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Prolyl oligopeptidase acts as a link between chaperone-mediated autophagy and macroautophagy

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ABSTRACT

The accumulation of aggregated α-synuclein (α-syn) has been identified as the primary component of Lewy bodies that are the pathological hallmarks of Parkinson's disease (PD). Several preclinical studies have shown α-syn aggregation, and particularly the intermediates formed during the aggregation process to be toxic to cells. Current PD treatments only provide symptomatic relief, and α-syn serves as a promising target to develop a disease-modifying therapy for PD. Our previous studies have revealed that a small-molecular inhibitor for prolyl oligopeptidase (PREP), KYP-2047, increases α-syn degradation by accelerating macroautophagy (MA) leading to disease-modifying effects in preclinical PD models. However, α-syn is also degraded by chaperone-mediated autophagy (CMA). In the present study, we tested the effects of PREP inhibition or deletion on CMA activation and α-syn degradation. HEK-293 cells were transfected with α-syn and incubated with 1 & 10 μM KYP-2047 for 24 h. Both 1 & 10 μM KYP-2047 increased LAMP-2A levels, induced α-syn degradation and reduced the expression of Hsc70, suggesting that the PREP inhibitor prevented α-syn aggregation by activating the CMA pathway. Similarly, KYP-2047 increased the LAMP-2A immunoreactivity and reduced the Hsc70 levels in mouse primary cortical neurons. When LAMP-2A was silenced by a siRNA, KYP-2047 increased the LC3BII/LC3BI ratio and accelerated the clearance of α-syn. Additionally, KYP-2047 induced CMA effectively also when MA was blocked by bafilomycin A1. Based on our results, we suggest that PREP might function as a core network node in MA-CMA crosstalk, and PREP inhibition can reduce α-syn levels via both main autophagy systems.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder mostly affecting the motor system. It is characterized by decreased dopamine levels due to the loss of dopaminergic (DAergic) neurons in the *substantia nigra pars compacta* [1]. The array of pharmacologic treatments available for PD is broader than for any other neurodegenerative diseases of the CNS. However, even the most effective treatment for PD, L-3, 4-dihydroxyphenylalanine (L-DOPA), does not stop or reduce the neurodegeneration and has a side effect of dyskinesia particularly at the progressed state of PD [2,3]. There is an urgent need for disease-modifying therapies for PD patients but the outcomes of clinical trials aiming for disease-modifying drugs for PD have been disappointing.

At the cellular level, loss of DAergic neurons is accompanied by abnormal aggregation and accumulation of α-synuclein (α-syn) within intracytoplasmic inclusions known as Lewy bodies which are also rich in lipid membrane fragments [4–6]. The progression of α-syn aggregation and cell-to-cell propagation of certain α-syn species is thought to underlie neuronal dysfunction, and this may also be associated with the progression of PD symptomology. Moreover, GWAS studies have linked α-syn both to familial and sporadic forms of PD [7]. Genetic

Abbreviations: α-syn, alpha-synuclein; DAergic, Dopaminergic; DMEM, Dulbecco's modified Eagle's medium; FBS, Fetal bovine serum; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; HRP, Horseradish peroxidase; Hsc70, Heat shock cognate chaperone of 70kDa; KO, Knock-out; KYP-2047, 4–phenylbutanoyl–*L*–prolyl-2 (*S*)–cyanopyrrolidine; LAMP-2A, Lysosome-associated membrane protein type 2A; LC3B, Microtubule-associated proteins 1A/1B light chain 3B; MA, Macroautophagy; NBM, Neurobasal medium; Nrf2, Nuclear factor erythroid 2-related factor; OD, Optical density; PD, Parkinson's disease; PP2A, Protein phosphatase 2A; PREP, Prolyl oligopeptidase; TBS-T, Tris-buffered saline-0.05% Tween 20; WB, Western blot; WT, Wild-type.

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polymorphic variants of the α-syn locus (A53T, A30P and E46K) are associated with α-syn aggregation and confer an increased risk for sporadic PD similar to SNCA duplications and triplications [8]. Thus, clearance pathways for aggregated proteins could be the key to control α-syn toxicity and to affect PD pathogenesis.

Autophagy plays an essential role in the clearance of misfolded proteins and damaged cellular organelles both under physiological and pathological conditions by transferring the debris to the lysosome for degradation and recycling [9]. There are three primary types of autophagy: macroautophagy (MA), microautophagy, and chaperone-mediated autophagy (CMA), each involving different mechanisms of substrate delivery to the lysosome. mA is a self-eating pathway which still remains unclear. MA is a unique bulk degradation mechanism capable of transporting large intracellular structures such as oligomeric α-syn for degradation [10]. In contrast, CMA is a unique pathway that selectively transfers cytosolic proteins into lysosome for degradation. In CMA, any misfolded proteins containing the KFERQ motif, such as α-syn, can be recognized by the heat shock cognate chaperone of 70 kDa (Hsc70) [11]. After binding of the chaperone/substrate complex to the lysosome-associated membrane protein type 2A (LAMP-2A) receptor, the unfolded substrate proteins are translocated into the lysosome for degradation by hydrolases [12]. It has been found that mutant and oligomeric α-syn cannot be degraded by CMA but instead they bind strongly to the CMA receptor LAMP-2A and impair CMA activation. However, monomers and dimers of non-mutated α-syn can be degraded by CMA [13,14]. Therefore, CMA impairment leads to promote the extracellular release of α-syn and subsequently α-syn pathology.

Prolyl oligopeptidase (PREP; POP; PO; EC 3.4.21.26) is a cytosolic serine protease with endopeptidase activity that cleaves post-proline bonds of small peptides [15]. It has been shown that PREP interacts directly with α-syn and enhances its aggregation *in vitro* and in a cellular model [16,17]. In addition, studies have indicated that PREP and α-syn co-localize in cellular models during α-syn aggregation and co-localization has also been found in the *substantia nigra pars compacta* of post-mortem PD brain [18]. Our earlier studies have revealed that a small-molecular inhibitor of PREP, KYP-2047, was able to interfere with the α-syn aggregation process, and KYP-2047 also induces MA by activating the protein phosphatase 2A (PP2A)-DAPK-Beclin1 pathway to degrade aggregated forms of α-syn [19–22]. However, it is not known whether PREP and PREP inhibitors have any effects on other autophagy mechanisms, such as CMA, to promote the α-syn disaggregation and degradation. To further explore the role of PREP in CMA mediated degradation of α-syn aggregates, we have now tested the effects of KYP-2047 and deletion of PREP on CMA activation in non-neuronal cell lines (wild type and PREP knock out (PREP KO) HEK-293) with transient α-syn overexpression and on mouse primary cortical neurons.

2. Materials and methods

2.1. Chemicals and Reagents

The PREP inhibitor, KYP-2047 [4–phenylbutanoyl–*L*–prolyl-2(*S*)–cyanopyrrolidine] was synthesized for us at the School of Pharmacy, University of Eastern Finland, as described earlier [52]. Reagents were purchased from Sigma-Aldrich (St-Louis, MO, USA) unless otherwise stated in the text. Ethanol was purchased from Altia (Helsinki, Finland).

2.2. Cell culture

Human embryonic kidney (HEK-293) wild type (WT) cells and HEK-293 PREP knock out (KO) cells were used in this study. HEK-293 PREP-KO cells were prepared by using CRISPR/Cas9n plasmid as described in [21]. Cells were used for experiments in passages 3–20, and the HEK-293 cell line was organically purchased from ATCC (Manassas, VA, USA) and authenticated by the Genomics Unit of Technology Centre, Institute for Molecular Medicine Finland (FIMM, Helsinki, Finland) with the Promega GenePrint24 at 2019. HEK-293 wt cells were grown in full Dulbecco's modified Eagle's medium (DMEM, #D6429) with 10% (v/v) fetal bovine serum (FBS; #16000–044, ThermoFisher Scientific, Waltham, MA, USA) and 1% (v/v) penicillin–streptomycin solution (#15140122, ThermoFisher Scientific). HEK-293 PREP KO cells were cultured in DMEM with 20% FBS. 400,000/cell per well were seeded on 6-well plates and were cultured for 24 h post plating for further experiments.

2.3. Primary culture of mouse cortical neurons

Pure primary mouse cortical neurons were obtained from C57BL/6J mice (WT) (1 day old; Envigo, Venray, The Netherlands) and from PREP KO mice (Deltagen, San Carlos, CA, USA) in a C57BL/6JRccHsd genetic background (5–10 back crossings). All animal handling and surgery experimental procedures were performed in accordance with the European Communities Council Directive 86/609/EEC and were approved by the Finnish National Animal Experiment Board. Cortical tissue was dissected out and dissociation in an ice-cold Hank's Balanced Salt Solution medium (#14175, ThermoFisher Scientific) containing trypsin (0.25 mg/mL) and DNAse (5U/mL). After centrifugation, cortical neurons were re-suspended in Neurobasal Medium (NBM; #21103049, ThermoFisher Scientific) containing 2% (v/v) B27 (#17504044, ThermoFisher Scientific), 1% (v/v) penicillin–streptomycin solution, 100 μg/mL Primocin (#ant-pm-1, InvivoGen, Toulouse, France) and 2 mM glutamine (#25030081, ThermoFisher Scientific) and seeded onto poly-L-lysine coated cell culture plates at an appropriate density (200,000 cells/well to 12-well plates). After 24 h seeding, the medium was half changed and neurons were maintained at 37 ℃ in a 5% CO_2 incubator. The neurons were cultured for 7 days for experimental use.

2.4. Cell treatment

HEK-293 WT/PREP KO cells were seeded on 6-well plates and transfected with pAAV-EF1a-V5-synuclein (2500 ng/well; Addgene #60057; Dr. Brandon Harvey, Intramural Research Program, National Institute on Drug Abuse, Baltimore, MD) by using Lipofectamine 3000 Transfection Reagent (L3000015; ThermoFisher Scientific) according to the manufacturer's instructions. LAMP-2A Stealth siRNA (#1299001; ThermoFisher Scientific) transfection in HEK-293 WT/PREP KO cells was done using the Lipofectamine RNAiMAX Transfection Reagent (13778–030; ThermoFisher Scientific) according to the manufacturer's instructions. After 24 h transfection, HEK-293 WT/PREP KO cells and neurons were incubated with the MA inhibitor Bafilomycin A1 (B1793; final concentration of 20 nM). For PREP inhibitor experiments, a concentration of 1 μM or 10 μM of KYP-2047 was used 24 h after transfection. Selected concentrations are based on our earlier studies with KYP-2047 in cell culture experiments [17,19,22]. KYP-2047 was diluted to cell culture medium (DMEM or NBM) from 100% DMSO stock (100 mM), and corresponding concentration of DMSO was used as a vehicle control.

2.5. Western blot (WB) analysis

Cells or primary neurons were washed with PBS and lysed in modified RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Sodium deoxycholate, 1% NP-40, pH 7.4) containing Halt Phosphatase (#87786, ThermoFisher Scientific) and Halt Protease Inhibitor cocktails (#78430, ThermoFisher Scientific). Cells lysates were quantified by Pierce BCA Protein Assay Kit (#23225, ThermoFisher Scientific). Equal amounts of protein (~30 μg) were separated by 7.5% (#4561023; Bio-Rad, Hercules, CA, USA) or 12% Mini-Protein TGX gels (#4561043; Bio-Rad) and then transferred onto Trans-Blot Turbo Midi PVDF (#1704157; Bio-Rad) membranes by using Trans-Blot Turbo Transfer System (#1704150; Bio-Rad). The membranes were blocked with the blocking buffer (5% (W/V) skim milk (Valio, Helsinki, Finland), Tris-

Table 1
Details of antibodies used.

Antibody	Species	Manufacturer	Product#	Dilution	
				WB	IHC
LAMP-2A	Rabbit	Abcam	ab18528	1/1000	1/200
Hsc70	Rat	Abcam	ab51052	1/5000	–
GAPDH	Mouse	Abcam	ab8245	1/5000	–
LC3B	Rabbit	Sigma	L7543	1/1000	–
α-Synuclein	Mouse	Abcam	ab80627	1/1000	–
Vinculin	Rabbit	Abcam	ab129002	1/10000	–
Cadherin	Mouse	Abcam	ab1416	–	1/200

buffered saline-0.05% Tween 20 (TBS-T)) at room temperature for 1 h and incubated overnight with primary antibodies at 4 ℃. A detailed list of primary antibodies and respective concentrations is presented in Table 1. Antibody specification was verified by their vendor, and we also verified that the antibodies gave a band with correct molecular weight. After washing with TBS-T three times, the membranes were incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at room temperature, goat anti-rabbit HRP (#31463; ThermoFisher Scientific); goat anti-mouse HRP (#31430; ThermoFisher Scientific) and donkey anti-rat HRP (#ab102182; Abcam, Cambridge, UK). The membranes were incubated with SuperSignal West Pico Plus Chemiluminescent Substrate (#34579; ThermoFisher Scientific) or SuperSignal West Femto Maximum Sensitivity Substrate (#34094; ThermoFisher Scientific). Immunoreactivity was visualized by the ChemiDoc XTS + System (Bio-Rad) and the relative optical densities (OD) of the bands were analyzed with image analysis software Image J (version 1.51; National Institute of Health, Bethesda, MD).

2.6. Immunofluorescent staining

Immunofluorescent staining (IF) was used to detect changes in LAMP-2A after PREP inhibitor treatment in primary cortical neurons. Primary cortical neurons were on PLL-coated coverslips in a 12-well plate with a density of 100,000 cells/well. 24 h after seeding, cells were treated with 1 and 10 μM KYP-2047 for 24 h, washed with PBS and fixed for 10 min in PBS containing 4% paraformaldehyde at room temperature. After permeabilizing the cells with 0.5% Triton X-100 for 5 min, the neurons were blocked with 10% normal goat serum (#S-1000, Vector laboratories, Burlingame, CA, USA) for 30 min at room temperature on shaker. Then, neurons were incubated overnight at 4 ℃ in PBS containing primary antibodies (details in Table 1). After washing with PBS three times, cells were incubated with PBS containing anti-rabbit Alexa Fluor 488 (1:800; #ab150077, Abcam) and anti-mouse Alexa Flour 568 (1:800; #ab175473, Abcam) at room temperature for 2 h. Finally, cells were washed twice with PBS and mounted with Vectashied containing DAPI (H-1200, Vector Laboratories).

All images were captured with a Leica TCS SP8 STED confocal fluorescence microscope (Leica Microsystems, Wetzlar, Germany). The same exposure time was used for LAMP-2A stainings, and the samples were analyzed by using ImageJ. The analysis was done similarly as in Rostami et al. (2020). Briefly, 8-bit images were set with the same threshold, and thereafter particle analysis was performed. Particle area was correlated with cell count based on DAPI staining. From each section, more than 10 cells were counted, and for each treatment, 6 section was analyzed. All analysis were performed blinded for the treatment.

2.7. Nuclear factor erythroid 2-related factor (Nrf2) transcription factor assay

For nuclear factor erythroid 2-related factor (Nrf2) transcription factor assay, a protocol described in Eteläinen et al. [49] was used. Briefly, HEK-293 wt or HEK-293 PREP KO cells were plated in T-25 flasks. After the KYP-2047 treatment (1 and 10 μM), the nuclear extracts of cells were prepared by using Nuclear Extraction Kit (#ab113474; Abcam) according to the manufacturer's instruction. The protein concentration of nuclear extract was measured with Protein Assay Dye Reagent Concentrate (#5000006; Bio-Rad). Nrf2 transcription factor assay was carried out by using Nrf2 Transcription Factor Assay Kit (#ab207223; Abcam) according to the manufacturer's instruction. In detail, the nuclear extracts and controls were added to the wells which were pre-coated with oligonucleotide containing Nrf2 consensus binding site, and incubated for 1 h at RT. After incubating with primary antibody for Nrf2 at RT for 1 h, anti-rabbit HRP-conjugated secondary antibody was added and incubated in the dark for 1 h at room temperature. The developing solution was added to wells, incubated 15 min at room temperature avoiding direct light, and then the stop solution was added when all the wells turned medium to dark blue. Absorbance (OD 450 nm and with a reference wavelength of OD 665 nm) was measured by using Varioskan LUX Multimode Microplate Reader (ThermoFisher Scientific).

2.8. Reverse transcription qPCR (RT-qPCR)

For RT-qPCR, total RNA was isolated from living HEK-293 wt cells using TRIzol Reagent solution (#15596026; ThermoFisher Scientific) and cDNA was prepared using iScript cDNA sytheses Kit for RT-qPCR (#1708890; Bio-Rad) with primers in 20 μL reaction according to the manufacturer's protocol (see details of primers in Table 2). The primer sequences were based on earlier publications, and RNAase free solutions were used in all steps. qPCR was carried out by using SsoAdvanced Universal SYBR Green Supermix (#1725271; Bio-Rad) on a MiniOpticon PCR system (#CFB3120EDU; Bio-Rad) with 1 ng of cDNA per reaction in 20 μL reaction with cycling conditions (activation 30 sec at 95℃, denaturation 15 sec at 95℃, annealing 30 sec at 60℃ for 40 cycles and

Table 2
Primers for RT-qPCR.

Gene	Forward	Reverse
LAMP-2A	TTATGACTCGCACTGAAGCG	ATGGTAGCCAGCAGACAAGT
Hsc70	CCTTCGTTATTGGAGCCAGG	GCATCTTTGGTAGCCTGACG
wt p53	CTCCTCAGCATCTTATCCG	AGCCTGGGCATCCTTG
mutant p53	TCAACAAGATGTTTTGCCAACTG	ATGTGCTGTGACTGCTTGTAGATG
Vinculin	CAACCAGTATTGCTCGTCGG	TGATGTCATTGCCCTTGCTG

65-95℃ for melt-curve analysis). White 0.2 ml 8-Tube PCR Strips (#TLS0851; Bio-Rad) and optical flat PCR Tube 8-Cap Strips (#TCS0803; Bio-Rad) were used for qPCR.

2.9. Data and statistical analyses

All the experiments were done at least in triplicate, with 3–7 individual samples on each, and the samples were not used to re-analyze same proteins. Data is expressed as mean value ± standard deviation (SD), and the negative control average was set as 100 % on each assay to reduce variability between repeats. Differences between two groups were analyzed by using two-tailed unpaired student's *t*-test. For multiple groups with one variable, One-way analysis of variance (ANOVA) was used followed by Tukey's post-hoc comparison if ANOVA assay gave the statistical significance ($p < 0.05$). Statistical analysis was performed by using PRISM GraphPad software (version 9.1.0, GraphPad Software, Inc., San Diego, CA).

3. Results

3.1. PREP inhibition activates CMA pathway and reduces the levels of GADPH, an endogenous substrate for CMA

Our previous studies have shown that PREP inhibition induces MA activity and reduces protein aggregation in PD models [19,21], and therefore, we wanted to characterize if PREP and PREP inhibition also regulates CMA. CMA activity has been detected in many different types of cell lines, such as HEK-293 cells, and we first characterized the impact of PREP deletion or inhibition on CMA in HEK-293 cell cultures [23]. HEK-293 cells were incubated with 1 & 10 μM KYP-2047 or vehicle (10 μM DMSO; 0.01%) for 4, 8, 12, 24 and 48 h (Fig. 1A-D), and we analyzed the levels of commonly used CMA marker proteins LAMP-2A and Hsc70, and a CMA substrate, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). At 4 h time-point, the LAMP-2A and Hsc70 levels were decreased with both KYP-2047 concentrations, and Hsc70 reduction was significant (Fig. 1A; $F = 54.33$, $p < 0.0001$; $p < 0.05$, NC vs. 1 μM KYP-2047; $p < 0.01$ NC vs. 10 μM KYP-2047; 1-way ANOVA with Tukey's post-test). At 8 h time-point no changes were seen, and after 12 h incubation, LAMP-2A was not altered but Hsc70 and GADPH were elevated with 1 μM KYP-2047 (Fig. 1B-C; Hsc70: $F = 23.04$, $p < 0.0151$; $p < 0.05$, NC vs. 1 μM KYP-2047; GADPH: $F = 13.79$, $p < 0.05$; $p < 0.05$, NC vs. 1 μM KYP-2047). Then again at the 24 h time-point, WB analysis showed that the LAMP-2A level significantly increased and the Hsc70 level decreased with KYP-2047 treatment (Fig. 1D; LAMP-2A: $F = 37.01$, $p < 0.0077$; $p < 0.05$, NC vs. 1 μM KYP-2047; $p < 0.01$ NC vs. 10 μM KYP-2047; Hsc70: $F = 37.48$, $p < 0.0075$; $p < 0.05$, NC vs. 1 μM KYP-2047; $p < 0.01$ NC vs. 10 μM KYP-20471-way ANOVA with Tukey's post-test) pointing to activated CMA, and therefore this time-point was selected for further experiments (Fig. 1D). Simultaneously, a decrease in the levels of a CMA substrate, GAPDH, further suggested that the maximal CMA activity occurred at 24 h PREP inhibition (Fig. 1D; not significant).

A selective method to study CMA activity is to silence or delete LAMP-2A which prevents the chaperone/substrate complex from entering into lysosomes. To verify the role of PREP inhibition in CMA pathway activation, we disrupted LAMP-2A expression by using siRNA to silence the LAMP-2A gene [24]. Moreover, PREP-KO HEK-293 cells were used to confirm that the effects are PREP-related. LAMP-2A levels were significantly reduced after 24 h of silencing, and 1 & 10 μM KYP-2047 did not alter LAMP-2A expression after LAMP-2A silencing although there was a significant effect by KYP-2047 on LAMP-2A in intact cells after 24 h exposure (Fig. 2A-B; $F = 34.95$, $p < 0.0001$; $p < 0.01$, NC vs. 1 μM KYP-2047; $p < 0.001$, NC vs. 10 μM KYP-2047; NC vs. LAMP-2A silenced +/- 1 or 10 μM KYP-2047; 1-way ANOVA with Tukey's multiple comparison test). In PREP-KO cells, LAMP-2A levels were decreased (not significant) compared to WT cells and LAMP-2A silencing effectively reduced LAMP-2A expression also in PREP-KO cells (Fig. 2A-B; $p < 0.001$, intact PREP-KO cells vs. LAMP-2A silenced PREP-KO cells; 1-way ANOVA with Tukey's multiple comparison test). Correlating with LAMP-2A changes, Hsc70 was downregulated by 1 and 10 μM KYP-2047 incubation, and by LAMP-2A silencing (Fig. 2A & C). After LAMP-2A silencing, 10 μM KYP-2047 did not influence Hsc70 levels but 1 μM KYP-2047 increased the Hsc70 levels (Fig. 2A & C; $F = 34.95$, $p < 0.0001$; $p < 0.0001$, NC vs. 1 μM KYP-2047; $p < 0.0001$, NC vs. 10 μM KYP-2047; NC vs. LAMP-2A silencing +/- 10 μM KYP-2047; 1-way ANOVA with Tukey's multiple comparison test). In PREP-KO cells, Hsc70 levels were significantly lowered in intact cells and also in LAMP-2A silenced cells (Fig. 2A & C; $p < 0.0001$, NC vs. PREP-KO cells and PREP-KO cells with LAMP-2A siRNA; 1-way ANOVA with Tukey's multiple comparison test). 1 and 10 μM KYP-2047 significantly decrease the GAPDH levels in HEK-293 cells when LAMP-2A was not silenced (Fig. 2A & D). Silencing of LAMP-2A decreased GAPDH levels (not significant), and 10 μM KYP-2047 did not affect the levels of GAPDH. However, similar to Hsc70, 1 μM KYP-2047 had increasing effect on GADPH (Fig. 2A & D, GAPDH: $F = 9.336$, $p < 0.0001$; $p = 0.0797$, NC vs. 1 μM KYP-2047; $p < 0.05$, NC vs. 10 μM KYP-2047; 1-way ANOVA with Tukey's multiple comparison test). Furthermore, the levels of GAPDH were not significantly changed with/without LAMP-2A silencing in PREP-KO HEK-293 cells. Since LAMP-2A and Hsc70 are two key regulators of CMA pathway activation and GAPDH is the endogenous CMA substrate, these results indicated that PREP inhibition induces the CMA pathway activation.

3.2. PREP inhibition reduces α-syn accumulation via both the CMA and MA pathways

Previous reports have shown that α-syn accumulation and aggregation impairs the CMA pathway, leading to dysfunction in α-syn degradation that further contributes to neurotoxicity. We speculated that PREP inhibition could reduce α-syn accumulation by regulating the CMA pathway activity. In order to study if PREP inhibition induces α-syn degradation via CMA activation *in vitro*, we performed a transient transfection of α-syn in WT HEK-293 cells followed by 24 h treatment with 1 & 10 μM KYP-2047. WB analysis of CMA markers revealed α-syn overexpression had no effect on LAMP-2A levels but when combined with 1 μM KYP-2047, the LAMP-2A levels were significantly elevated (Fig. 3A & B, LAMP-2A: $F = 9.718$, $p < 0.0001$; $p < 0.01$, NC vs. α-syn + 1 μM KYP-2047; $p < 0.01$, α-syn vs. α-syn + 1 μM KYP-2047; 1-way ANOVA with Tukey's multiple comparison test). LAMP-2A was reduced by siRNA and α-syn transfection had no additional effect on this with or without KYP-2047 incubation (Fig. 3A & B).

α-syn overexpression significantly reduced Hsc70 levels and KYP-2047 increased the levels in dose-dependent manner (Fig. 3A & C; Hsc70: $F = 4.624$, $p = 0.0015$; $p < 0.01$, NC vs. α-syn; 1-way ANOVA with Tukey's multiple comparison test). When LAMP-2A was silenced, the effect of α-syn transfection on Hsc70 levels was not significant but when combined with 1 μM KYP-2047, the Hsc70 levels significantly decreased ($p < 0.05$, NC vs α-syn + LAMP-2A siRNA and 1 μM KYP-2047). A CMA substrate, GAPDH, was performing similar to Hsc70, and the levels were significantly decreased by α-syn overexpression but increasing when incubated with KYP-2047 (Fig. 3A & D, GAPDH: $F = 9.059$, $p < 0.0001$; $p < 0.0001$, NC vs. α-syn; $p < 0.01$ NC vs. α-syn + 1 μM KYP-2047). LAMP-2A silencing combined with α-syn transfection significantly decreased GAPDH levels, and KYP-2047 no further effect on this (Fig. 3A & D, GAPDH: $F = 9.059$, $p < 0.0001$; $p < 0.0001$, NC vs. α-syn; $p < 0.01$ NC vs. α-syn + 1 μM KYP-2047; $p < 0.01$ NC vs. LAMP-2A siRNA + α-syn; $p < 0.0001$, NC vs. LAMP-2A siRNA + α-syn + 1/10 μM KYP-2047).

Both 1 and 10 μM KYP-2047 significantly decreased the levels of α-syn in HEK-293 cells when incubated for 24 h after transfection (Fig. 3A & E, α-syn: $F = 5.125$, $p = 0.0043$; $p < 0.05$ α-syn vs. α-syn + 1 μM KYP-2047; $p < 0.01$ α-syn vs. α-syn + 10 μM KYP-2047). LAMP-2A is

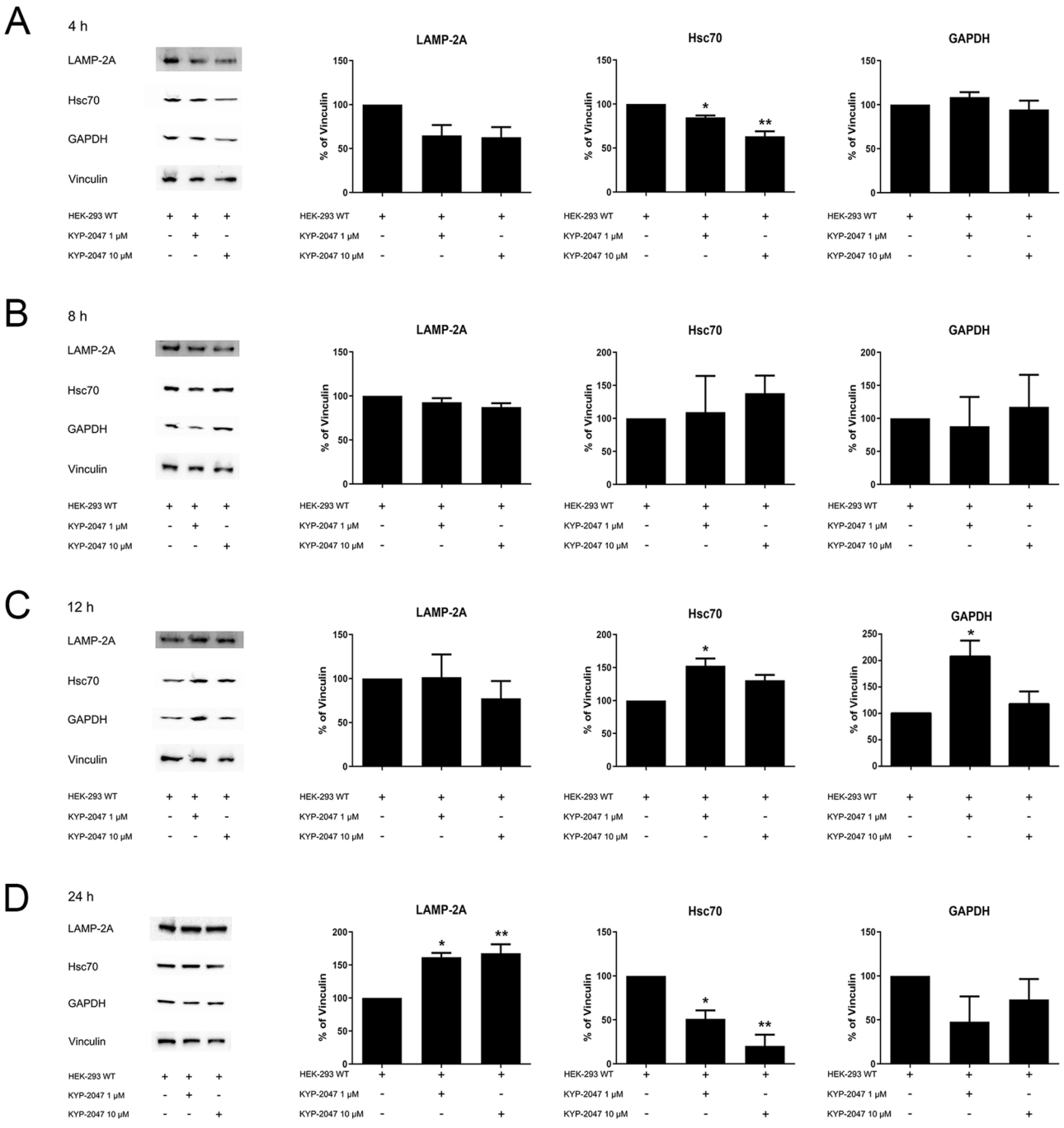

Fig. 1. The effect of 4, 8, 12 and 24 h PREP inhibition by KYP-2047 on CMA markers. LAMP-2A protein levels were decreased in HEK-293 cells after 4 h 1 and 10 μM KYP-2047 incubation (A; absolute values (av): NC, 0.94; 1 μM KYP-2047, 0.62; 10 μM KYP-2047, 0.60; not significant), but after 8 h incubation the decrease was not as evident (B; av: NC, 0.79; 1 μM KYP-2047, 0.73; 10 μM KYP-2047, 0.69. Hsc70 levels were significantly decreased after 4 h KYP-2047 incubation both with 1 and 10 μM concentration (A; av: NC, 0.55; 1 μM KYP-2047, 0.47; 10 μM KYP-2047, 0.35) but no significant changes were seen after 8 h incubation (B; av: NC, 0.35; 1 μM KYP-2047, 0.38; 10 μM KYP-2047, 0.49). The levels of GADPH were not altered either 4 or 8 h KYP-2047 incubation (A-B; av 4 h: NC, 0.30; 1 μM KYP-2047, 0.32; 10 μM KYP-2047, 0.29; av 8 h: NC, 0.24; 1 μM KYP-2047, 0.21; 10 μM KYP-2047, 0.29). At 12 h time-point, KYP-2047 had no effect on LAMP-2A levels but Hsc70 and GAPDH were significantly elevated by 1 μM KYP-2047 (C; av LAMP-2A: NC, 0.87; 1 μM KYP-2047, 0.88; 10 μM KYP-2047, 0.67; av Hsc70: NC, 0.31; 1 μM KYP-2047, 0.49; 10 μM KYP-2047, 0.67; av GAPDH: NC, 0.26; 1 μM KYP-2047, 0.53; 10 μM KYP-2047, 0.30). After 24 h incubation with KYP-2047 (D), Western blot analysis showed that the LAMP-2A levels were significantly increased (av: NC, 0.54; 1 μM KYP-2047, 0.88; 10 μM KYP-2047, 0.91) and the Hsc70 levels decreased dose-dependently with KYP-2047 treatment (av: NC, 0.36; 1 μM KYP-2047, 0.18; 10 μM KYP-2047, 0.07). GADPH was decreased by KYP-2047 at 24 h time-point but this was not significant (av: NC, 0.24; 1 μM KYP-2047, 0.11; 10 μM KYP-2047, 0.17). Vinculin was used as a loading control. Data is expressed as means (±SEM) from three independent experiments. ** $p < 0.01$ and * $p < 0.05$ vs negative control (NC), 1-way ANOVA with Tukey's multiple comparison test.

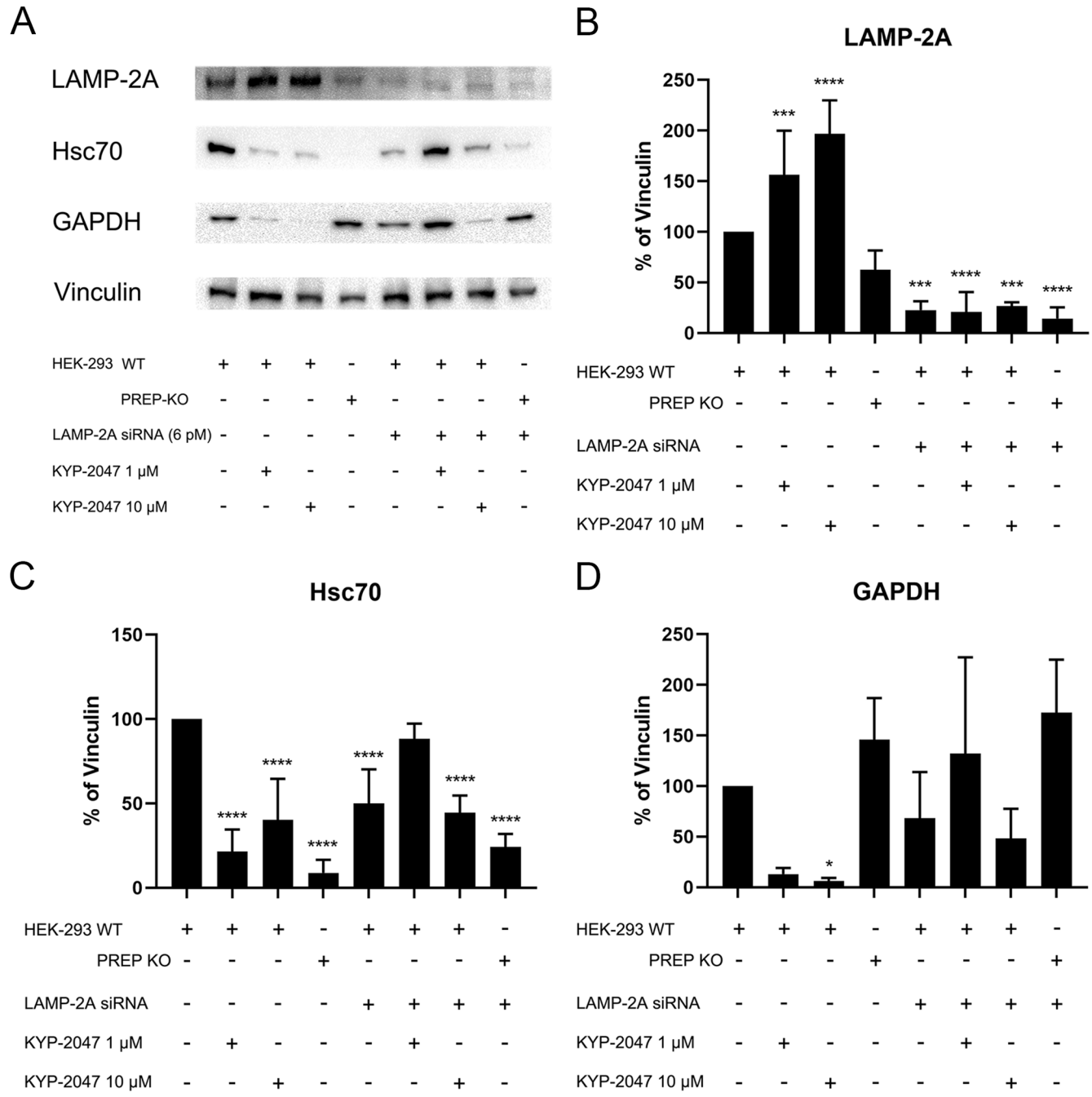

Fig. 2. PREP inhibition induces CMA pathway activation and reduces the level of CMA substrate GAPDH in HEK-293 cells, and LAMP-2A siRNA restores the CMA pathway activation induced by PREP inhibition. HEK-293 wild-type (WT)/PREP KO HEK-293 cells were incubated with 1 & 10 μM KYP-2047 or vehicle (10 μM DMSO; 0.01%) for 24 h after disrupting LAMP-2A expression by using siRNA, and the levels of CMA markers (LAMP-2A & Hsc70) and a substrate (GAPDH) were determined by Western blot (A). With 1 & 10 μM KYP-2047, the LAMP-2A levels were increased in the HEK-293 cells (B; absolute values (av): NC, 1.01; 1.54 and 2.00, respectively) but after silencing the LAMP-2A with a SiRNA (SiRNA, 0.22), no effect by KYP-2047 was seen (SiRNA + 1 μM KYP-2047, 0.21; SiRNA + 10 μM KYP-2047, 0.26). In PREP KO HEK-293 cells, the LAMP-2A level was not significantly altered without siRNA (B; av: PREP-KO, 0.63; PREP-KO + SiRNA, 0.15). The levels of Hsc70 were significantly decreased with 1 & 10 μM KYP-2047 exposure (C; av: NC, 1.37; 0.29 and 0.55, respectively) and with 10 μM KYP-2047 after LAMP-2A siRNA treatment (SiRNA, 0.67; 1 μM KYP-2047 + SiRNA, 1.20; 10 μM KYP-2047 + SiRNA, 0.60). In PREP -KO HEK-293 cells, the level of Hsc70 was also significantly decreased (C; 0.12; PREP-KO + SiRNA, 0.33). LAMP-2A deficiency did not affect the level of GAPDH but 10 μM KYP-2047 decreased the GAPDH expression in HEK-293 cells (D; av: NC, 1.33; 1 μM KYP-2047, 0.17; 10 μM KYP-2047, 0.08; SiRNA, 0.90; SiRNA + 1 μM KYP-2047, 1.76; SiRNA + 10 μM KYP-2047, 0.64). In PREP KO HEK-293 cells, GAPDH levels were not significantly changed (D; PREP-KO, 1.93; PREP-KO + SiRNA, 2.29). Vinculin was used as a loading control. Data is expressed as means (±SEM) from three independent experiments. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ vs negative control, 1-way ANOVA with Tukey's multiple comparison test (B-D).

implicated in the α-syn degradation via CMA activation as previously reported, and therefore, it would be expected that the interruption of LAMP-2A gene expression increases in α-syn protein level. However, we observed that LAMP-2A deficiency had no effect on α-syn levels in α-syn transfected HEK-293 cells, and it even decreased the α-syn levels (Fig. 3A & E; $p < 0.05$, α-syn vs. α-syn + LAMP-2A siRNA; 1-way ANOVA with Tukey's multiple comparison test). KYP-2047 had no additional effect on the α-syn levels after LAMP-2A silencing (Fig. 3A & E).

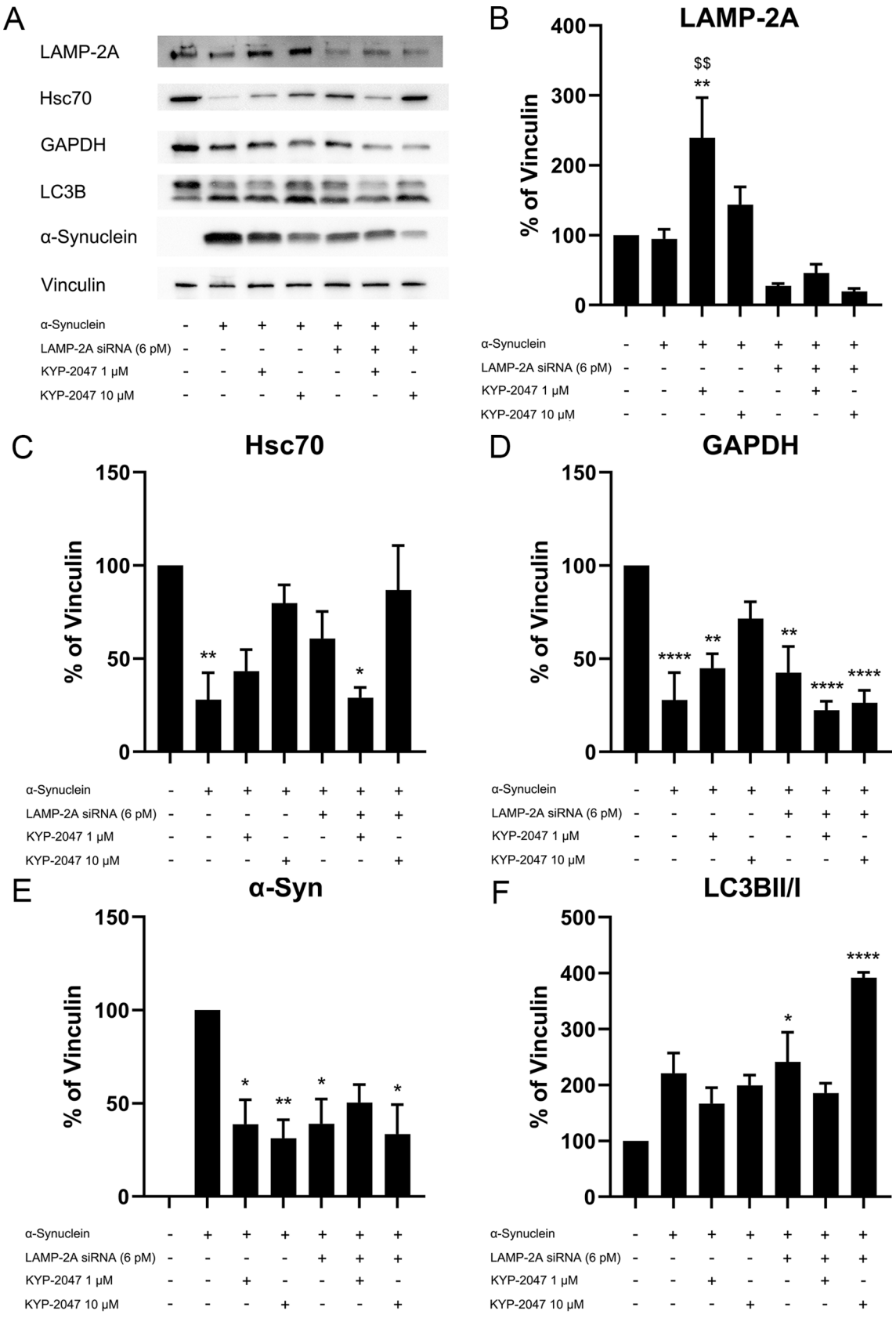

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Fig. 3. PREP inhibition reduces WT α-syn accumulation via CMA and MA activation. (A). Western blot analysis of CMA markers after transient α-syn transfection revealed that LAMP-2A was significantly increased by KYP-2047 (B; absolute values (av): NC, 1.48; α-syn + DMSO, 1.41; α-syn + 1 μM KYP-2047, 3.55; α-syn + 10 μM KYP-2047, 2.13), and the level of Hsc70 and GAPDH were decreased in KYP-2047 treated cells (C; av Hsc70: NC, 1.31; α-syn + DMSO, 0.35; α-syn + 1 μM KYP-2047, 0.56; α-syn + 10 μM KYP-2047, 1.04; D; av GADPH: NC, 2.06; α-syn + DMSO, 0.58; α-syn + 1 μM KYP-2047, 0.93; α-syn + 10 μM KYP-2047, 1.46), while the α-syn level was significantly reduced in α-syn overexpressing cells compared to the cells with vehicle (E; av: α-syn + DMSO, 2.57; α-syn + 1 μM KYP-2047, 1.00; α-syn + 10 μM KYP-2047, 0.80). LAMP-2A silencing countered the KYP-2047-induced LAMP-2A increase (B; av: SiRNA + α-syn + DMSO, 0.40; SiRNA + α-syn + 1 μM KYP-2047, 0.68; SiRNA + α-syn + 10 μM KYP-2047, 0.28), but LAMP-2A deficiency had no effect on α-syn degradation in α-syn overexpressing cells (E; av: SiRNA + α-syn + DMSO, 1.00; SiRNA + α-syn + 1 μM KYP-2047, 0.57; SiRNA + α-syn + 10 μM KYP-2047, 0.67), and the level of GAPDH also decreased in KYP-2047 treatment groups after SiRNA (D; av: SiRNA + α-syn + DMSO, 0.88; SiRNA + α-syn + 1 μM KYP-2047, 0.45; SiRNA + α-syn + 10 μM KYP-2047, 0.53). LAMP-2A silencing with α-syn overexpression did not have significant effect on Hsc70 but this was decreased with 1 μM KYP-2047 (C; av: SiRNA + α-syn + DMSO, 0.80; SiRNA + α-syn + 1 μM KYP-2047, 0.38; SiRNA + α-syn + 10 μM KYP-2047, 1.13), Of the MA markers the LC3BII/I ratio was increased in KYP-2047 treated cells, especially after LAMP-2A silencing (F; av: NC, 0.28; α-syn + DMSO, 0.63; α-syn + 1 μM KYP-2047, 0.48; α-syn + 10 μM KYP-2047, 0.57; SiRNA + α-syn + DMSO, 0.69; SiRNA + α-syn + 1 μM KYP-2047, 0.53; SiRNA + α-syn + 10 μM KYP-2047, 1.12). Vinculin served as a loading control. Data is expressed as means (±SEM) from three independent experiments. **** $p < 0.0001$, ** $p < 0.01$ and * $p < 0.05$ vs negative control (NC). $$ $p < 0.05$ NC vs α-syn, 1-way ANOVA with Tukey's multiple comparison test (B-F).

GAPDH and α-syn have been reported to be degraded via CMA but they can also be cleaved by MA [25]. Since both GAPDH and α-syn levels were decreased even when LAMP-2A was silenced, we deduced that MA may be activated when the CMA pathway has been blocked. To address this possibility, we detected MA markers light chain 3 beta I&II (LC3BI & LC3BII) in HEK-293 cells after α-syn overexpression, and observed that the LC3BII/I ratio was slightly increased after α-syn transfection (not significant) and KYP-2047 treatment did not have additional effects on these markers (Fig. 3A & F, LC3BII/I: $F = 10.00$, $p < 0.0001$; 1-way ANOVA with Tukey's multiple comparison test). Interestingly, the LC3BII/I ratio was significantly increased after LAMP-2A silencing in α-syn transfected cells, and 10 μM KYP-2047 incubation further elevated this ratio (Fig. 3A & F, LC3BII/I: $F = 10.00$, $p < 0.0001$; $p < 0.05$, NC vs. α-syn + LAMP-2A siRNA; $p < 0.0001$, NC vs. α-syn + LAMP-2A siRNA + 10 μM KYP-2047; 1-way ANOVA with Tukey's multiple comparison test).

3.3. PREP inhibition induces CMA pathway activation in mouse primary cortical neurons

We also wanted to confirm the main results from HEK-293 cells with neuronal cells since the role of PREP and its inhibition has been studied particularly in the context of CNS diseases. Matured primary cortical neurons (DIV7) from both WT mice were treated with 1 and 10 μM KYP-2047 for 24 h, and the levels of the CMA markers Hsc70 and GADPH in neurons were detected by WB. Moreover, primary cortical neurons from PREP-KO mice were used as a control for PREP-specific effects. LAMP-2A levels were not detectable by WB, and therefore, immunofluorescence staining was used to study the changes in LAMP-2A. Similar to non-neuronal cell cultures, PREP inhibition (10 μM KYP-2047) significantly elevated the immunoreactive LAMP-2A signal (Fig. 4A& B; $F = 3.562$, $p < 0.05$ NC vs. 10 μM KYP-2047, 1-way ANOVA with Tukey's multiple comparison test).

We observed a significant decrease in the Hsc70 levels in the KYP-2047 treatment group compared to the vehicle group which indicated that KYP-2047 induced CMA pathway activation in neuronal cells. Interestingly, Hsc70 levels were significantly elevated in PREP-KO cells compared to WT neurons (Fig. 4C & D, Hsc70: $F = 294.7$, $p < 0.0001$; $p < 0.0001$, NC vs. 1 μM KYP-2047; $p < 0.0001$, NC vs. 10 μM KYP-2047; $p < 0.0001$, NC vs. PREP KO; 1-way ANOVA with Tukey's multiple comparison test). Correspondingly, we also found that the expression of GAPDH was associated with CMA activation in KYP-2047 treatment groups as the GAPDH levels tended to decrease but the levels were significantly elevated in PREP-KO cells (Fig. 4C & E, GAPDH: $F = 44.86$, $p < 0.0001$; no significance compared to 1 μM KYP-2047 or 10 μM KYP-2047; $p < 0.0001$, NC vs. PREP KO; 1-way ANOVA with Tukey's multiple comparison test).

Moreover, we studied the MA markers to investigate whether the PREP inhibition activated also the MA pathway in neuronal cells. The LC3BII/I ratio was significantly increased after KYP-2047 treatment with both concentrations, and the LC3BII/I ratio was also slightly increased in PREP KO cells, confirming that PREP inhibition induces MA also in primary neuronal cells (Fig. 4C & F, LC3BII/I: $F = 8.301$, $p = 0.0029$; $p < 0.001$, NC vs. 1 μM KYP-2047, $p < 0.05$, NC vs. 10 μM KYP-2047, no significance compared to PREP KO; 1-way ANOVA with Tukey's multiple comparison test).

3.4. PREP serves as a regulator between MA and CMA pathways

Increasing evidence suggests that CMA and MA directly interact with each other during the clearance process of protein accumulations to maintain cell viability [26,27]. Based on the results above, we showed that the PREP inhibition by KYP-2047 activated both CMA and MA, as KYP-2047 increased the LC3BII/I ratio in primary cortical neurons, and after LAMP-2A silencing in HEK-293 cells KYP-2047 increased the LC3BII/I ratio, pointing to an increase in autophagic flux and MA activation. It has been shown that the blockage of MA can lead to CMA up-regulation and subsequent decrease in the fraction of cellular proteins degraded through CMA, which means that CMA can compensate MA and vice versa [28]. To explore whether PREP inhibition affects the interaction between CMA and MA, we used bafilomycin A1 that blocks the fusion of autophagosomes to lysosomes to inhibit MA. When PREP inhibitor was used in the presence of bafilomycin A1, it led to significantly elevated LAMP-2A levels (Fig. 5A & B, LAMP-2A: $F = 314.3$, $p < 0.0001$; $p < 0.0001$, NC vs. bafilomycin A1 + 1 μM KYP-2047; $p < 0.05$, NC vs. bafilomycin A1 + 10 μM KYP-2047; 1-way ANOVA with Tukey's multiple comparison test) and decreased levels of Hsc70 and GAPDH, pointing that the CMA pathway has been activated (Fig. 5A & C, Hsc70: $F = 71.23$, $p < 0.0001$; $p < 0.0001$, NC vs. bafilomycin A1 + 1 μM KYP-2047; $p < 0.0001$, NC vs. bafilomycin A1 + 10 μM KYP-2047; Fig. 5A & D, GAPDH: $F = 76.79$, $p < 0.0001$; $p < 0.0001$, NC vs. bafilomycin A1 + 1 μM KYP-2047; $p < 0.0001$, NC vs. bafilomycin A1 + 10 μM KYP-2047; 1-way ANOVA with Tukey's multiple comparison test).

Furthermore, we also tested the impact of transient α-syn overexpression with KYP-2047 and bafilomycin A1 on CMA markers. The LAMP-2A levels were significantly increased when KYP-2047 was combined with bafilomycin A1 treatment in α-syn transfected HEK-293 cells, whereas there were no significant effects on LAMP-2A by α-syn transfection or bafilomycin A1 alone (Fig. 6A & B, LAMP-2A: $F = 27.95$, $p < 0.0001$; $p < 0.001$, NC vs. α-syn + bafilomycin A1 + 1 μM KYP-2047, $p < 0.0001$, NC vs. α-syn + bafilomycin A1 + 10 μM KYP-2047; 1-way ANOVA with Tukey's multiple comparison test). Interestingly, compared with vehicle group, the levels of Hsc70 and GAPDH were reduced significantly in all groups (Fig. 6A & C, Hsc70: $F = 18.89$, $p < 0.0001$; $p < 0.0001$, NC vs. α-syn; $p < 0.0001$, NC vs. bafilomycin A1; $p < 0.01$, NC vs. α-syn + bafilomycin A1 + 1 μM KYP-2047; $p < 0.0001$, NC vs. α-syn + bafilomycin A1 + 10 μM KYP-2047; GAPDH: $F = 75.5$, $p < 0.0001$; $p < 0.0001$, NC vs. α-syn; $p < 0.0001$, NC vs. bafilomycin A1; $p < 0.01$, NC vs. α-syn + bafilomycin A1 + 1 μM KYP-2047; $p < 0.0001$, NC vs. α-syn + bafilomycin A1 + 10 μM KYP-2047; 1-way ANOVA with

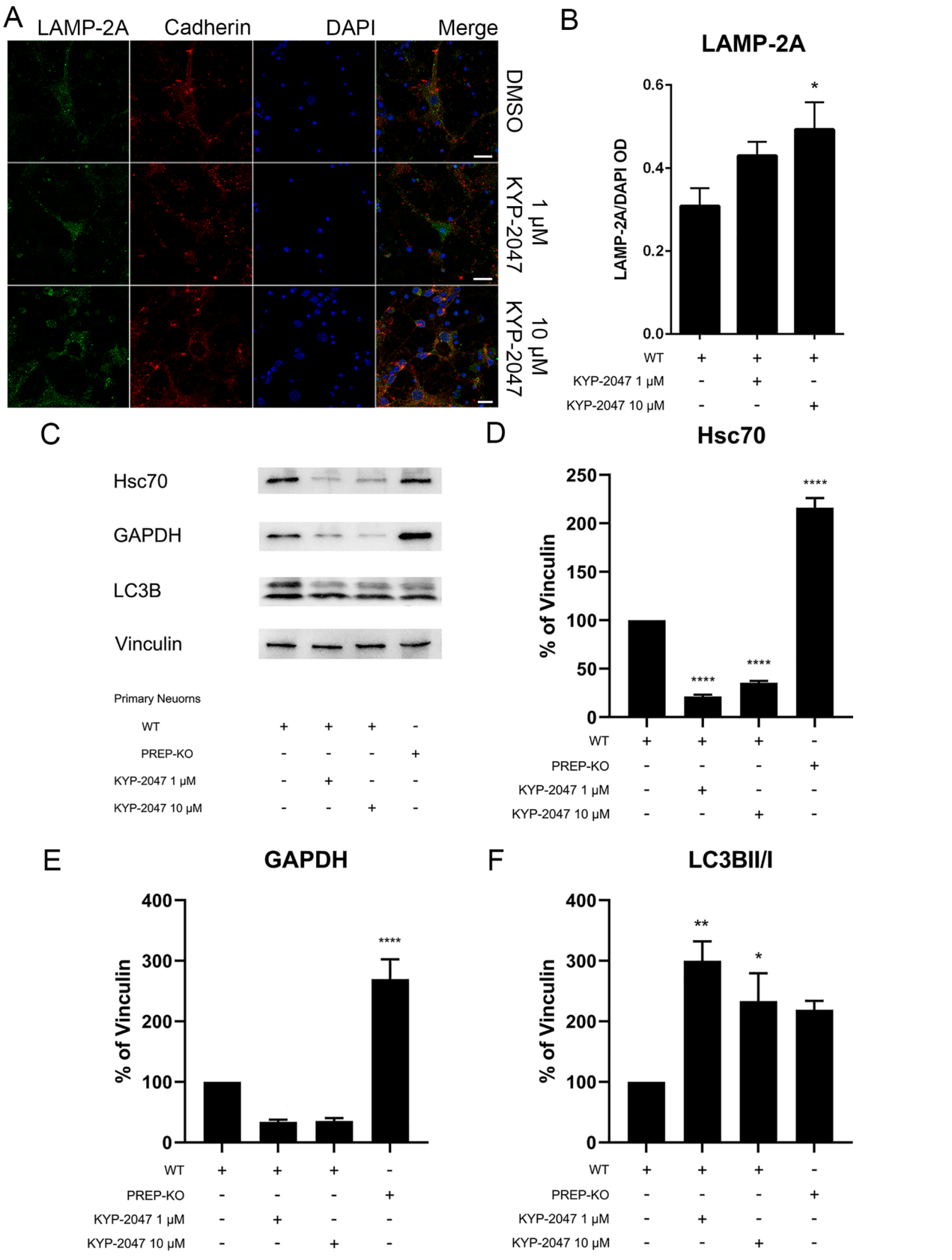

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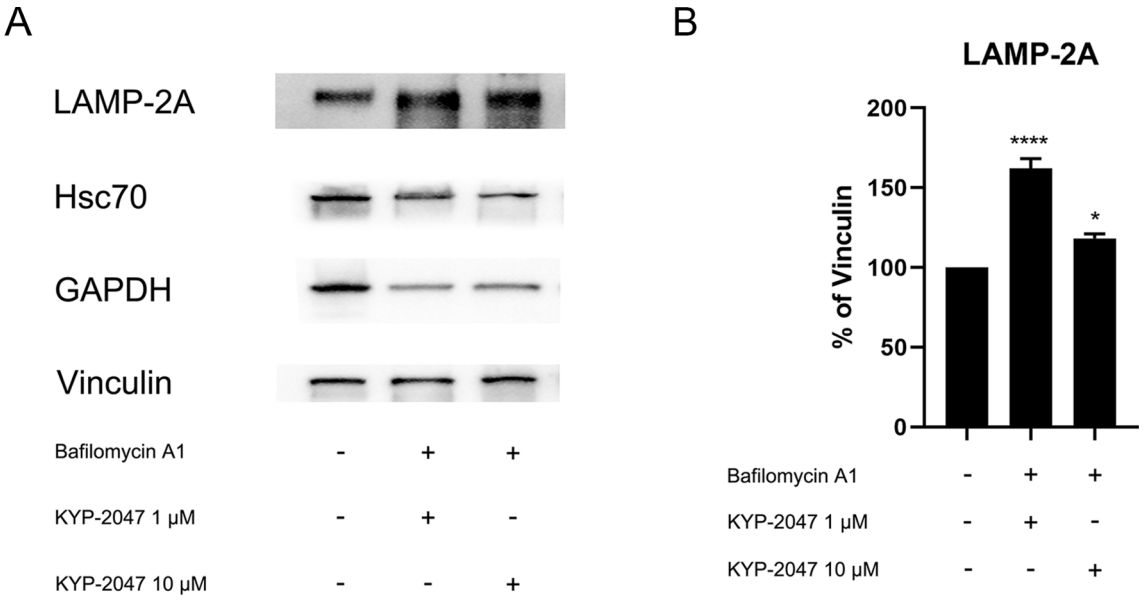
Fig. 4. PREP inhibition induces CMA pathway activation in WT mice primary cortical neurons. The levels of LAMP-2A in WT/PREP KO mice primary cortical neurons were studied by using immunofluorescence staining (A). 10 μM KYP-2047 significantly elevated the immunoreactive LAMP-2A signal, and cadherin served as a marker for cell membranes and DAPI as a marker for cell nuclei (B; DMSO, 0.31; 1 μM KYP-2047, 0.43; 10 μM KYP-2047, 0.49). The levels of the CMA markers Hsc70 and GAPDH were detected by WB after treatment with KYP-2047 for 24 h in both WT and PREP KO neurons (C). The Hsc70 levels were decreased after KYP-2047 incubations and inreased in PREP KO primary neurons (D; av: NC, 1.01; 1 μM KYP-2047, 0.21; 10 μM KYP-2047, 0.35; PREP-KO, 2.16), which indicated KYP-2047 induced CMA pathway activation in the neuronal cells. The levels of GAPDH was associated with CMA activation in the KYP-2047 treatment group compared with the PREP KO group (E; av: NC, 0.34; 1 μM KYP-2047, 0.08; 10 μM KYP-2047, 0.12; PREP-KO, 0.91). The LC3BII/I ratio was also increased in both KYP-2047 treatment groups and PREP KO groups (F; av: NC, 4.40: 1 μM KYP-2047, 13.21; 10 μM KYP-2047, 10.26; PREP-KO, 9.64). Vinculin served as a loading control. Data is expressed as means (±SEM) from three independent experiments. **** $p < 0.0001$, ** $p < 0.01$ and * $p < 0.05$ vs negative control (NC), 1-way ANOVA with Tukey's multiple comparison test (B-D).



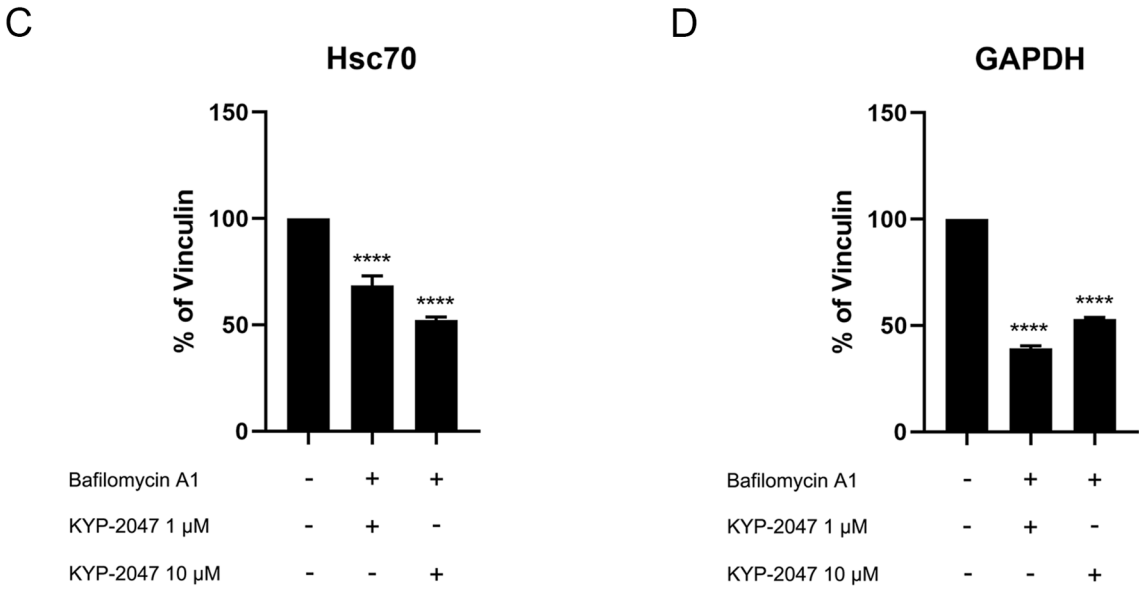


Fig. 5. PREP serves as a regulatory link between MA and CMA. The MA blockage by bafilomycin A1 can lead to CMA activation when combined with KYP-2047 treatment for 24 h (A). 1 & 10 μM KYP-2047 significantly elevated LAMP-2A levels in the presence of bafilomycin A1 (B; absolute values (av): NC, 1.58; Bafilomycin A1 + 1 μM KYP-2047, 2.57; Bafilomycin A1 + 10 μM KYP-2047, 1.87), and KYP-2047 reduced the levels of Hsc70 and GAPDH (C; av: Hsc70: 1.70; Bafilomycin A1 + 1 μM KYP-2047, 1.17; Bafilomycin A1 + 10 μM KYP-2047, 0.89; D; GADPH: NC, 2.00; Bafilomycin A1 + 1 μM KYP-2047, 0.78; Bafilomycin A1 + 10 μM KYP-2047, 1.06). Vinculin served as a loading control. Data is expressed as means (±SEM) from three independent experiments. **** $p < 0.0001$ and * $p < 0.05$ vs negative control (NC), 1-way ANOVA with Tukey's multiple comparison test (B-D).

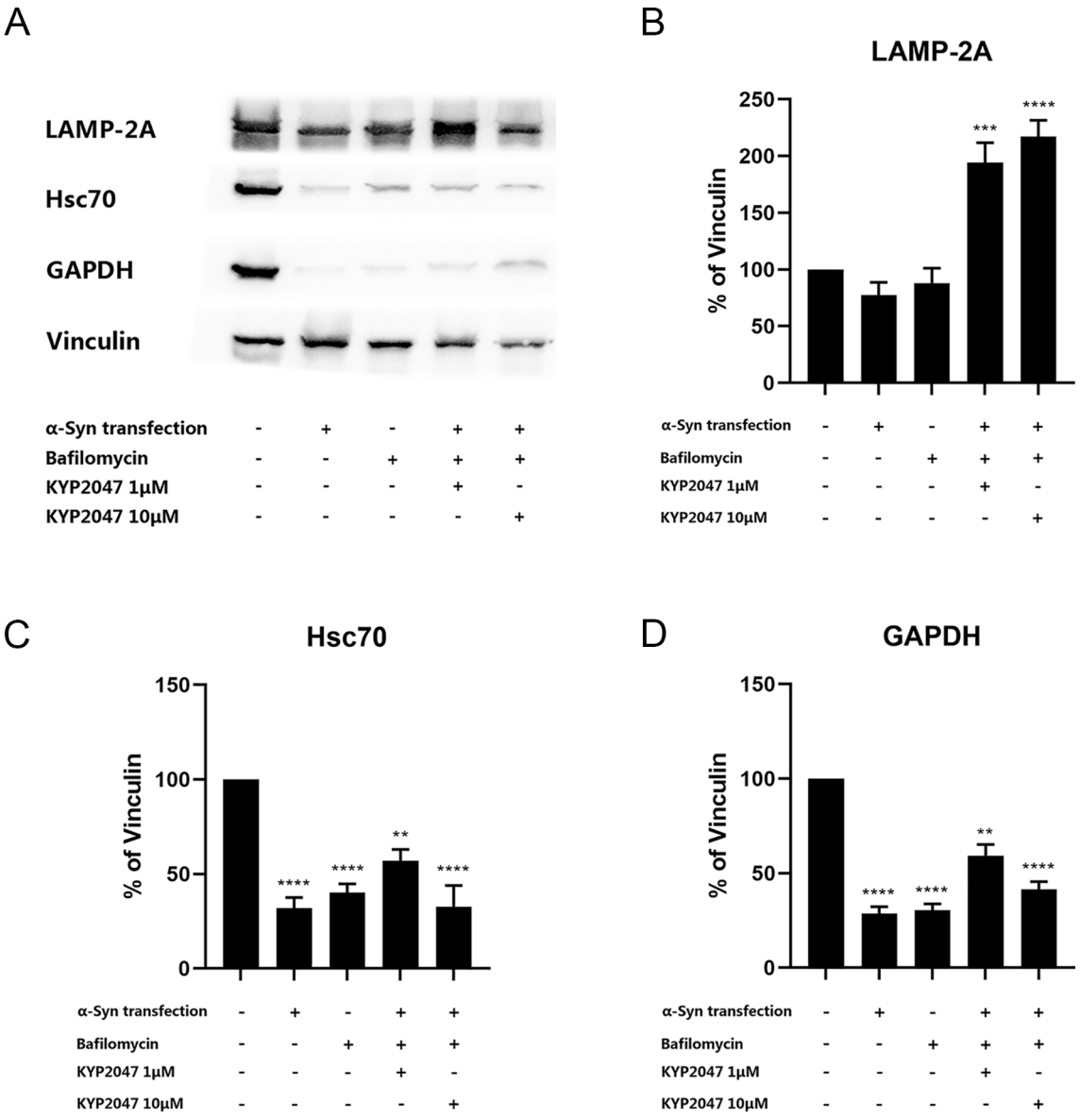


Fig. 6. PREP inhibition links MA and CMA pathways during α-syn overexpression. The LAMP-2A levels were elevated when KYP-2047 was combined with bafilomycin A1 in α-syn transfection cells, whereas there were no significant effects by α-syn transfection and Bafilomycin A1 alone (A, B; av: NC, 1.16; α-syn, 0.89; bafilomycin A1, 1.02; α-syn + bafilomycin A1 + 1 μM KYP-2047, 2.25; α-syn + bafilomycin A1 + 10 μM KYP-2047, 2.52%). Correspondingly, compared to vehicle group, the levels of Hsc70 and GAPDH were significantly reduced (C; av Hsc70: NC, 1.44; α-syn, 0.46; bafilomycin A1, 0.58; α-syn + bafilomycin A1 + 1 μM KYP-2047, 0.82; α-syn + bafilomycin A1 + 10 μM KYP-2047, 0.48; D; GADPH: NC, 1.38; α-syn, 0.40; bafilomycin A1, 0.41; α-syn + bafilomycin A1 + 1 μM KYP-2047, 0.81; α-syn + bafilomycin A1 + 10 μM KYP-2047, 0.56). Vinculin served as a loading control. Data is expressed as means (±SEM) from three independent experiments. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ vs negative control (NC), 1-way ANOVA with Tukey's multiple comparison test (B-D).

Tukey's multiple comparison test). Taken together, our data suggests that PREP/PREP inhibitor could serve as a regulator linking MA to CMA.

3.5. PREP inhibition has effects on two main core network nodes in CMA-MA crosstalk, Nrf2 and p53

Nuclear factor erythroid 2-related factor (Nrf2) regulates the expression of antioxidant proteins that protect against oxidative damage, and it has been reported to play a role against the α-syn aggregation toxicity and it serves as an upstream signal to regulate the activities of these two autophagic pathways [29]. Under stress conditions, Nrf2 disassociates from its inhibitor protein Kelch-like ECH-associated protein 1 (Keap1) and induces CMA pathway activation in the cytoplasm. Then again, after translocating into the nucleus, Nrf2 binds to the antioxidant response element (ARE) and activates the MA pathway via the ULK gene [29]. Therefore, we wanted to test if PREP inhibition together with α-syn overexpression and MA inhibition with bafilomycin A1 has an impact on the Nrf2 translocation into nucleus. Our results show that the Nrf2 transcription activity in the nucleus was markedly increased in α-syn overexpressing cells with KYP-2047 treatment when compared to negative control or α-syn overexpression alone (Fig. 7A, Nrf2 transcription assay: $F = 45.25$, $p < 0.0001$; $p < 0.0001$, NC vs. α-syn + 1 μM KYP-2047, $p < 0.001$; NC vs. α-syn + 10 μM KYP-2047; 1-way ANOVA with Tukey's multiple comparison test). However, a similar effect was not seen when MA was blocked by bafilomycin A1, and interestingly, the Nrf2 transcription activity in the nucleus was reduced in the PREP KO HEK-293 cells (Fig. 7A, Nrf2 transcription assay: $F = 45.25$, $p < 0.0001$; $p < 0.05$, NC vs. PREP KO; 1-way ANOVA with Tukey's multiple comparison test).

In addition to Nrf2 signaling, it has been suggested that p53, the

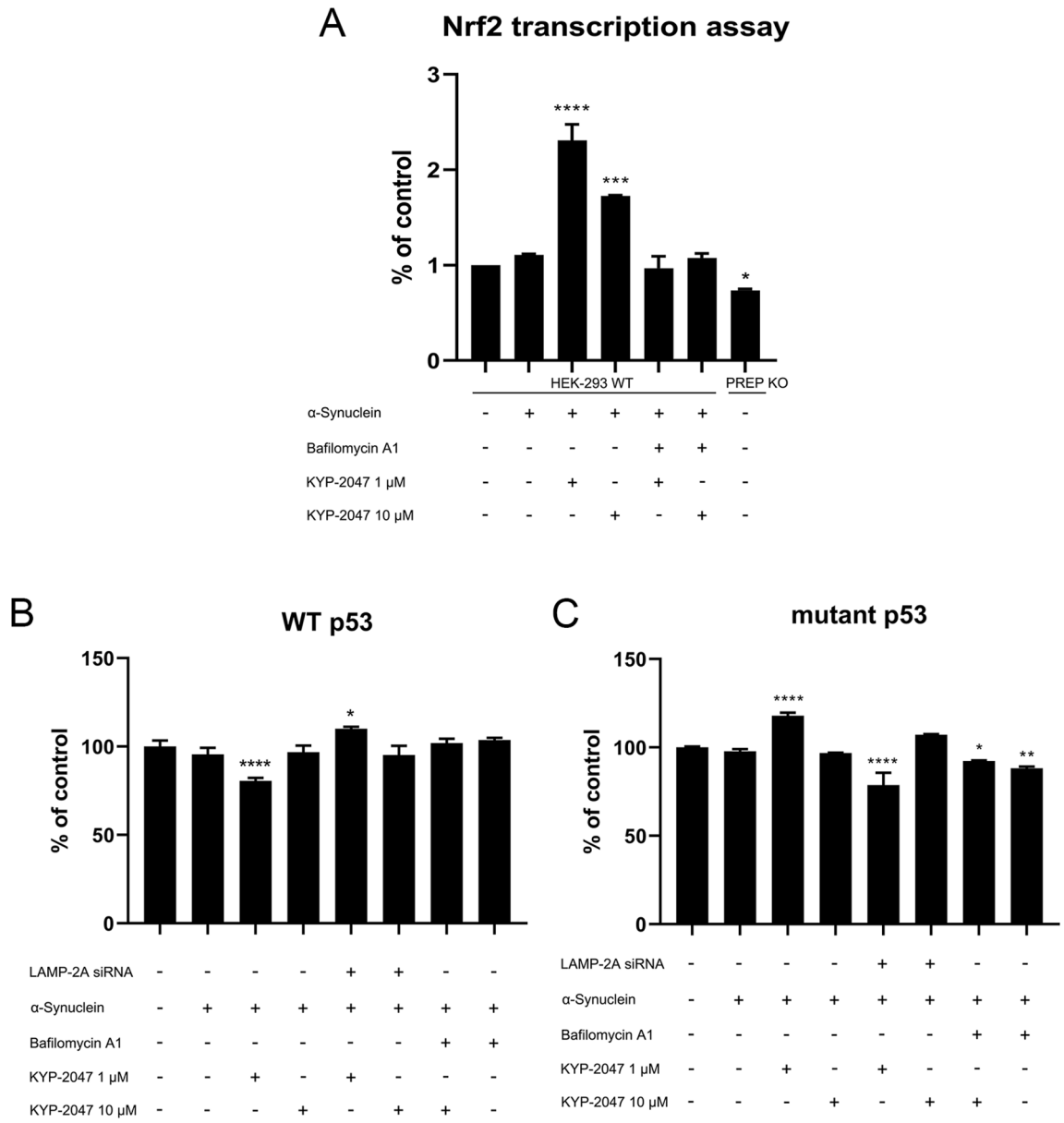


Fig. 7. PREP inhibition has effects on two main core network nodes in CMA-MA crosstalk. The Nrf2 transcription activity in the nucleus were markedly increased in α-syn transfection HEK-293 cells with KYP-2047 treatment, whereas, the transcription activity was reduced in the PREP KO HEK-293 group (A; α-syn, 1.11; α-syn + 1 μM KYP-2047, 2.31; α-syn + 10 μM KYP-2047, 1.72; α-syn + bafilomycin A1 + 1 μM KYP-2047, 0.97; α-syn + bafilomycin A1 + 10 μM KYP-2047, 1.07; PREP-KO, 0.61). Compared to the vehicle and α-syn transfection groups, the mRNA level of WT p53 was decreased with α-syn transfected HEK-293 cells with 1 μM KYP-2047 (B). When LAMP-2A was silenced, 1 μM KYP-2047 with α-syn transfection elevated p53 levels (B; av: NC, 0.45; α-syn, 0.43; α-syn + 1 μM KYP-2047, 0.36; α-syn + 10 μM KYP-2047, 0.43; α-syn + SiRNA + 1 μM KYP-2047, 0.49; α-syn + SiRNA + 10 μM KYP-2047, 0.42; α-syn + bafilomycin A1 + 1 μM KYP-2047, 0.46 α-syn + bafilomycin A1 + 10 μM KYP-2047, 0.46). Mutant p53 was then again increased in α-syn transfected HEK-293 cells after 1 μM KYP-2047 treatment, and decreased in α-syn transfection in LAMP-2A silenced HEK-293 cells with 10 μM KYP-2047 group (C; av: NC, 0.55; α-syn, 0.53; α-syn + 1 μM KYP-2047, 0.65; α-syn + 10 μM KYP-2047, 0.53; α-syn + SiRNA + 1 μM KYP-2047, 0.43; α-syn + SiRNA + 10 μM KYP-2047, 0.59; α-syn + bafilomycin A1 + 1 μM KYP-2047, 0.51; α-syn + bafilomycin A1 + 10 μM KYP-2047, 0.48). Data is expressed as means (±SEM) from three independent experiments. For qPCR assay, data were expressed as means (±SD) from three independent experiments.**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ vs negative control (NC), 1-way ANOVA with Tukey's multiple comparison test (A-C).

sequence-specific DNA-binding transcription factor, might play a critical role in mediating CMA-MA crosstalk in CNS diseases. A study by Tasdemir et al. described that p53 directly regulates autophagy genes which activate the MA pathway [30]. Then again, MA inhibition increases the cytosolic levels of mutant p53, leading to enhanced mutant p53 degradation by a CMA-dependent manner [28]. Therefore, we tested the mRNA levels of p53 and mutant p53 in HEK-293 cells by using the qPCR assay. Compared to the vehicle and α-syn overexpression groups, the mRNA level of WT p53 was decreased and mutant p53 was increased when α-syn overexpression was combined with 1 μM KYP-2047 treatment. Interestingly, a similar effect was not seen with 10 μM KYP-2047 (Fig. 7B, WT p53: $F = 22.2$, $p < 0.0001$; $p < 0.0001$, NC vs. α-syn + 1 μM KYP-2047; $p = 0.9135$, NC vs. α-syn + 10 μM KYP-2047; 1-way ANOVA with Tukey's multiple comparison test; Fig. 7C, mutant p53: $F = 60.99$, $p < 0.0001$; $p < 0.0001$, NC vs. α-syn + 1 μM KYP-2047; 1-way ANOVA with Tukey's multiple comparison test).

When α-syn overexpression was combined with LAMP-2A silencing, the impact of PREP inhibition turned over. 1 μM KYP-2047 increased WT

p53 levels and reduced the mutant p53 mRNA (Fig. 7B, WT p53: $p < 0.05$, NC vs. α-syn + LAMP-2A siRNA + 1 μM KYP-2047; Fig. 7C, mutant p53: $p < 0.0001$, NC vs. α-syn + LAMP-2A siRNA + 1 μM KYP-2047; 1-way ANOVA with Tukey's multiple comparison test). There were no significant changes in WT p53 mRNA levels when MA was blocked and combined with α-syn overexpression and additionally with KYP-2047 treatments, but mutant p53 was decreased by bafilomycin A1 (Fig. 7B, p53; Fig. 7C, mutant p53: $p = 0.0416$, NC vs. α-syn + bafilomycin A1 + 10 μM KYP-2047; $p = 0.001$, NC vs. α-syn + bafilomycin A1; 1-way ANOVA with Tukey's multiple comparison test).

4. Discussion

In recent years, numerous studies have suggested that autophagy is an important mechanism in the pathophysiology of neurodegenerative diseases, and it has been considered as the major potential target to improve the treatment of such diseases, including synucleinopathies [31]. Therefore, cellular clearance pathways could be the key targets to control the α-syn aggregation, thus affecting the disease progression. Our previous studies have indicated that KYP-2047, a small-molecular PREP inhibitor, decreases α-syn aggregation and activates MA *in vitro* and *in vivo* via activation of PP2A. There is a growing body of evidence that in addition to MA, CMA has an important role in α-syn degradation and could be a possibility for PD treatment [32]. However, the interaction between PREP and PREP inhibition and CMA has not been studied earlier. In this study, we used two different cellular models, HEK-293 cells with transient α-syn overexpression and mouse primary cortical neurons, and showed that PREP inhibition can also activate CMA and induce α-syn degradation, and importantly, we presented here that PREP can act as a switch between MA and CMA.

Unlike MA, CMA was the first studied autophagy process that indicated that the intracellular components degraded by the lysosomes can be selected [23]. Substrate proteins for CMA are not sequestered in autophagosome-lysosome lumen but translocate into the lysosomal lumen after directly crossing the lysosomal membrane. CMA activity is difficult to measure and quantify as it is a highly dynamic process. Only LAMP-2A acts as a receptor and the rate-limiting step for CMA, and therefore, the level of LAMP-2A in the lysosomal membrane can be directly correlated with CMA activity [33]. As another essential factor for CMA, Hsc70 remains the only chaperone proved to directly bind with the KFERQ-like motif which is capable of activating CMA [34]. In our study, we found that both when HEK-293 and primary cortical neurons were incubated with KYP-2047, LAMP-2A levels were increased and the Hsc70 expression decreased. Moreover, we found the level of endogenous CMA substrate, GAPDH, was also reduced by KYP-2047, suggesting that the PREP inhibition enhanced the CMA pathway activation. However in contrast, the LAMP-2A level was significantly decreased in PREP KO HEK-293 cells. Our former research has revealed that the PREP inhibition and deletion up-regulated MA [22,35] and in this study we observed that the CMA pathway is a counterbalance for MA, indicating that the PREP deletion downregulated the levels of LAMP-2A by inducing MA in HEK-293 cells.

α-syn plays a major role in PD pathogenesis. It has been shown that α-syn can be degraded by the ubiquitin–proteasome system (UPS) and the autophagy-lysosomal pathway (ALP), including CMA and MA pathways [36]. Evidence from post-mortem patient brain tissue suggests that ALP plays a critical role in synucleinopathies, since PD post-mortem samples have shown that the levels of LAMP-2A significantly reduced in the *substantia nigra pars compacta* and amygdala compared with control brains, pointing to impaired CMA [12,31]. These findings highlight the role of CMA in the pathological process of PD. α-syn, which contains a KFERQ motif in its sequences, can be delivered to lysosomes for its degradation via the CMA pathway [14]. Most of the previous studies using different cellular models of synucleinopathies have reported that CMA pathway inhibition increased α-syn accumulation, and that CMA activation reduces the levels of α-syn [37,38]. We observed a reduction in the α-syn levels in KYP-2047 treated α-syn overexpressing cells combined with a significant increase in LAMP-2A levels. This indicated that PREP inhibition induced α-syn degradation via activated CMA pathway.

There are several compounds described in the literature as regulators for the MA pathways [39]. However, the inhibitors of MA such as PI3K pathway blockers (3-methyladnine (3-MA), wortmannin, and LY294002) have no effect on CMA pathway activation, and the classical activator of MA, rapamycin, has also no effect on CMA [40]. The lack of small molecular inhibitors or activators for the CMA pathway has been the major limitation for specific drug development targeting CMA. Currently, the most efficient and selective way to block the CMA pathway is to silence LAMP-2A by using siRNA which can prevent substrate binding/translocation to the lysosomal surface [40]. In order to eliminate the possibility of off-target actions, we silenced LAMP-2A by using RNA interference, and as expected, the PREP inhibition had no effect on LAMP-2A levels after silencing. When the CMA pathway was blocked by silencing LAMP-2A, the CMA substrates can be degraded by other autophagic pathways, especially by MA. LC3BII, converted from LC3BI in the genesis of autophagosomes can be used as a marker to quantify autophagosome and MA activation particularly with proper controls [41]. Increased LC3BII levels can result from increased autophagic flux or impaired degradation of autophagosomes after fusion with the lysosome [42]. Interestingly, in the LAMP-2A-deficient α-syn overexpressing cells, the α-syn levels were decreased along with the LC3BII/I ratio, and KYP-2047 further increased this. This indicates that when CMA was blocked, α-syn degradation was induced by activating the MA pathway and up-regulating the LC3BII/I ratio, and KYP-2047 enhanced this effect. Although we have shown earlier that PREP inhibition induces autophagy, it was not studied in the presence of CMA inhibition, and our results highlight the impact of PREP inhibition as a switch between CMA and MA.

Recent studies have shown that the down-regulation of LAMP-2A in the primary rat cortical neurons increases endogenous α-syn levels, followed by a MA activation and increased α-syn degradation, which suggests the presence of a compensatory mechanism between these two autophagic mechanisms [38]. To elucidate further the contribution of PREP and PREP inhibition in the CMA pathway, we used cultured primary mouse cortical neurons. We found that the immunoreactive LAMP-2A levels were increased after KYP-2047 incubation in cortical neurons similar to HEK-293 cells. The results of the WB analyses showed that the levels of Hsc70 and GAPDH reduced in KYP-2047 incubated cells correlating with the increased LAMP-2A levels and with the results in HEK-293 cells. In addition, the assessment of MA markers by measuring the LC3II/I ratio revealed an increase by PREP inhibition and PREP deletion, indicating that MA pathway has also been activated as we have shown earlier in cell cultures and *in vivo*.

Our data showed that PREP links to CMA-MA pathways crosstalk not only in non-neuronal cells but also in neuronal cells. To study this interplay further, we used MA inhibitors. In the current study, bafilomycin A1, which inhibits vesicular H^+-ATPase/endoplasmic reticulum Ca^{2+}-ATPase and prevents the fusion between the autophagosome and lysosome, was used to block the late-stage of MA pathway [43]. When the effect of KYP-2047 on CMA markers was evaluated in the presence of bafilomycin A1 in HEK-293 cells, the results showed that when the MA pathway was blocked, the CMA pathway was activated by PREP inhibition. To assess this further, we studied the effects of MA inhibition and PREP inhibition on α-syn degradation. Our results revealed that KYP-2047 up-regulated the LAMP-2A levels and activated the CMA pathway after co-treatment with bafilomycin A1 and α-syn transfection in HEK-293 cells. This is in line with the previous reports showing that the CMA pathway is a counterbalance for the MA pathway [29], but our current report, however, suggests that PREP can be in the crossroads of CMA and MA pathways.

Dysfunction in the CMA and MA pathways are implicated in numerous pathophysiological processes such as neurodegenerative

diseases, cancer and infectious or metabolic diseases [35,44,45]. The cellular and molecular mechanisms regulating the CMA and MA pathways are highly complex and involve different active kinases and several other proteins and signaling cascades, and there is an interplay between MA and CMA [46]. Some molecular mechanisms underlying the crosstalk between the CMA pathway and the MA pathway have been found, but how they communicate with each other and link together is still unknown. As stated above, we suggest that PREP is a novel regulator between the CMA and MA pathways. The signaling mechanisms that control CMA are not fully understood yet but might involve responses to cellular stressors, such as starvation, DNA damage, lipotoxicity hypoxia, and oxidative stress [47]. The deficiency and overexpression of Nrf2 has been linked to reduced and increased LAMP-2A levels, respectively, providing a new strategy to activate CMA through Nrf2 inducers to facilitate elimination of damage and toxic proteins [48]. In addition, a study by Eteläinen et al. (2021) showed that PREP inhibition might induce Nrf2 activation during oxidative stress [49]. We present in the current study that KYP-2047 increased Nrf2 transcription activity in α-syn transfected HEK-293 cells. Furthermore, the MA inhibitor bafilomycin A1 has been reported to cause a significant decrease in the production and activation of Nrf2 [50]. This is in line with our results of the Nrf2 transcription assay when MA was blocked by bafilomycin A1, which indicated that the inhibiting effect of PREP on MA might be induced by Nrf2 transcription activity. In addition to Nrf2, another critical component of stress signaling and adaptation is the tumor suppressor protein p53. A component of p53-mediated response to these stressors is activation of autophagy, and in turn, autophagy represses p53 levels and function [51]. Mutant p53 is the substrate of CMA, and since MA suppresses p53 activation, this indicates that p53 might serve as a regulator between CMA-MA crosstalk. Interestingly, KYP-2047 regulated the mRNA levels of WT/mutant p53 in a concentration-dependent manner in α-syn overexpressing HEK-293 cells, which is corresponding to the WB analysis results of LAMP-2A levels with KYP-2047 treatment in the α-syn overexpressing HEK-293 cells. KYP-2047 also increased and decreased the mRNA levels of WT p53 and mutant p53 in LAMP-2A silenced α-syn transfected HEK-293 cells, respectively. However, further studies will be needed to clarify the mechanism how PREP/PREP inhibition regulates CMA-MA crosstalk. Our findings demonstrated the mutual regulation of the CMA pathway and the MA pathway integrated by PREP inhibition. In summary, our data proved that PREP is a novel regulator linking these pathways and that PREP inhibition has a significant role in maintaining the balance of different types of autophagy. In the future, the function of PREP/PREP inhibitor in different stages and types of autophagy should be analyzed and elucidated.

To conclude, we found that PREP inhibition by KYP-2047 activates CMA activation in the neuronal and non-neuronal cells, and induces α-syn degradation. After blocking CMA by RNA interference of LAMP-2A, KYP-2047 could still decrease the α-syn levels by activating the MA pathway. Furthermore, when MA was blocked, KYP-2047 induced CMA activation to maintain autophagic processes. Our results suggest that PREP is a molecule which serves as a novel regulator in the crosstalk between CMA and MA pathways. Moreover, PREP inhibition can activate both major ALP pathways to degrade excess α-syn, promoting its possibilities in drug development for PD.

CRediT authorship contribution statement

H. Cui: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **S. Norrbacka:** Conceptualization, Investigation, Methodology, Validation. **T.T. Myöhänen:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Visualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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