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Electronic Supplementary Information

CHIP-Mediated Hyperubiquitylation of Tau Promotes Its Self-Assembly into the Insoluble Tau Filaments

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Experimental Procedures

Antibodies and reagents

Antibodies and dilution factors used in this study included the following: anti-Ac-Lys (MA1-2021, Invitrogen, USA, 1/5,000), anti-β-actin (A1978, Sigma, 1/10,000), anti-CHIP (A301-572A, Bethyl, 1/5,000), anti-GST (MA4-004, Invitrogen, 1/1,000), anti-His (A03001, IgTherapy, Korea,

1/2,000), anti-PSMD2 (PA527663, Pierce, 1/5,000), anti-PSMA4 (PW8115, Enzo Life Science, USA, 1/5,000), anti-tau (clone Tau-5; Invitrogen, 1/10,000), anti-tau^{ser396} (ab109390, Abcam, 1/5,000), anti-tau^{ser199} (ab81268, Abcam, 1/5,000), anti-tau$^{Ser202/Thr205}$ (AT8 clone; MN1020, Invitrogen. 1/3,000, 1/100 for immunostaining), anti-NeuN (Abcam; ab177487, 1/100 for immunostaining), and anti-ubiquitin (clone P4D1, Santa Cruz Biotechnology, USA, 1/5,000). Secondary antibodies (horseradish peroxidase–conjugated anti–mouse IgG and anti–rabbit IgG antibodies) were acquired from Millipore. Major biochemical reagents employed in this study were MG132 (Bachem) and ATP (Calbiochem). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and phosphate-buffered saline (PBS; pH 7.4) were purchased from WelGENE. Okadaic acid and Coomassie Brilliant Blue R250 were purchased from Sigma.

Purification of recombinant tau and post-translational modification (PTM) enzymes

The longest isoform of human tau-441, which has a code-optimized recombinant sequence, was expressed in *Escherichia coli* BL21 (DE3) cells. The cells were incubated at 37 °C; isopropyl-β-D-thiogalactopyranoside (IPTG; 0.5 mM) was added to each culture until the optical density at 600 nm reached 0.5, after which each culture was incubated for additional 3 h at 37 °C. After that, the cells were harvested and sonicated in lysis buffer (50 mM NaH_2PO_4 [pH 7.4] and 300 mM NaCl) containing a protease inhibitor cocktail. Next, the lysates were heated at 85 °C for 15 min, cleared by centrifugation, and passed through a 0.22-μm polypropylene filter. His-tagged human tau was then purified on ÄKTA pure (GE Healthcare) using a HiTrap TALON crude column (5 mL, GE Healthcare). Proteins were eluted with 150 mM imidazole (50 mM NaH_2PO_4 [pH 7.4], 300 mM NaCl, and 150 mM imidazole). The eluted tau protein was supplemented with dithiothreitol (DTT) to a final concentration of 0.1 mM for reducing disulfide bonds and was stored at −80 °C. The purified His-tau proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue R250 to determine the size and purity.

The purification of CHIP was performed mostly as previously described.[1] Briefly, pGEX4T-1-CHIP-transformed BL21 (DE3) cells were cultured at room temperature for 15 h, harvested in lysis buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, and 10 % of glycerol), and lysed via sonication. After the lysates were centrifuged and filtered, the supernatants were incubated with Glutathione Separose Resin (GE Healthcare) at 4 °C for 12 h. After a wash with

washing buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 10 % of glycerol, and 0.2 % of Triton X-100), GST-CHIP proteins were eluted with 10 mM L-glutathione (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, and 10 % of glycerol).

Mouse GSK3β gene was synthesized into pFastBac^{TM}HT A vector (GenScript) and expressed using the Bac-to-Bac® Baculovirus system (Invitrogen). The resulting construct was expressed in *Spodoptera frugiperda* (Sf9) insect cells at 27 °C for 3 days and cells were harvested by centrifugation and stored at 80 ℃ until use. The cells were thawed in a buffer (20 mM HEPES [pH 7.5], 500 mM NaCl, 20 mM imidazole, 3 mM β-mercaptoethanol and 10 % glycerol) and disrupted by homogenization. The cell lysate was applied to Ni-NTA affinity chromatography and His6-tagged protein was pooled with a buffer (20 mM HEPES [pH 7.5], 500 mM NaCl, 300 mM imidazole, 3 mM β-mercaptoethanol and 10 % glycerol). Then, mouse GSK3β was purified by gel filtration chromatography using a HiPrep^{TM} 26/10 Desalting column (Cytiva) with a buffer (20 mM Tris-HCl [pH 7.5], 500 mM NaCl). Fractions containing recombinant mouse GSK3β were collected and concentrated using a centrifugal concentrator (Millipore). The catalytic core of the Usp2 (Usp2-cc) was purified as described previously.[2] Recombinant UBA1, UbcH5b, and p300/EP300 (catalytic domain) were purchased from UBPBio (B1101), Boston Biochem (E2-622), and Enzo Life Sciences (BML-SE451), respectively.

Purification of 20S and 26S human proteasomes

Human 20S and 26S proteasomes were affinity-purified from a stable HEK293 cell line expressing biotin-tagged human PSMB2 as previously described.[3] Briefly, the cells were cultured in 15 cm culture dishes, homogenized in lysis buffer (50 mM NaH_2PO_4 [pH 7.5], 100 mM NaCl, 10 % glycerol, 5 mM $MgCl_2$, 0.5 % NP-40) containing protease inhibitors, 5 mM ATP, and 1 mM DTT, by using Dounce tissue grinders (Wheaton).. Then the lysates were centrifuged at 10,000 × g and the cleared supernatants were incubated with BioMag Streptavidin resin (Qiagen) for 6 h at 4 °C. For 26S purification, the beads were incubated in TEV protease-containing elution buffer (50 mM Tris-HCl [pH 7.5], 1 mM $MgCl_2$, 10 % glycerol, and 1 mM ATP) for 1 h at 30 °C. For 20S, the resin was vigorously washed with 300 mM NaCl before elution. The TEV-eluted proteasomes were concentrated using Amicon ultracentrifugal filters

and stored at -80 °C in 10 % glycerol.

In vitro phosphorylation and acetylation assays

These assays were performed on the tau protein. For the phosphorylation, 270 nM tau and 625 nM GSK3β (amino acids 35-380) were incubated in phosphorylation buffer (50 mM Tris-HCl [pH 7.4], 10 mM $MgCl_2$, 5 mM DTT, and 1 mM ATP) at 30 °C for various periods. Acetylation reactions were conducted by means of 270 nM tau, 555 nM p300/EP300, and 125 nM acetyl-coenzyme A in acetylation buffer (10 mM HEPES-KOH [pH 7.4], 50 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 2.5 mM ethylene diamine tetraacetic acid, and 0.1 mM ethylene glycol tetraacetic acid) for 4 h at room temperature. Both assays were carried out in a total 40 μL reaction volume. The modifications were monitored by IB with cognate antibodies.

The tau ubiquitylation assay and ub-tau degradation *in vitro*

In vitro ubiquitin reconstitution was performed on the tau protein. In a test tube, the ubiquitylation reaction mixture consisted of 450 nmol tau, 70 nmol UBA1, 890 nmol UbcH5b, 3 μmol CHIP, and 1.2 nmol ubiquitin in ubiquitylation buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 1 mM ATP, and 0.2 mM DTT). Reactions were conducted at 37 °C for various periods. To examine the aggregation of ubiquitylated tau, the reaction was allowed to proceed for more than 4 d. To identify the degradation of modified- and unmodified-tau species, purified human proteasomes (5 nM) were incubated with phospho-ub-tau (100 nM) in proteasome assay buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 10 % of glycerol, 2 mM ATP, 10 mM $MgCl_2$, and 1 mM DTT). The degradation rate was monitored by IB with an anti-tau antibody.

In vitro aggregation and deubiquitylation of tau

Tau aggregation was monitored by means of ThT as previously described.[4] Briefly, 3.33 μM tau (intact, phospho-, ub-, or phospho-ub-tau) was incubated with 50 μM ThT (Enzo Life Sciences) in ThT assay buffer (50 mM glycine-NaOH [pH 8.5]). The oligomerization kinetics were determined by means of fluorescence (480 nm for excitation and 535 nm for emission) at various time points between hours 6 and 96 of ThT incubation (TECAN infinite m200 fluorometer, Männedorf, Switzerland). Tau aggregation was also monitored using a filter trap assay. To

examine the deubiquitylation of ub-tau, purified catalytic domains of USP2-cc (0.3 μM) were incubated with ubiquitin-modified tau (100 nM) in a buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 1 mM ATP, and 0.2 mM DTT). Deubiquitylation of tau was monitored by SDS-PAGE/IB with an anti–tau 5 antibody.

Size-exclusion chromatography and tau seeding assay

Hyper-ub-tau proteins were generated through *in vitro* phosphorylation (for 12 h) and ubiquitylation (for 48 h) reactions as described above. Total reaction mixtures were loaded onto a column and eluted with tau SEC buffer (20 mM Tris-HCl [pH 7.5], 10 mM NaCl, 5 mM $MgCl_2$, 5 mM ATP, and 1 mM DTT). SEC was carried out on a Superose 200 Increase 10/300 GL column by fast protein liquid chromatography (ÄKTA; GE Healthcare); 0.25 mL fractions were collected, and 10% glycerol was added to each fraction. Four fractionations of tau proteins were collected depending on their sizes (Group 1 with > 250 kDa; Group 2 with ~ 70 kDa-sizes), mixed with ~ 1 mg/mL tau monomers (mass ratio between tau seeds and monomer is ~ 1:50) for 24 h or 48 h, and analyzed with SDS-PAGE and subsequent negative-staining electron microscopic analysis. All experiments were performed at 37 ℃.

Filter Trap Assay

For the filter trap assay, protein samples in the PTM reaction buffer were mixed with 2 × boiling buffer (4% SDS, 40 mM EDTA, 200 mM β-mercaptoethanol) and boiled at 85 °C for 10 min. The samples were then passed through the cellulose acetate membrane (Advantec, 0.2 μm pore size),[5] which was pre-wet with 0.1% SDS for 5–10 min, using a 96-well dot-blot apparatus (SCL-D96, Cleaver Scientific) with vacuum aspiration. The membrane retaining SDS-insoluble proteins was washed twice using 200 μL of 0.1% SDS. Proteins trapped by the filter were analyzed by immunoblotting.

Transmission Electron Microscopy Imaging

Formvar-carbon copper grids (size 200 mesh) were prepared by glow discharge using the easiGlow system (Ted Pella) for 30 sec at 15 mA, and then tau protein samples (4 μL) were loaded to grid. After ~1 min, the sample solution was removed with filter paper, and 2% uranyl

acetate (EMS) was immediately added to grid. After additional ~1 min incubation, uranyl acetate staining solution was removed, and the grid was allowed to air dry. Electron micrographs were recorded at a magnification of ×9,600 – ×25,000 with Tecnai 20 transmission electron microscope (Thermo Fisher Scientific) which was operated at 120 kV and equipped with a US1000X-P camera 200 (Gatan).

Liquid chromatography coupled with tandem mass spectrometry (MS/MS)

Protein digestion was performed via the 2-step FASP procedure as described before,[6] with some modifications. In brief, samples were mixed (1:1, v/v) with SDT buffer (2 % of SDS, 10 mM TCEP, and 50 mM CAA in 0.1 M Tris [pH 8.0]) and loaded onto a 30K Amicon filter (Millipore). Buffer exchanges were performed with the UA solution (8 M urea in 0.1 M Tris [pH 8.5]) via centrifugation at 14,000 × *g* for 15 min. Following an exchange of the buffer with 40 mM ammonium bicarbonate, protein digestion was performed at 37 ℃ overnight using a trypsin/LysC mixture (Promega) at a 100:1 protein-to-protease ratio. The digestion-generated peptides were collected by centrifugation. After the filter units were washed with 40 mM ammonium bicarbonate, a second digestion was performed at 37 °C for 2 h with trypsin (enzyme-to-substrate ratio [w/w] of 1:1000). All the resulting peptides were acidified with 10 % trifluoroacetic acid and desalted in homemade C18-StageTips as described previously.[6] Desalted samples were completely dried in a vacuum dryer and stored at −80 °C.

Liquid chromatography coupled with MS/MS was performed on a hybrid quadrupole Orbitrap mass spectrometer, Q-exactive plus (Thermo Fisher Scientific, Waltham, MA), coupled to an Ultimate 3000 RSLC system (Dionex) via a nanoelectrospray source, as described elsewhere,[7] with some modifications. Namely, fractionated peptide samples (1 μg) were separated via the two-column setup with a trap column (300 μm internal diameter × 5 mm, C18 3 μm, 100 Å) and an analytical column (50 μm internal diameter × 50 cm, C18 1.9 μm, 100 Å). After the samples were loaded onto the nano-liquid chromatography system, a 120-min gradient from 8 % to 26 % solvent B (100% acetonitrile with 0.1 % of formic acid) was applied to all samples. Spray voltage was 2.0 kV in positive-ion mode, and the temperature of the heated capillary was set to 320 °C. Mass spectra were acquired in data-dependent mode by a top 15 method. The Orbitrap analyzer was set to scan precursor ions with a mass range of m/z 300–

1650 and a resolution of 70,000 at m/z 200. Higher-energy collisional dissociation scans were run at a resolution of 17,500 with a normalized collision energy of 27. The maximum ion injection time for the survey and MS/MS scans was 25 and 50 ms, respectively.

MS raw files were processed using the Maxquant software, version 1.6.1.0.[8] MS/MS spectra were subjected to searches against the sequence of the human tau protein with an N-terminal 6xHis-tag using the Andromeda search engine.[9] Primary searches were performed at 6 ppm precursor ion tolerance. MS/MS ion tolerance was set to 20 ppm. Cysteine carbamido-methylation was chosen as a fixed modification. N-terminal acetylation, oxidation on methionine, acetylation on lysine, lysine with a diGly remnant, and phosphorylation on serine, threonine, and tyrosine were selected as variable modifications. A false discovery rate of 1 % was applied at peptide, protein, and modification levels. The minimum score for the modified peptides was set to 40. To assign phosphorylation, acetylation, and ubiquitylation sites, localization probability of 0.75 was chosen as the threshold.

Biophysical screening and synthesis of CHIP inhibitors

To identify CHIP-interacting compounds, ^{1}H ligand-observed STD NMR screening was performed using the BIONET Premium Fragment Library as previously reported.[10] The STD NMR experiments were carried out at 293 K on an 800 MHz Bruker NMR spectrometer. NMR samples (500 μL) for the STD experiments were prepared as a solution consisting of 1 mM each fragment in 50 mM NaCl, 10 mM potassium phosphate, and 0.1 mM NaN_3 at pH 7.0 in D_2O either containing or devoid of 1 □M CHIP in screening buffer (50 mM NaCl, 10 mM potassium phosphate, and 0.1 mM NaN_3 [pH 7.0]). Secondary STD NMR experiments were conducted with the single fragment itself to confirm the binding.

To synthesize compound #153 from the eligible fragments, 5-bromo-6-methylpyridin-2-amine (100 mg, 0.54 mmol), 3-chloro-5-(trifluoromethyl)phenyl boronic acid (100 mg, 0.45 mmol), and a 1 M aqueous solution of potassium carbonate (1.78 mL) were added into a microwave vial at ambient temperature. The resulting suspension was purged with nitrogen for 3 min, and bis(triphenylphosphine)palladium(II) dichloride (31.6 mg, 0.045 mmol) was added. The microwave vial was sealed with a microwave cap. After microwaves were applied to the mixture at 120 °C for 3 h, the mixture was cooled down, diluted with ethyl acetate, and washed with

water. The organic layer was dried with magnesium sulfate, filtered, and concentrated *in vacuo*. The residue was purified by preparative thin layer column chromatography (ethyl acetate–hexanes at 1:1) to obtain 5-(3-chloro-5-(trifluoromethyl)phenyl)-6-methylpyridin-2-amine (compound #153). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 7.78 (s, 1H), 7.72 (s, 1H), 7.62 (s, 1H), 7.33 (d, 1H, J = 8.0 Hz), 6.37 (d, 1H, J = 8.0 Hz), 6.11 (s, 2H), 2.25 (s, 3H).

Cell cultures and transfection

Mammalian cells including HEK293and HT22 cells were purchased from the Korea Cell Line Bank and were grown in DMEM supplemented with 10 % of FBS, 2 mM L-glutamine, and 100 U/mL penicillin/streptomycin. The HEK293-pre1-HTBH cell lines were generated as previously described.[3] Cells were placed in a humidified incubator with 5 % CO_2 at 37 °C. For transient overexpression, cells were transfected with 1–2 μg of total plasmid DNA in a 12-well culture plate (at >95 % confluence or a density of 2.0 × 10^5 cells/well) for 48 h via Lipofectamine 3000 (Invitrogen) according to the manufacturer's guideline. Fluorescent signals from live cells were obtained by using an Olympus fluorescence microscope (IX71) or laser scanning confocal microscope LSM 700 (Zeiss). All images were captured using identical microscope settings and representative of the whole cell population.

Primary rat cortical and hippocampal neurons and tau oligomer transduction

Primary neuron cultures were prepared from rat embryonic cortex or hippocampus at embryonic day 17 as previously reported.[24] Briefly, isolated cortical and hippocampal neurons were cultured on poly-D-lysine-coated plates and coverslips, respectively, and treated with okadaic acid (30 nM), MG132 (10 μM), and compound #153 (25 or 50 μM) for 6 h at day 7 *in vitro*. For immunoblotting, cortical neurons were lysed using RIPA buffer to prepare the soluble and insoluble fractions of whole cell extracts. For immunostaining, hippocampal neurons were fixed with 4 % paraformaldehyde for conventional immunostaining. F-actin staining was performed by incubating the fixed cells for 20 min with phalloidin-Alexa Fluor 568 dye.

Cell viability assays

The effects of compound #153 on cell viability were assessed by the CellTiter-Glo Luminescent

Cell Viability Assay (Promega) kit and the live cell counting using the ImageXpress Micro system (Molecular Devices), both in accordance with the manufacturers' protocols. For CellTiter-Glo assay, cells were grown in black-wall/clear-bottom 96-well plates, treated with various concentrations of compounds #153 or #154 (up to 200 μM) for 12 h, and then luminescence substrates were added in the same volume as the cell culture medium. The mixture was incubated for 10 min at room temperature on a shaker, followed by luminescence measurement. For the high-content screening approach, cells were treated with compounds #153 or #154 (50 μM) in the presence and absence of MG132 (10 μM) for 6h, co-stained with Hoechst 33342 nuclear dye and propidium iodide (each 4 μg/mL) for 6 h. More than 4,000 cells per well were imaged using the 10× widefield microscope and analyzed using the standard Live/Dead application module in MetaXpress software (Molecular Devices).

Isolation of soluble and insoluble tau aggregates from HEK293 cells stably overexpressing tau

To separate the soluble and insoluble tau aggregates from cultured cells, HEK293-htau40 cells were used. Cells were washed with ice-cold PBS and lysed with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 0.1 % of SDS, 1 % of NP-40, 0.5 % of deoxycholate, and a protease inhibitor cocktail). Lysates were centrifuged at 16000 × *g* for 30 min. After separation of the supernatants, the pellets were washed several times with RIPA buffer and then added to an SDS sample buffer. All the samples were prepared in equal volumes and heated for 10 min at 80 °C. To detect tau aggregates, cells were treated with proteasome inhibitor MG132 (5 μM), phosphatase PP1/PP2A inhibitor okadaic acid (30 nM), the CHIP inhibitor (compound #153; 100 mM), or their combinations. After 6 h of incubation, WCLs were prepared in RIPA buffer and subjected to IB under reducing conditions with the anti–tau-S199 antibody.

Statistical analysis

Statistical significance of differences between various groups was determined by Student's t-test or one-way ANOVA followed by the Bonferroni *post hoc* test for most data. All experiments were performed in triplicated and data represent the mean standard deviation (SD). Differences

were considered significant when p-values were less than 0.05.

Supporting figures and figure legends

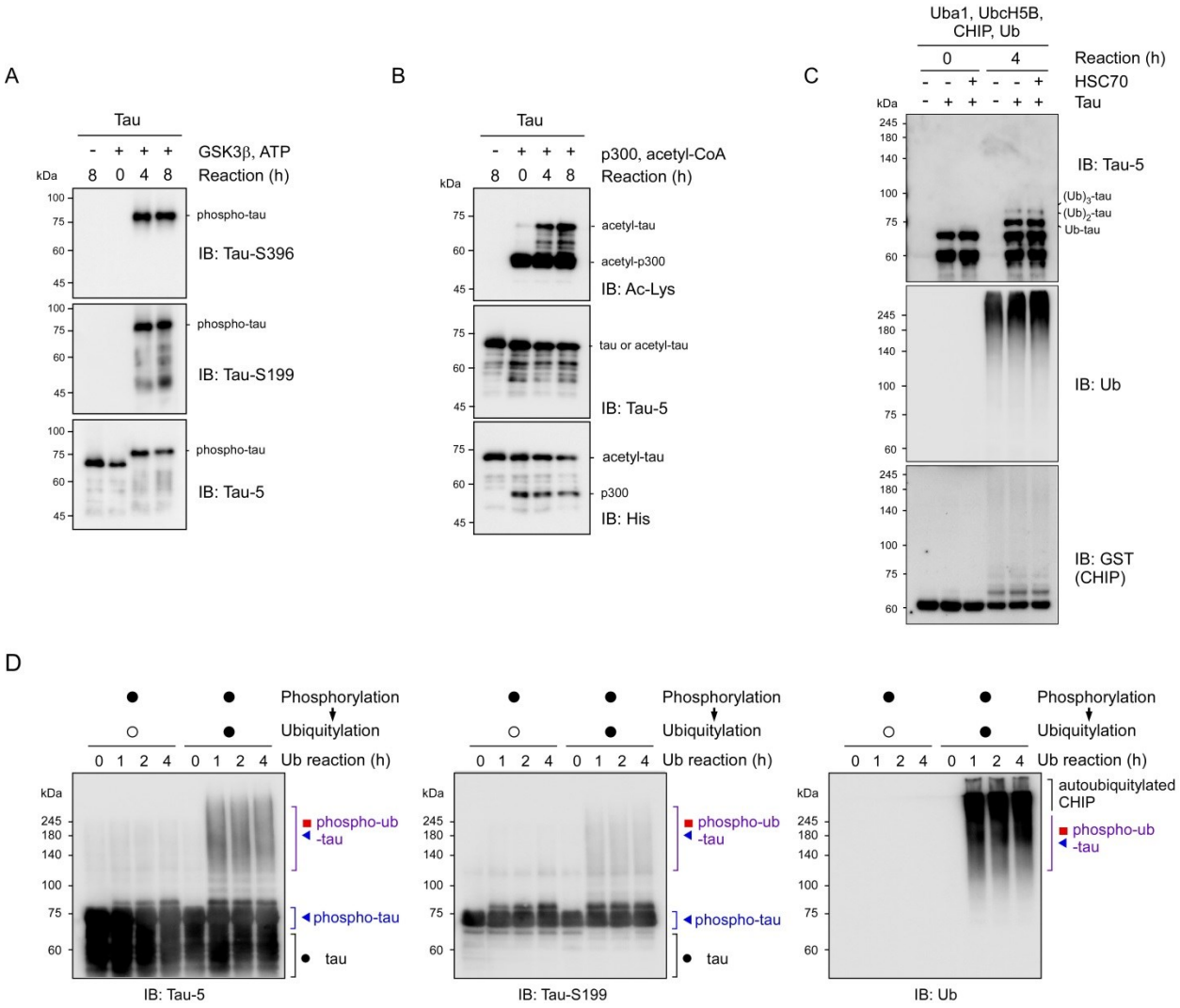

Figure S1. ***In vitro*** **reconstitution of tau phosphorylation, acetylation, and ubiquitylation reactions.** (**A–C**) Recombinant His-tau (0.5 μg) was phosphorylated by GSK3β (1 μg with 1 mM ATP, **A**), acetylated by the catalytic domain of p300/EP300 (1 μg with 125 μM acetyl-coenzyme A, **B**), or ubiquitylated by enzymes E1 (UBA1, 200 ng), E2 (UBE2D2/UbcH5b, 400 ng), and E3 (GST-tagged CHIP/STUB1, 4 μg) in the presence or absence of HSC70 (1 μg, **C**) at

37 °C. These biochemical reactions largely finished in less than 8 h. Notably, chaperone HSC70 was not essential for tau ubiquitylation, and at most 2–3 ubiquitin moieties were found to be attached to tau proteins after the 4-h ubiquitylation reaction. Tau proteins were monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis/immublotting (SDS-PAGE/IB) analysis with antibodies against total tau (tau 5), phospho-tau (tau-S396), and acetyl-Lys. (**D**) A similar *in vitro* ubiquitylation reaction as in Fig. 1A was carried out by either heat-inactivated (open circle) or active CHIP (filled circle). Tau proteins were analyzed by non-reducing SDS-PAGE followed with IB using anti-tau-5, tau-S199, and ubiquitin (Ub) antibodies.

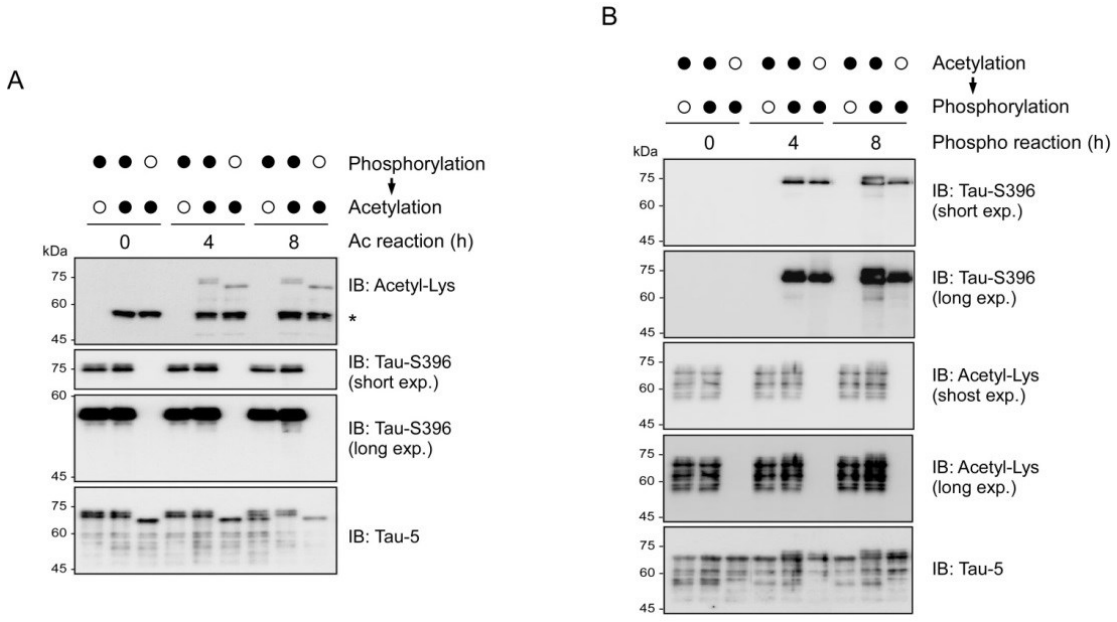

Figure S2. Tau acetylation has a minimal effect on phosphorylation and vice versa. (**A**) Purified tau proteins were phosphorylated *in vitro* and then acetylated for the indicated reaction periods at 37 °C. (**B**) As in (A), except that the tau acetylation reaction was carried out prior to phosphorylation. Tau species were monitored SDS-PAGE/IB with anti–tau 5 (total tau), anti–tau-S396 (phospho-tau), and anti–acetyl-Lys (acetyl-tau) antibodies. Open and filled circles indicate the post-translational modification reactions involving heat-inactivated and active enzymes, respectively.

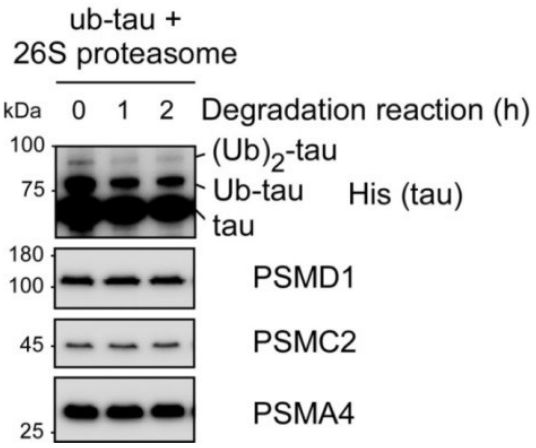

Figure S3. Slow degradation of ub-tau by 26S proteasomes. Affinity-purified human 26S proteasomes (5 nM) were incubated for the indicated periods with ub-tau species (100 nM), which had mainly one or two ubiquitin moieties after *in vitro* ubiquitylation reactions. These data complement Figure 2B.

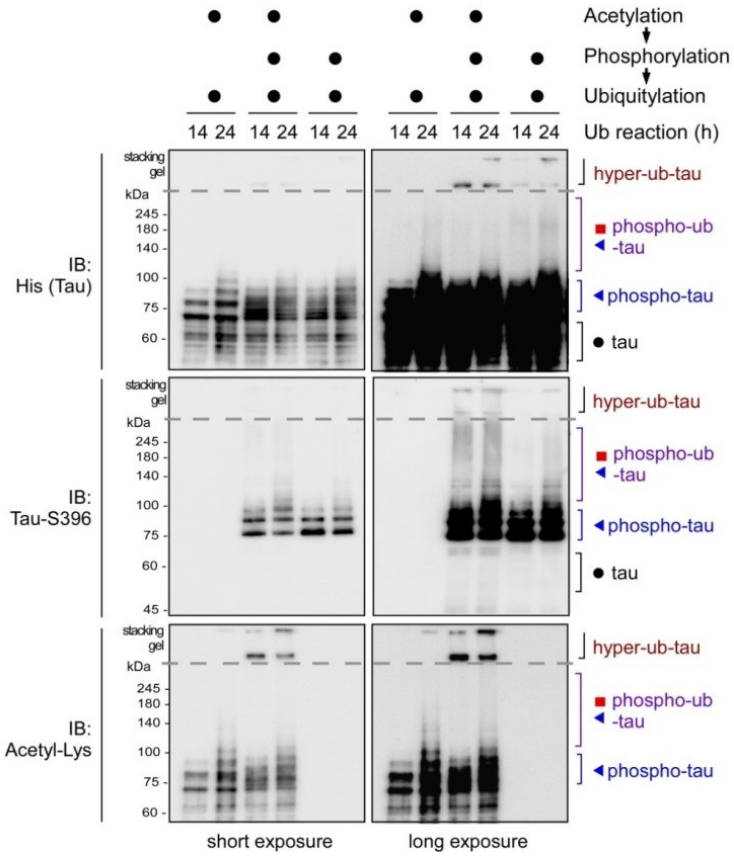

Figure S4. Little effect of acetylation on tau accumulation into the insoluble fraction after the extended *in vitro* ubiquitylation reaction. *In vitro* acetylation or phosphorylation reactions of tau were conducted for 1 h. The products were utilized for subsequent *in vitro* ubiquitylation reconstitution for the indicated periods at 37 °C. Less soluble hyperubiquitylated tau species (hyper-ub-tau) were indicated above the separating gel (dotted line). These data complement Figure 4A.

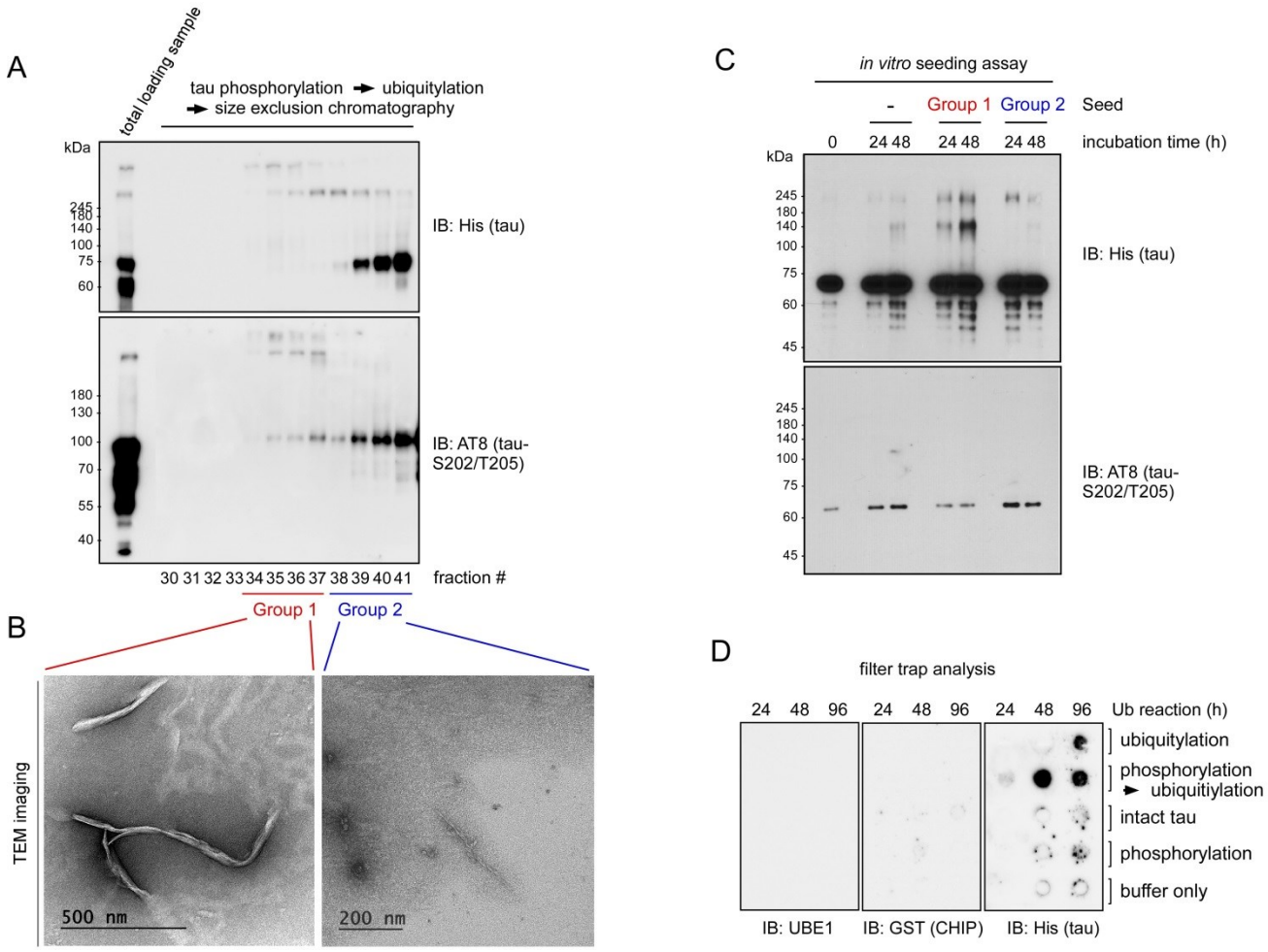

Figure S5. Hyper-ub-tau accumulates as insoluble aggregates both *in vitro* and *in vivo*. (A) Recombinant tau proteins after phosphorylation (for 12 h) and ubiquitylation (for 48 h) reactions were fractionated using size-exclusion chromatography using a Superose 200 Increase 10/300 GL column. (**B**) Fractions containing hyper-ub-tau species (> 250 kDa; Group 1) and monomeric tau (Group 2) were pooled and analyzed with negative-staining transmission electron microscopy (TEM). (**C**) *In vitro* seeding activity using the fractioned pools from (A). More than 50-fold of

tau monomers were incubated with either the Group 1 or Group 2 seeds for 24 h and 48 h at 37 ℃. Generation of tau oligomers was analyzed with SDS-PAGE/IB. Note that the phospho-tau (from the seeds) exhibited only weak and monomeric signals. **(D)** Samples prepared as described in Figure 4C were subjected to a filter trap assay (4 μg tau in a 96-well) using GST (for possible CHIP aggregation) or His (for tau) antibodies. These data supplement Figures 4C and 4D.

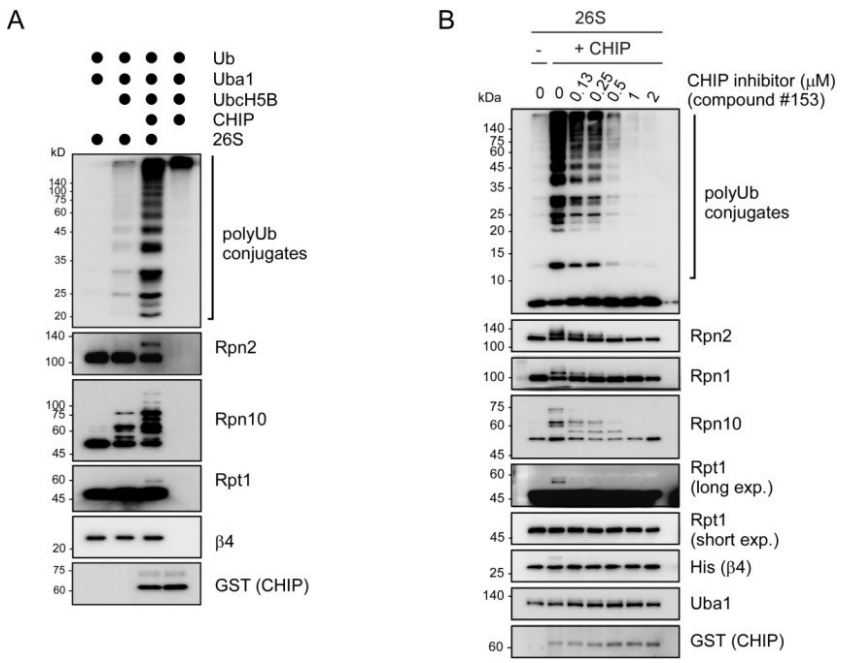

Figure S6. The CHIP inhibitor identified via biophysical screening efficiently delays proteasome ubiquitylation. (**A**) *In vitro* polyubiquitylation of human proteasomes by CHIP. The 26S proteasomes were affinity-purified and incubated with recombinant Ub, Uba1, UbcH5B, and CHIP proteins in different combinations. The ubiquitylation of 26S proteasomes was monitored with SDS-PAGE/IB using anti-Ub and other subunit antibodies. (**B**) As in (**A**), except that the CHIP inhibitor (compound #153) was subjected to *in vitro* ubiquitination of the purified proteasomes in a dose-course manner.

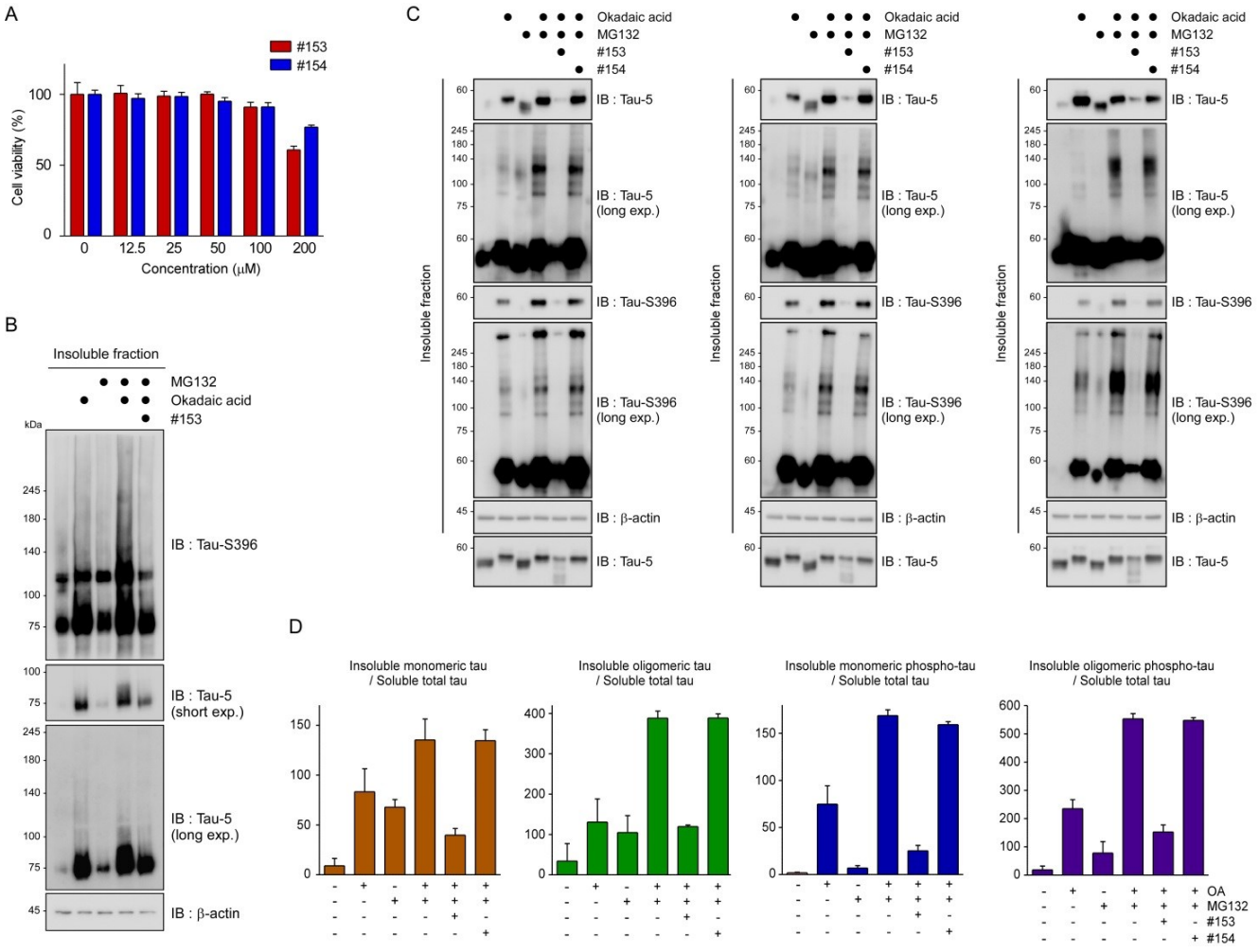

Figure S7. The compound #153 reduced the level of insoluble tau and delayed its accumulation in both neuronal and non-neuronal cells. (**A**) Limited cytotoxicity of compound #153, as measured by the CellTiter-Glo assay. The compounds were added at various concentrations to HEK293 cells for 12-h incubation, showing no significant cytotoxicity at concentration up to 100 μM. These data supplement Fig. 5F. (**B**) Reduced amounts of insoluble tau proteins after treatment with the CHIP inhibitors. A plasmid encoding tau was transfected

into HEK293 cells, which were incubated with MG132 (5 μM), okadaic acid (30 nM), or compound #153 (100 μM) for 6 h. RIPA-insoluble, pellet fractions from whole-cell lysates were isolated and analyzed by SDS-PAGE/IB (**C**) Triplicate experiments of Figure 6C. (**D**) Quantification of monomeric/oligomeric or total/phosphorylated endogenous tau in the insoluble fraction. The values were normalization to those of soluble total tau. Plots are presented as mean ± SD from three independent experiments as shown in C. OA, okadaic acid.

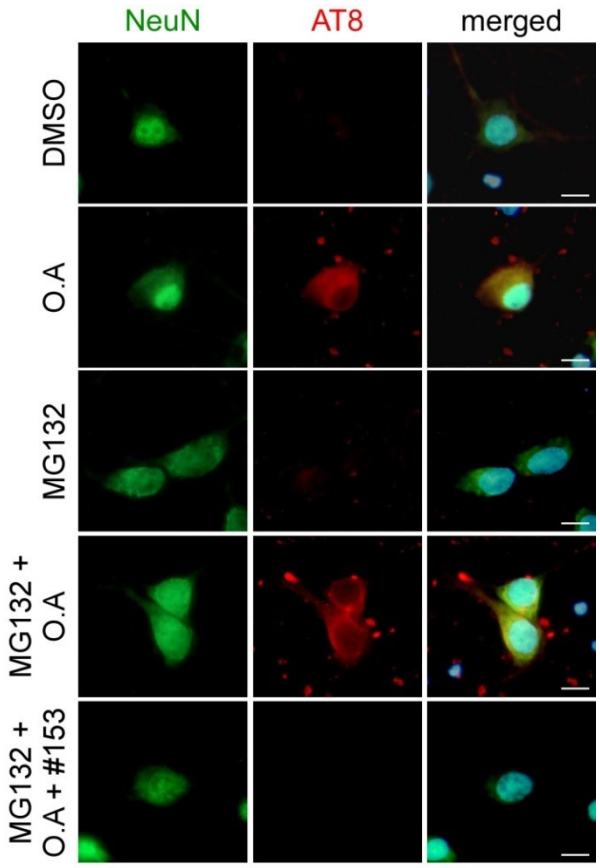

Figure S8. Effects of CHIP inhibition on tau aggregation in rat primary hippocampal cells. As in Figure 6F, except that 25 μM of compound #153 was treated and that neuronal nuclei (NeuN; green), a postmitotic neuron marker, was co-immunostained with phosphorylated tau (using the AT8 antibody; red). AT8 signals were significantly elevated in the presence of okadatic acids and MG132, which was effectively abolished by CHIP inhibition with compound #153. Scale bar, 10 μm. These data supplement Fig. 6G.

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