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Cycloheximide inhibits starvation-induced autophagy through mTORC1 activation

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ABSTRACT

Protein synthesis inhibitors such as cycloheximide (CHX) are known to suppress protein degradation including autophagy. The fact that CHX inhibits autophagy has been generally interpreted to indicate that newly synthesized protein is indispensable for autophagy. However, CHX is also known to increase the intracellular level of amino acids and activate mTORC1 activity, a master negative regulator of autophagy. Accordingly, CHX can affect autophagic activity through inhibition of *de novo* protein synthesis and/or modulation of mTORC1 signaling. In this study, we investigated the effects of CHX on autophagy using specific autophagy markers. We found that CHX inhibited starvation-induced autophagy but not Torin1-induced autophagy. CHX also suppressed starvation-induced puncta formation of GFP-ULK1, an early-step marker of the autophagic process which is regulated by mTORC1. CHX activated mTORC1 even under autophagy-inducible starvation conditions. Finally, the inhibitory effect of CHX on starvation-induced autophagy was cancelled by the mTOR inhibitor Torin1. These results suggest that CHX inhibits starvation-induced autophagy through mTORC1 activation and also that autophagy does not require new protein synthesis at least in the acute phase of starvation.

1. Introduction

Macroautophagy, hereafter referred to simply as autophagy, is a major intracellular degradation system [1–3]. Cytoplasmic materials are engulfed by a double-membrane autophagosome and delivered to the lysosome, resulting in degradation of the enclosed contents together with the inner autophagosomal membrane. Autophagy is typically induced by starvation and is thought to be mainly regulated by post-translational modifications of autophagy-related proteins and lipid modifications [4,5], although recent studies also suggest involvement of transcriptional regulation [6–12].

Early studies demonstrated that inhibitors of protein synthesis such as cycloheximide (CHX) also inhibits starvation-induced protein degradation [13–16]. More recently, the specific effects of CHX on autophagy were tested and controversial results were obtained. In budding yeast, Takeshige et al. showed that starvation-induced accumulation of autophagic bodies in the vacuole was strongly suppressed by CHX treatment [17]. By contrast, Abeliovich et al. reported that pretreatment of CHX did not affect induction of autophagy by rapamycin, although the size of autophagosomes became smaller [18]. In mammalian cells, CHX was shown to inhibit autophagosome formation [19,20]. However, Lawrence et al. reported that pretreatment with CHX for 3 h inhibited autophagosome–lysosome fusion without affecting autophagy induction [21]. These studies showed that CHX inhibits autophagy, although the step affected seems to be different among studies. Most of the studies suggested that newly synthesized protein is required for autophagy. On the other hand, it was reported that CHX has no inhibitory effect on glucagon-induced autophagy [22] or virus infection-induced autophagy [23].

It should be noted that inhibition of protein synthesis can reduce consumption of amino acids and thereby increase the level of intracellular amino acids [13,24,25]. It is also known that CHX activates mTORC1, a master negative regulator of autophagy, through accumulation of intracellular amino acids following inhibition of protein synthesis [24,26]. Therefore, CHX can affect autophagic activity through inhibition of *de novo* protein synthesis and/or modulation of mTORC1 signaling.

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In this study, using specific markers, we investigated whether CHX indeed inhibits autophagy, and if so, at which step autophagy is blocked.

2. Methods

2.1. Cell culture and transfection

Mouse embryonic fibroblasts (MEFs) and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 Units/ml penicillin, and 50 μg/ml streptomycin (regular medium) in a 5% CO_2 incubator. MEFs stably expressing GFP-fused microtubule-associated protein 1 light chain 3 (GFP-LC3) or GFP-ULK1 were generated previously [27]. For amino acid starvation treatment, cells were washed with phosphate-buffered saline (PBS) and incubated in amino acid-free DMEM with 10% dialyzed FBS. Serum was dialyzed against PBS (6 L) for 24 h using Slide-A-Lyzer 3500 MWCO dialysis cassette (Thermo Scientific).

2.2. Antibodies and reagents

Antibodies against 4E-BP1 (#9452), S6 kinase 1 (S6K1) (#9202), S6K1 pT389 (#9234), general amino acid control non-repressible 2 (GCN2) (#3302), Akt (#9272), and Akt pT308 (#9275) were purchased from Cell Signaling Technology. Antibody against GCN2 pT899 (ab75836) was purchased from Abcam. Mouse monoclonal anti-β-actin antibody was purchased from Sigma–Aldrich (A2228). Anti-LC3 rabbit polyclonal antibody was previously generated [28]. CHX and chloroquine (CQ) were purchased from Sigma–Aldrich.

2.3. Fluorescence microscopy of GFP-LC3 punctate structures

Cells grown on coverslips were washed with PBS and fixed in 4% paraformaldehyde (PFA) in PBS for 10 min. Cells were observed with a fluorescence microscope (IX81; Olympus) or a confocal laser microscope (FV1000D; Olympus) using a 60x PlanApoN oil immersion lens (1.42 NA; Olympus). For final output, images were processed using Photoshop 7.0.1 software (Adobe Systems). The number of punctate structures was determined as follows: punctate structures were extracted using the top hat operation (parameter: 100 × 100 pixel area), and a binary image was created. Small structures (less than 3 × 3 pixel area) were removed using an open operation. The puncta were counted using the integrated morphometry analysis program. False dots were removed by comparison with the original image.

2.4. GFP-ULK1 immunohistochemistry

MEFs expressing GFP-ULK1 grown on coverslips were washed with PBS and fixed in 4% PFA in PBS for 10 min. Fixed cell were permeabilized with 50 μg/ml digitonin in PBS for 5 min, blocked with 3% bovine serum albumin in PBS for 30 min, and incubated with anti-GFP antibody for 1 h. After washing, cells were incubated with Alexa-Fluor-488-conjugated goat anti-rat IgG secondary antibodies (Invitrogen) for 1 h.

2.5. Western blot and quantification

Cell lysates were prepared in a regular lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 mM NaF, 0.4 mM Na_3VO_4, 10 mM sodium pyrophosphate, and Complete EDTA-free protease inhibitor (Roche Applied Science)). The lysates were clarified by centrifugation at 17,400*g* for 5 min and then boiled in sample buffer. Samples were separated by SDS–PAGE and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore). Immunoblot analysis was performed and visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce) or Immobilon Western (Millipore). The signal intensities were analyzed using an LAS-3000mini imaging analyzer and Multi Gauge software version 3.0 (Fujifilm). Contrast and brightness adjustment was applied uniformly to the images using Photoshop 7.0.1.

2.6. Statistical analyses

Data were expressed as mean ± SE. Unpaired Student *t* test and analysis of variance (ANOVA) was used to determine differences between two and three groups, respectively.

3. Results

3.1. Translation inhibitors block starvation-induced autophagy but not Torin1-induced autophagy

We first determined whether CHX inhibits autophagy by monitoring the modification of LC3 [29,30]. LC3 is one of the mammalian homologs of autophagy-related 8 (Atg8) and is usually present in the cytosol in its LC3-I form. When autophagy is induced, LC3 localizes on the autophagosomal membrane in its lipid-conjugated form LC3-II. When autophagy was induced by withdrawal of amino acids or treatment with the mTOR inhibitor Torin1, the amount of LC3-II was increased (Fig. 1A). However, when cells were pretreated with 50 μg/ml CHX for 5 min, starvation-induced LC3-II formation was suppressed (Fig. 1A). By contrast, pretreatment of CHX did not inhibit Torin1-induced LC3-II formation. These observations are consistent with findings from previous studies in yeast that CHX inhibits autophagy induced by starvation [17] but not by rapamycin [18]. Similar results came from cells treated with puromycin, another translation inhibitor (Fig. 1A).

Inhibition of autophagy by CHX was further confirmed by fluorescence microscopy of MEFs stably expressing GFP-LC3. Amino acid starvation or Torin1 treatment induced formation of GFP-LC3 punctate structures (Fig. 1B), which represented autophagosomes/autolysosomes [29,30]. However, the induction of GFP-LC3 puncta by starvation but not by Torin1 treatment was inhibited by pretreatment with CHX. These data suggest that blockage of protein translation inhibits starvation-induced autophagy but not Torin1-induced autophagy.

3.2. CHX restores the mTORC1 activity under autophagy-inducible starvation conditions

It has been reported that CHX can activate mTORC1 by accumulation of intracellular amino acids [24]. Therefore, we measured the mTORC1 activity based on phosphorylation status of its substrates S6K and 4E-BP1 under autophagy-inducible conditions. Torin1 treatment caused strong dephosphorylation of S6K and 4E-BP1 regardless of CHX treatment, suggesting that mTORC1 was inhibited (Fig. 1A). Upon amino acid starvation, mTORC1 activity was also inhibited. However, when cells were pretreated with CHX, phosphorylation of both S6K and 4E-BP1 was maintained even after 1-h amino acid-starvation treatment as previously reported (Fig. 1A) [24]. These data suggest that the inhibitory effect of CHX on autophagy could be due to modulation of mTORC1 activity rather than inhibition of protein synthesis.

Next we tested whether CHX can reactivate mTORC1 and suppress autophagy in cells that had been starved. MEFs expressing GFP-LC3 were deprived of amino acids for 45 min, and then treated

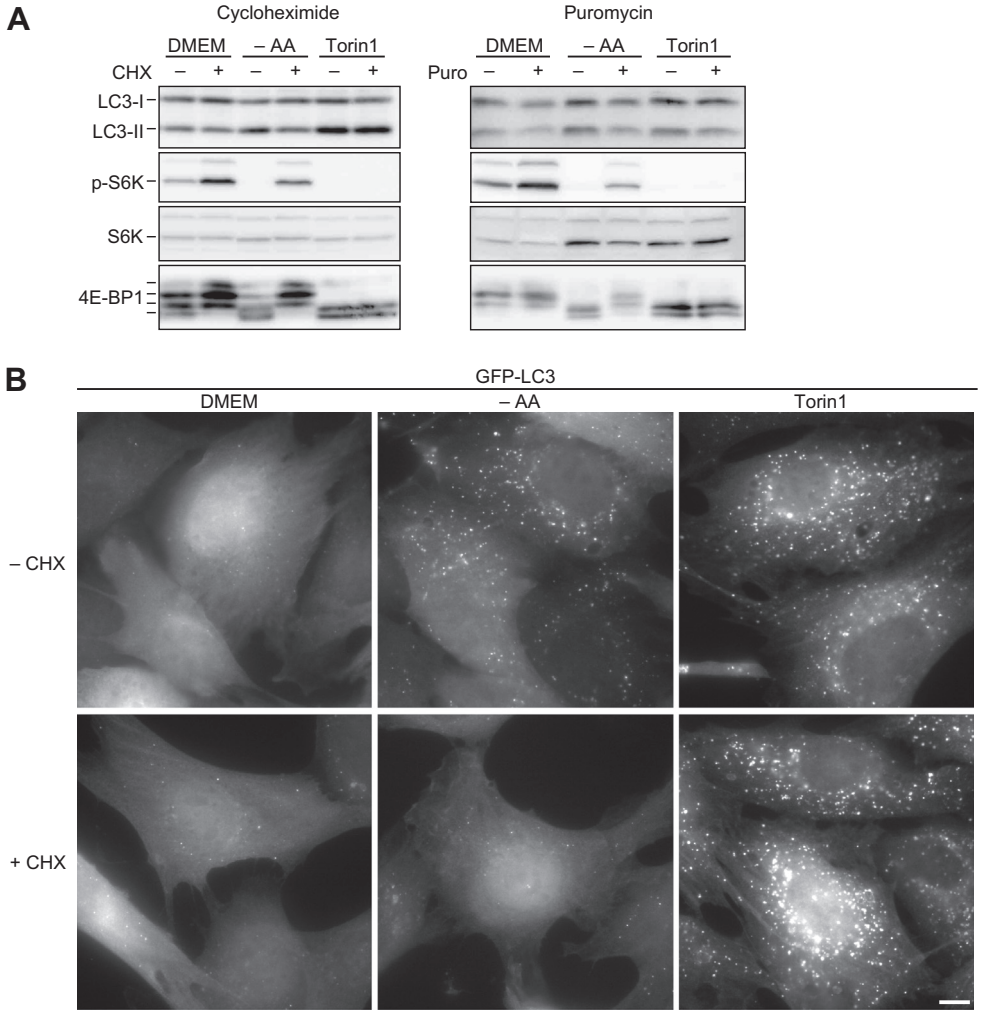

Fig. 1. Protein synthesis inhibitors block starvation-induced autophagy but not Torin1-induced autophagy. (A) HEK293 cells were pretreated with 50 μg/ml CHX or 100 μg/ml puromycin for 5 min before incubation with Torin1 or without amino acids for 60 min in the presence of CHX or puromycin. (B) MEFs stably expressing GFP-LC3 were pretreated with 50 μg/ml CHX for 5 min before addition of Torin1 or depletion of amino acid for 60 min. Bar indicates 10 μm.

with CHX for an additional 45 min in the same medium. The addition of CHX suppressed GFP-LC3 puncta formation in amino acid-deprived medium (Fig. 2A and B). We also evaluated the effect of CHX addition on autophagic flux by LC3 turnover assay. Without CHX, the level of LC3-II was further increased by treatment with the lysosomal inhibitor chloroquine (CQ) in amino acid-starved cells because LC3-II was degraded in the lysosome in an autophagy-dependent manner (Fig. 2C). By contrast, CHX treatment diminished the LC3-II accumulation by CQ treatment in amino acid-deprived cells, suggesting that CHX addition decreases autophagic flux (Fig. 2C). The phosphorylation levels of S6K and 4E-BP1 were increased by CHX addition even during starvation.

GCN2 is an amino acid depletion-sensing kinase. GCN2 binds to uncharged tRNAs, resulting in kinase activation through homodimerization and autophosphorylation [31]. As expected, amino acid deprivation increased the phosphorylation level of threonine 898 in GCN2, which was markedly repressed by CHX treatment (Fig. 2C). We found a good correlation between reactivation of mTORC1 and inactivation of GCN2; both were observed within 10 min after CHX treatment (data not shown). These data suggest that CHX treatment increases the level of the intracellular amino acid pool, which leads to reactivation of mTORC1 and suppression of autophagy in starved cells.

3.3. CHX inhibits an early step in autophagy

To investigate which autophagy step was inhibited by CHX, we examined the formation of GFP-ULK1 puncta. ULK1 functions at an early step in autophagosome formation and is present on isolation membranes (unclosed autophagosomes) but not on completely sealed autophagosomes [27]. ULK1 is one of the mTORC1 substrates and is negatively regulated by mTORC1 [32–34]. When MEFs stably expressing GFP-ULK1 were incubated in amino acid-deprived medium, GFP-ULK1 puncta, representing isolation membranes, were generated (Fig. 3A and B). These GFP-ULK1 puncta were markedly repressed by addition of CHX under starvation conditions (Fig. 3A and B). These data suggest that CHX inhibits an early step in autophagy.

3.4. Torin1 rescues autophagic inhibition following CHX treatment

If CHX indeed inhibits starvation-induced autophagy through reactivation of mTORC1, autophagy should be reactivated by mTORC1 inhibition. Thus, we tested whether Torin1 treatment restores the suppression of autophagy following CHX treatment. MEFs stably expressing GFP-LC3 were starved for 45 min and treated with CHX for an additional 30 min in the same medium to suppress autophagy. Then, Torin1 was added and cultured for another

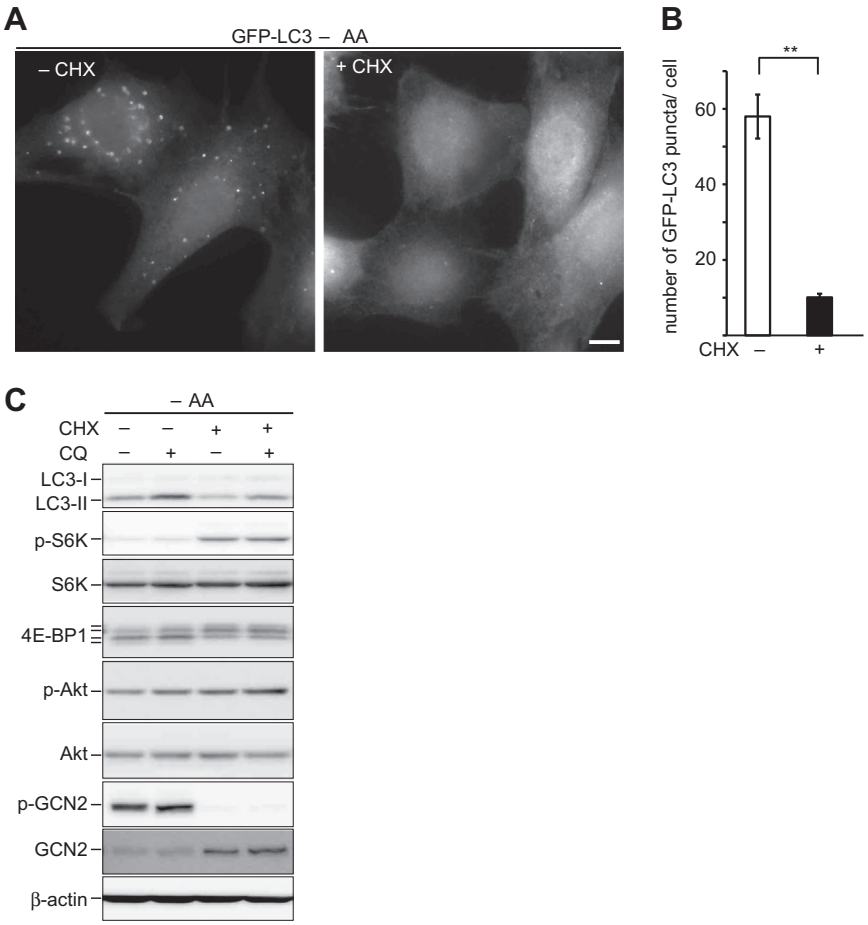

Fig. 2. CHX inhibits autophagy flux and causes reactivation of mTORC1 signaling and suppression of GCN2 activity. (A) MEFs stably expressing GFP-LC3 were incubated in amino acid-derived medium for 45 min, followed by incubation with 50 μg/ml CHX for 45 min. Bar indicates 10 μm. (B) Quantification of the number of GFP-LC3 puncta per cell in (A). At least 30 different cells were analyzed. ** indicates $p < 0.001$. (C) MEFs were incubated in amino acid-deprived medium for 45 min, followed by incubation with 50 μg/ml CHX for 45 min. Next, 20 μM CQ was added 15 min before analysis.

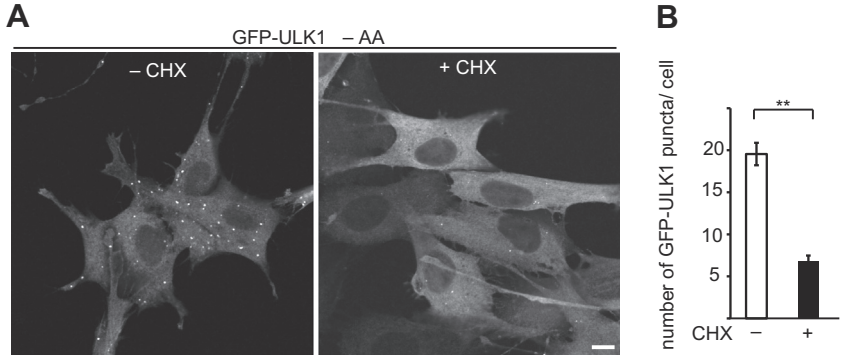

Fig. 3. CHX inhibits an early step of autophagy. (A) MEFs stably expressing GFP-ULK1 were incubated in amino acid-deprived medium for 75 min followed by treatment with or without 50 μg/ml CHX for 45 min. Cells were then fixed, permeabilized, and subjected to immunofluorescence microscopy using anti-GFP antibody. Bar indicates 10 μm. (B) Quantification of GFP-ULK1 punctate structures in (A). At least 30 different cells were analyzed. ** indicates $p < 0.001$.

45 min to determine whether autophagy was reactivated. As shown above, CHX suppressed starvation-induced GFP-LC3 puncta formation (Fig. 4, (1)). However, further addition of Torin1 restored the formation of GFP-LC3 puncta even in the presence of CHX (Fig. 4, (2)) to a level comparable to that in amino acid-starved cells without CHX treatment (Fig. 4, (3)). These results suggest that CHX inhibits starvation-induced autophagy through activation of mTORC1 and that starvation-induced autophagy does not require *de novo* protein synthesis.

4. Discussion

Based on the findings of this study, we propose that CHX inhibits starvation-induced autophagy through activation of mTORC1 due to the increase in the intracellular amino acid pool. This hypothesis is different from the previous assumption that inhibitors of protein synthesis suppress autophagy by inhibition of synthesis of short-lived protein(s) essential for autophagy. Our finding that Torin1 can induce autophagy even in the presence of CHX

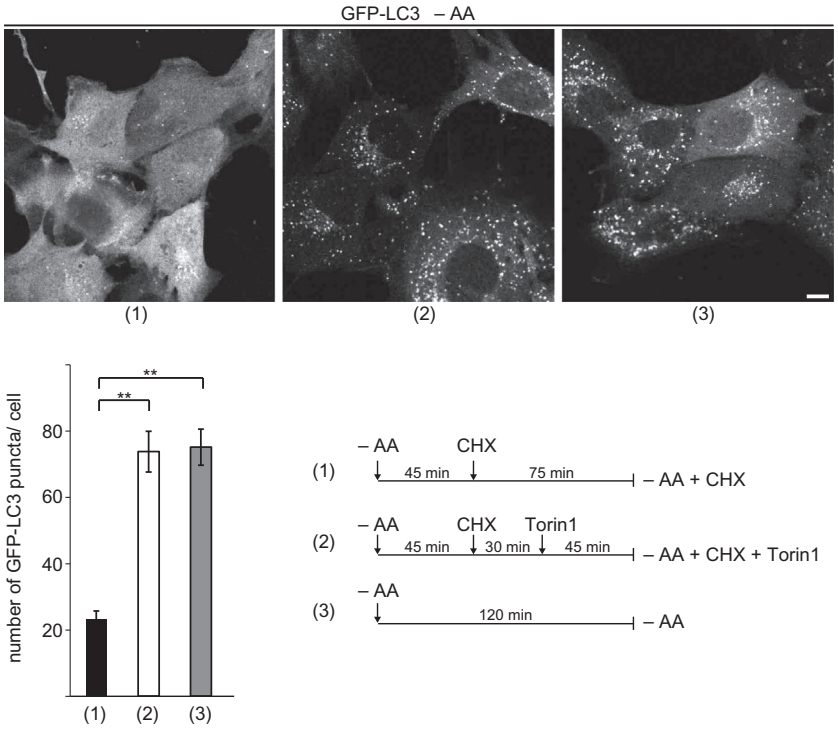

Fig. 4. CHX inhibits autophagy at a step upstream of mTORC1. MEFs stably expressing GFP-LC3 were incubated in amino acid-deprived medium for 45 min followed by treatment with (1 and 2) or without 50 μg/ml CHX (3) for 30 min. Then, cells were added with Torin1 and incubated for another 45 min (2). All the samples were collected at 120 min after the starvation treatment. At least 30 different cells were analyzed and the results are shown in the graph. Bar indicates 10 μm. Statistical analysis used one-way ANOVA with Tukey's test. ** indicates $p < 0.001$.

(Fig. 4) rather suggests that starvation-induced autophagy does not require new protein synthesis at least in our experimental setting. However, it does not rule out the possibility that transcriptional and/or translational regulation is important for autophagy during prolonged starvation. Recently, several transcription factors were shown to be involved in autophagy gene regulation. For example, FoxO family transcription factors modulate autophagy [7,10]. TFEB, ZKSCAN3, p53, E2F1, and NF-κB also induce some autophagy genes [6,8,9,11,12]. These transcription factors may be important to regulate basal autophagy or starvation-induced autophagy at more advanced stages. In particular, as Atg8/LC3, which is essential for autophagy, is degraded by autophagy, it would be important to restore its expression level [35–37].

The findings of this study also reemphasize that CHX should be carefully used in protein turnover assays. Besides conventional "pulse-chase experiments" using radioisotopes, protein half-life is often determined by monitoring protein degradation in cells treated with CHX to eliminate newly synthesized proteins. However, as shown here and in previous studies, CHX strongly inhibits autophagy and could artificially prolong the half-life of proteins that are preferentially degraded by autophagy. Thus, we recommend that such experiments should be performed only if the contribution of autophagy to degradation of the proteins of interest is negligible.

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