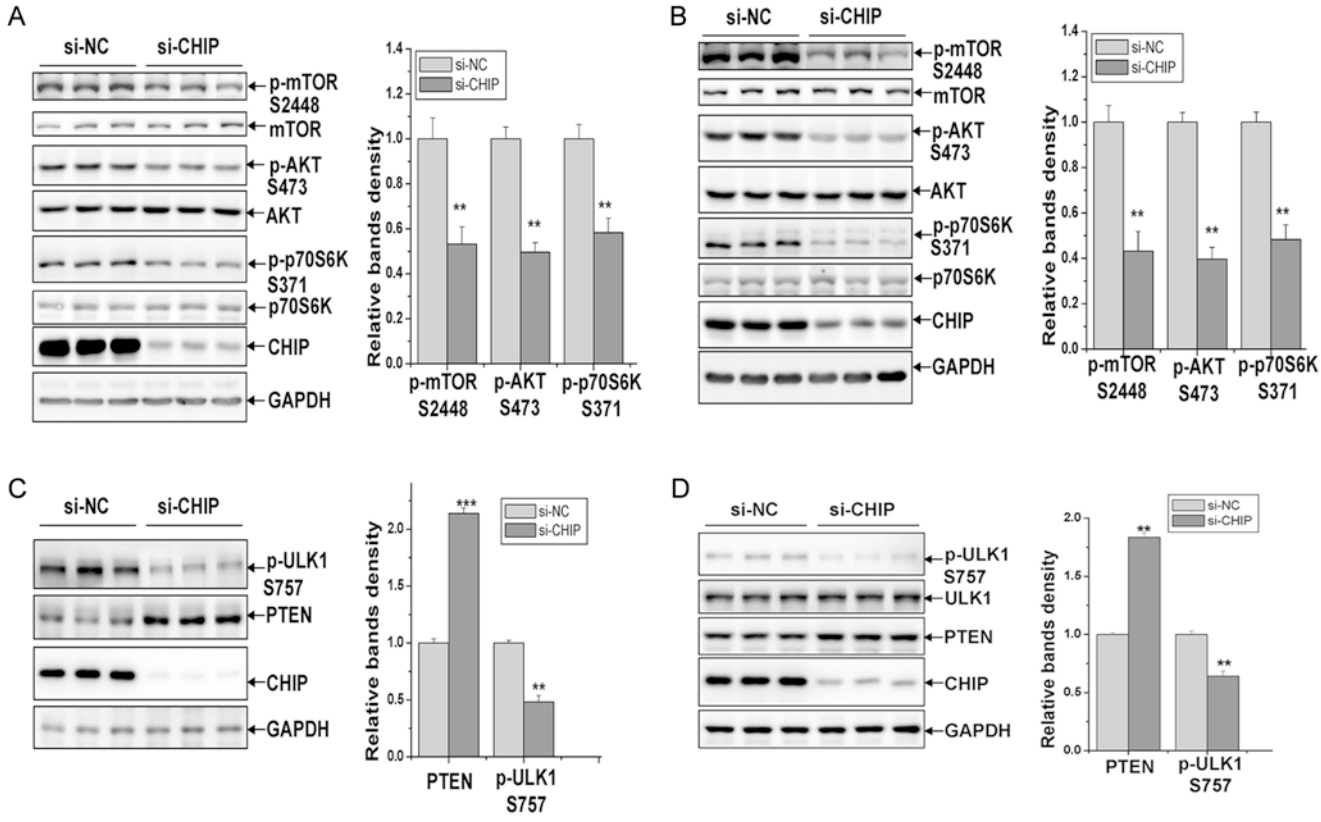

Fig. 2. Down-regulation of AKT/mTOR phosphorylation by knockdown of CHIP. (A–D) Left panels, HEK293 cells (A, C) and N2a cells (B, D) were transfected with the indicated siRNAs. Seventy-two hours later, cells were lysed and the lysates subjected to immunoblot analysis using the indicated antibodies. Right panels, relative ratios of phosphorylation of AKT/mTOR/p70S6K to total protein (A, B) and phosphorylation of ULK1 S757 as well as the protein level of PTEN to GAPDH (C, D) (mean ± SEM; *****P* **<0.01, ******P*** **<0.001 *vs* si-NC, one-way ANOVA).**

to autophagy inhibitors such as Baf A1[45,46]. Therefore, we examined the effect of CHIP on cell survival with or without Baf A1 treatment, and found that knockdown of CHIP alone did not significantly influence cell survival despite accumulation of LC3-II (Fig. 4A and B). However, under Baf A1 treatment, more robust caspase-3 cleavage and LC3-II accumulation were found in CHIP-depleted cells than in control cells (Fig. 4A and B). Besides, although knockdown of CHIP alone had little effect on cell viability, it significantly decreased viability in response to Baf A1 treatment (Fig. 4C and D). These results suggested increased susceptibility to cell death by knockdown of CHIP in response to inhibition of autophagic flux.

DISCUSSION

CHIP is ubiquitously expressed in tissues and has multiple functions[11]. CHIP is a U-box-type E3 ligase containing tetratricopeptide repeat (TPR) domains, with a co-chaperone property that functions in combination with several chaperones such as HSP70 and HSP90[47-52]. CHIP plays a crucial role in cytoplasmic homeostasis and cell viability by decreasing or stabilizing its substrates[11,12]. It participates in a number of biological processes, especially in neurodegeneration including AD, PD, HD, ALS, and several types of ataxia[12,13, 24-28]. Here, we showed that the neurodegenerative disease-related protein CHIP plays a role in the regulation of autophagic flux. CHIP deficiency resulted in autophagic flux inhibition and autophagosome accumulation by increasing autophagosome formation through the AKT/mTOR/p70S6K pathway and by inhibiting autolysosome maturation.

Impairment of autophagic flux with increased

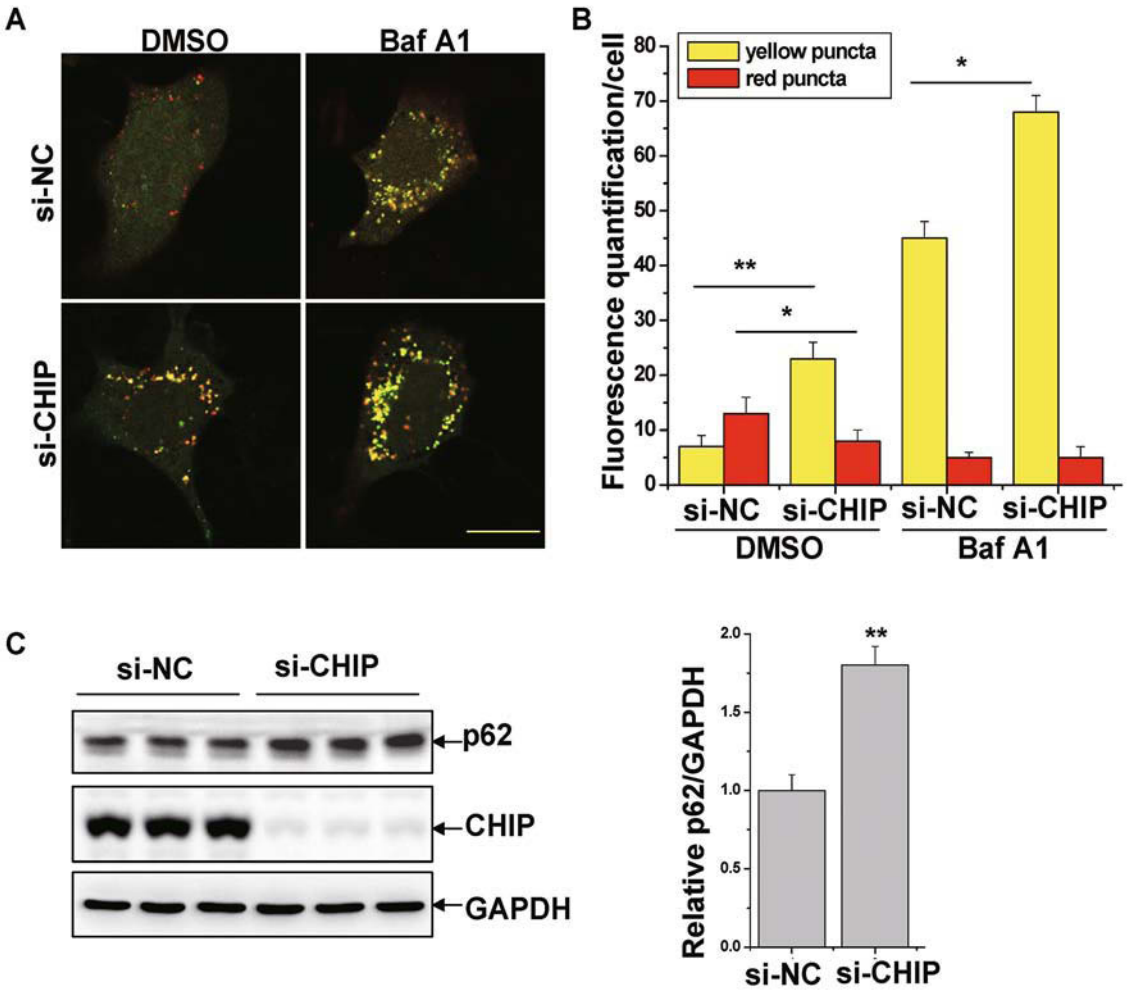

Fig. 3. Inhibition of autophagosome-lysosome fusion and p62 degradation by knockdown of CHIP. (A) Confocal images of HEK293 cells expressing mCherry-EGFP-LC3B, transfected with indicated siRNAs. Seventy-two hours later, cells were treated with DMSO or 100 nmol/L Baf A1 for 4 h as indicated. Scale bar, 10 μm. (B) Quantitative data presented yellow and red puncta in control or CHIP knockdown with or without Baf A1 treatment (mean ± SEM; *P <0.05, **P<0.01, one-way ANOVA). (C) Left panel, HEK293 cells were transfected with indicated siRNAs. Seventy-two hours later, cells were collected and the total cell lysates were subjected to immunoblot analysis using the indicated antibodies. Right panel, relative ratios of p62 to GAPDH (mean ± SEM; *P <0.05, **P <0.01, one-way ANOVA).

autophagosome formation and impaired autolysosome clearance occurs frequently in neurodegenerative diseases[3,4]. For example, massive autophagosome accumulation occurs in AD brains because of increased initiation of autophagy but decreased maturation of autophagosomes[53-55]. In PD, the dysregulation of autophagy occurs at multiple steps[3,4,9]. In HD, a deficit in cargo recognition leads to inefficient autophagy although the autophagosome formation and clearance are normal[56]. Increased autophagosomes with an impaired autophagic flux also occur in ALS patients and animal models[10, 57-59].

Besides CHIP, many E3 ligases including parkin, SCF (β-TrCP), MDM2, RNF5, TRAF6, and TRIM13 are involved in autophagy by modifying different targets[60]. For example, the PD-associated protein parkin is an E3 ligase that inhibits autophagy by stabilizing Bcl-2 through mono-ubiquitination[61]. SCF inhibits autophagy through activating mTOR signaling by ubiquitinating DEPTOR, an mTOR inhibitor[62]. TRIM13 induces autophagy by interacting with p62/SQSTM1 during ER stress[63]. Here, we showed that

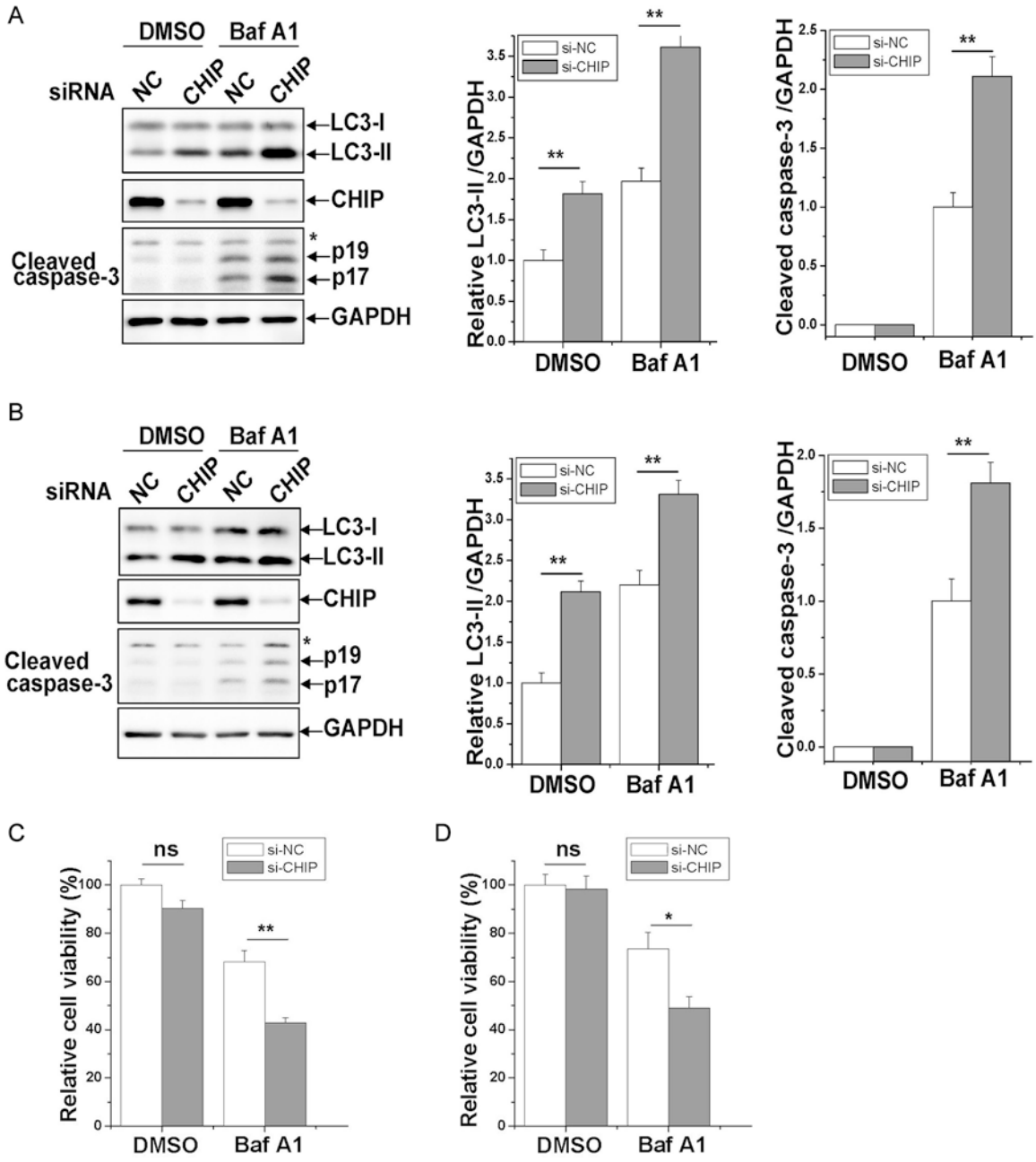

Fig. 4. Increased cell death in response to autophagic flux inhibition by CHIP knockdown. (A–B) Left panels, HEK293 cells (A) and N2a cells (B) were transfected with indicated siRNAs. Forty-eight hours later, cells were treated with DMSO or 100 nmol/L Baf A1 for 4 h as indicated. Then total cell lysates were subjected to immunoblot analysis using the indicated antibodies (*nonspecific band). Right panels, relative ratios of LC-II (left) and cleaved caspase-3 (right) to GAPDH (mean ± SEM; **P <0.01, one-way ANOVA). (C–D) HEK293 cells (C) and N2a cells (D) were transfected with indicated siRNAs. Forty-eight hours later, cells were treated with DMSO or 100 nmol/L Baf A1 for 4 h as indicated. Then cells were subjected to MTT assay (ns, not significant; *P <0.05; **P <0.01).

knockdown of CHIP increases autophagosome formation by decreasing AKT/mTOR signaling. mTOR signaling is considered as the master regulator of autophagy initiation. mTOR acts as a sensor of cellular nutrients and growth

factors, promoting cell growth by activating biosynthetic pathways and inhibiting autophagy under nutritional sufficiency[37,38,64]. Inactivation of mTOR by starvation or mTOR inhibitors such as rapamycin promotes autophagy initiation and autophagosome nucleation by affecting the phosphorylation of multiple autophagy-related proteins including ULK1/2, ATG13, and FIP200[65-67]. The ULK1/2-ATG13-FIP200 complex acts as the major autophagy initiator[68]. Under starvation, the mTOR-dependent phosphorylation site Ser757 in mouse ULK1 (equivalent to Ser758 of human ULK1) is dephosphorylated and this subsequently leads to phosphorylation of ULK1 at Ser 317 and Ser777, as well as phosphorylation of ATG13 and FIP200. Then the activated complex initiates autophagosome formation[41,42,68]. p70S6K, a downstream target of mTOR, also acts as a negative regulator of autophagy[69]. In our studies, the phosphorylation of p70S6K was decreased in CHIP-depleted cells. AKT is a crucial positive mediator of mTOR activity and a downstream effector of PI3K[70]. Decreased phosphorylation of AKT in CHIP-depleted cells indicates that CHIP may influence the upstream of AKT. Interestingly, several findings have suggested that CHIP binds to and degrades PTEN, which is a major negative regulator of the PI3K/AKT/mTOR pathway[39,40,71]. CHIP-regulated autophagy may depend on its E3 ligase activity by targeting PTEN[39,40]. Our results also verified that knockdown of CHIP increases PTEN protein levels (Fig. 2C and D). Taken together, we propose that knockdown of CHIP triggers autophagosome formation by increasing PTEN protein levels and negatively regulating AKT/mTOR activity.

Abnormal activity of the PI3K/AKT/mTOR pathway commonly occurs in neurodegenerative diseases[38]. In AD, a significant loss of PTEN and hyperactivation of the PI3K/AKT/mTOR pathway are associated with impaired clearance of Aβ and tau[72]. Inhibition of mTOR signaling with rapamycin improves cognitive impairment and promotes Aβ and tau clearance by inducing autophagy[73]. However, in PD brains, as well as in SOD1 G93A transgenic mice, the PI3K/AKT/mTOR activity is downregulated[57,74,75].

CHIP is an HSP90/HSP70-interacting E3 ligase that ubiquitinates many unfolded proteins to enhance their degradation and mitigate severe endoplasmic reticulum (ER) stress[76,77]. Given that the unfolded protein response (UPR)-induced ER stress can lead to the induction of autophagy[78], the increased autophagy initiation by CHIP deficiency may also be caused by its effects on the UPR and ER stress.

Although autophagosome formation is increased after CHIP knockdown, the autophagic flux is apparently blocked, as indicated by the tandem mCherry-GFP-LC3B fluorescence and p62 level, similar to the results of Baf A1 treatment, suggesting the impairment of fusion of autophagosomes with lysosomes, or interference with lysosomal degradation. Inhibition of autophagic flux can trigger cell death[45,46]. However, knockdown of CHIP alone does not decrease cell viability. We reason that inhibition of autophagosome-lysosome fusion by CHIP deficiency is limited compared with Baf A1 treatment. However, significant cell death can be induced in CHIP-depleted cells compared with control cells under Baf A1 treatment, suggesting that loss of CHIP increases susceptibility to autophagic cell death.

In summary, we demonstrated that CHIP deficiency leads to abnormal autophagy followed by susceptibility of cells to autophagic cell death.

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