The dual fates of exogenous tau seeds: Lysosomal clearance versus cytoplasmic amplification

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Tau assembly movement from the extracellular to intracellular space may underlie transcellular propagation of neurodegenerative tauopathies. This begins with tau binding to cell surface heparan sulfate proteoglycans, which triggers macropinocytosis. Pathological tau assemblies are proposed then to exit the vesicular compartment as “seeds” for replication in the cytoplasm. Tau uptake is highly efficient, but only ~1 to 10% of cells that endocytose aggregates exhibit seeding. Consequently, we studied fluorescently tagged full-length (FL) tau fibrils added to native U2OS cells or “biosensor” cells expressing FL tau or repeat domain. FL tau fibrils bound tubulin. Seeds triggered its aggregation in multiple locations simultaneously in the cytoplasm, generally independent of visible exogenous aggregates. Most exogenous tau trafficked to the lysosome, but fluorescence imaging revealed a small percentage that steadily accumulated in the cytosol. Intra-cellular expression of Gal3-mRuby, which binds intravascular galactosides and forms puncta upon vesicle rupture, revealed no evidence of vesicle damage following tau exposure, and most seeded cells had no evidence of endolysosome rupture. However, live-cell imaging indicated that cells with pre-existing Gal3-positive puncta were seeded at a slightly higher rate than the general population, suggesting a potential predisposing role for vesicle instability. Clearance of tau seeds occurred rapidly in both vesicular and cytosolic fractions. The lysosome/autophagy inhibitor bafilomycin inhibited vesicular clearance, whereas the proteasome inhibitor MG132 inhibited cytosolic clearance. Tau seeds that enter the cell thus have at least two fates: lysosomal clearance that degrades most tau, and entry into the cytosol, where seeds amplify, and are cleared by the proteasome.

Results

Tau binds tubulin and is recruited to aggregates in the cytosol

Wildtype full-length (FL) tau undergoes seeded aggregation inefficiently in cultured cells, and thus, we studied FL (2N4R) tau containing a disease-associated P301S mutation, fused to mClover3 (FL tau-Clo) (Fig. 1A). We stably expressed FL tau-Clo in U2OS cells, where it colocalized with tubulin (Fig. 1B). We then directly imaged U2OS biosensor cells that were exposed to exogenous FL wildtype (2N4R) tau fibrils covalently labeled with Alexa Fluor 647 (AF647), tracking FL tau-Clo puncta formation over time. Similar to our original observations (7), a minority of cells exhibited induced aggregation of tau, which predominated in the cytoplasm (Fig. 1C). Time-lapse imaging (IN Cell Analyzer 6000, GE) also revealed intracellular aggregation in the cytoplasm (Fig. 1D), and in imaging dynamic inclusion formation in ~50 cells, we often observed aggregates forming simultaneously throughout the cytoplasm (Movie S1). For nascent intracellular aggregates, we observed no significant colocalization with AF647-labeled exogenous tau. This raised the question of how tau seeds traffic into the cytoplasm.

Internalized tau aggregates traffic to the endolysosomal system

To study the fate of internalized tau, we exposed U2OS to FL tau fibrils labeled with AF647, imaging them repeatedly...
over 2 days with high-content microscopy. We first tested for colocalization with vesicles by stably expressing mRuby3 fusions to Rab5 (to mark early endosomes), Rab7 (to mark late endosomes), and lysosomal-associated membrane protein 1 (LAMP1) (to mark lysosomes). Tau progressively colocalized with these markers, especially LAMP1. We concluded that most internalized tau entered the endolysosomal pathway.

When we tracked seeding in U2OS biosensor cells expressing FL tau using LAMP1, we observed no significant colocalization between emergent tau puncta with the lysosome, which was largely in a separate compartment versus the induced FL-tau-Clo aggregates (Fig. 3A). FL tau seeding is inefficient enough to make capture of large numbers of seeding events relatively difficult. We therefore performed the same experiment using repeat domain tau-Clo containing a P301S mutation to image a high number of seeded cells (Fig. 3B). Similar to our observation with FL tau, we observed no significant colocalization between RD tau-Clo aggregates and LAMP1 (Fig. 3C). Thus, although most internalized tau wound up in the lysosome, this seemed unlikely to be the primary location of seeding.

**Tau seeding is independent of the lysosome**

Since we did not observe significant colocalization of newly formed tau puncta with vesicle markers, we hypothesized that tau seeds might be released into the cytoplasm from a vesicular pool. After exposing U2OS cells to FL tau fibrils labeled with AF647, we detected diffuse fluorescence in the cytoplasm at 20 h (Fig. 4A). We next used live-cell imaging to monitor hundreds of cells exposed to FL tau fibrils tagged with AF647. We observed a small but steady increase of cytosolic AF647 signal, whereas transferrin-AF647 did not increase in signal following...
a similar exposure protocol (Fig. 4B). To test our observations by a different approach, we used cell fractionation based on differential centrifugation to measure tau seed levels in cytosol versus organelle (vesicle) fractions (Fig. 4C). We confirmed the accuracy of the fractionations using Western blot against GAPDH (cytosol), voltage-dependent anion channel (organelle), LAMP1 (organelle), and lamin B1 (nucleus) (Fig. 4D). We attempted to quantify tau protein levels via Western blot, ELISA, and mass spectrometry, but they were too low for reliable measurements. We next monitored seeding activity in the cytoplasm by transducing lysate into a well-characterized biosensor assay based on a next-generation cell line, v2L (8, 9). We observed a steady increase in cytosol seeding activity over time (Fig. 4E). Tau seeds thus steadily moved from vesicles to the cytosol.

**Tau fibrils do not damage vesicles**

Prior studies have proposed that tau-mediated damage to vesicles might allow leakage into the cytoplasm to initiate seeding (6, 10). To test this hypothesis, we expressed the galectin-3 β-galactoside–binding protein fused to mRuby3 (Gal3-Rub), to observe the relationship of vesicle rupture and tau seeding. β-galactosides localize to the outer leaflet of the cell membrane and are present in the lumen of endocytic vesicles. Gal3-Rub expressed intracellularly is normally diffusely distributed. However, if an endosome is damaged, Gal3-Rub binds β-galactosides, creating puncta (11, 12). We stably expressed Gal3-Rub in U2OS cells and tracked puncta formation using high-content microscopy. Gal3-Rub diffusely distributed in most cells (Fig. 5A). Following exposure of cells to FL tau fibrils tagged with AF647, we observed tau in every cell. However, we observed no change in Gal3-Rub puncta formation (Fig. 5A). We contrasted this with L-leucyl-L-leucine O-methyl ester (LLOMe; Cayman Chemical) treatment to disrupt vesicles, which strongly induced Gal3-Rub puncta formation (Fig. 5B). In summary, we observed no detectable change in overall vesicle permeability upon tau exposure, suggesting that tau fibrils do not significantly damage endocytic vesicles.

**Vesicle lysis slightly increases tau seeding**

We used live-cell imaging to track seeding into cells using expression of FL tau-Clo as a biosensor. We observed seeding into cells with and without coincident Gal3-Rub–positive puncta (Fig. 6, A and B). Seeding efficiency onto FL tau is relatively low; so to more easily quantify Gal3-Rub puncta in relation to seeding events, we used the RD tau-clo biosensor.
This enabled recording of hundreds of seeding events. We began by observing in an endpoint assay that ~12% of untreated cells exhibited Gal3-Rub puncta (Fig. 6C). By contrast, ~35% of cells with seeding exhibited coincident Gal3-Rub puncta, whereas ~65% did not (Fig. 6C). The higher association of Gal3-Rub puncta with seeded cells led us to test the relationship more rigorously. We used dynamic imaging of cells in culture to identify those that developed tau inclusions after exogenous seeding. We then tracked the cells backward ~12 h prior to the appearance of tau inclusions to determine the percentage that showed no Gal3-Rub puncta at any time (82%), transient Gal3-Rub puncta (2%), or pre-existing puncta (16%) (Fig. 6D). The large majority of seeded cells did not show any prior evidence of vesicle rupture. The number with pre-existing evidence of vesicle instability (18% total) was slightly higher than the total we observed in untreated cells (12%). To directly compare the seeding efficiency after direct rupture of vesicles, we tested the effect of vesicle rupture by LLOMe versus seed transduction via Lipofectamine 2000 (Thermo Fisher Scientific). We observed a modest increase in seeding after LLOMe treatment (from ~1% to ~5%); however, Lipofectamine treatment, which presumably delivers aggregates directly to the cytoplasm, increased seeding to ~32% (Fig. 6E). We concluded that while vesicle integrity may influence the frequency of seeding events, this is not the primary determinant.

**Distinct mechanisms of tau seed clearance in vesicles versus cytosol**

Our observations were consistent with escape of tau seeds from the vesicular compartment to the cytosol, where seeding occurs. The persistence of tau in each compartment would thus determine the relative efficiency of seeding. Consequently, we evaluated the kinetics of tau seed clearance using purification of seeds from each fraction, coupled with
detection using standard v2L biosensor cells (9). We exposed U2OS cells to recombinant FL tau fibrils, followed by fractionation of cells into organelle (vesicle) versus cytosol fractions. We used immunoprecipitation to test for seeding within the organelle fraction (to avoid toxicity on biosensor cells) and directly assayed the cytosol fraction. In the organelle fraction, tau had virtually disappeared by 12 h (Fig. 7A). In the cytosol fraction, clearance was slightly slower, with tau seeding detectable even at 48 h (Fig. 7B). We attempted to use ELISA and mass spectrometry to monitor tau clearance directly, but levels were too low for accurate measurement without using inordinately large amounts of recombinant tau in cultured cells.

We next tested the effect of inhibitors of the lysosome/autophagy (bafilomycin Sigma–Aldrich) and proteasome (MG132, Sigma-Aldrich) on seed clearance over 4 h, a maximum time point picked to minimize secondary effects. Bafilomycin halted degradation in the organelle fraction (Fig. 7C) but had no effect on the cytosol fraction (Fig. 7D). By contrast, MG132 had no effect on organelle clearance (Fig. 7E) but prevented clearance of tau seeds from the cytosol (Fig. 7F). We observed a rapid seed clearance in the organelle fraction.

**Figure 4. Exogenous tau enters the cytosol.** U2OS cells expressing FL tau-Clo (green) were exposed to tau fibrils labeled with AF647 (red), and fluorescence was tracked over time using live-cell imaging. A, representative images illustrate the increase in the cytosolic AF647 fluorescence between 30 min and 20 h. Zoomed-in regions are marked in yellow box and pictured in the second row. Quantification was based on imaging n >500 cells. Scale bars = 20 μm. B, tau-AF647 signal in the cytosol was compared with transferrin (Tfn)-AF647 added to cells. Tau signal increased over time, whereas Tfn signal did not. Fluorescence is indicated in arbitrary units (A.U.). Error bars represent SD. C, diagram indicating steps used to quantify exogenous tau seeding in different fractions. Immunoprecipitation was used to measure tau seeding in vesicle fractions, as crude lysate was toxic. D, fractions were analyzed by Western blot to assess fractionation fidelity. Antibodies against the indicated proteins were used to probe different cell fractions. C: cytosolic; O: organelle; and N: nuclear. E, cytosol tau seeding was measured by transducing v2L biosensors with the cytosol fraction from untransfected U2OS cells (after tau fibril addition). Representative graph shows the increase in cytosol seeding over time. The background seeding value from a negative control (cytosol from untreated cells) was subtracted from each data point. The seeding assay was performed on three independent biological replicates, and each individual measurement was carried out in triplicate. Error bars indicate SD. AF647, Alexa Fluor 647.
Discussion

It is unknown how a tau assembly propagates a unique structure from the outside to the inside of a cell. This study has investigated the trafficking kinetics of tau seeds into the cytosol and mechanisms of degradation. For ease of labeling and tracking, we used recombinant heparin-induced FL tau fibrils, which undoubtedly lack the same seed conformation that occurs in AD, or post-translational modifications. Second, rather than primary neurons, which might more accurately reflect disease processes, we used an immortalized cell line, U2OS, because it is highly adherent, has a large cell body, and is useful for live-cell imaging over days. We observed that most tau taken up by the cell traffics to the endolysosomal system, where it is degraded fairly rapidly. A small percentage of assemblies entered the cytosol to seed intracellular aggregation, which appeared to occur often simultaneously at multiple sites. Seeding increased after experimental rupture of endosomes; however, this did not appear to be the predominant mechanism, and tau assemblies did not measurably disrupt vesicles. Most assemblies appeared to be degraded via bafilomycin-sensitive mechanisms in the lysosome, whereas cytosolic seeds were degraded primarily by the proteasome. In summary, our data indicate two major routes into the cell for a tau seed: one into the lysosomal degradation pathway, and the other to the cytosol, where recruitment of native tau and template-based replication occur (Fig. 8).

Cytoplasmic tau seeding

In cultured cells, despite widespread tau uptake by macropinocytosis, seeding is relatively inefficient. To overcome this problem in experimental systems, we (8) and others (13) have used lipid-based transduction reagents to improve cytoplasmic delivery of tau assemblies. In biosensor cells expressing FL tau-Clo, we observed seeding in ~1% of exposed cells, despite ~100% of the cells taking up labeled tau assemblies into the endolysosomal system. We expressed FL tau-Clo constructs that bind the cytoplasmic tubulin network. Within hours after tau seed exposure, however, we observed simultaneous evolution of tau puncta throughout the cytoplasm. The simultaneous appearance of puncta within the cell suggests that there may be a regulatory pathway controlling this process. In data not presented, we tested for a role for the cell cycle but did not observe any. We have concluded that seeds not visible by microscopy escaped the endolysosomal compartment to recruit endogenous FL tau-Clo away from the tubulin network. Taken together, our results imply that, following macropinocytosis, only a small subset of tau assemblies wind up in the cytosol, where they serve as templates for amplification.

Endolysosome rupture only partially explains tau seeding

Prior studies have suggested that tau enters the cytosol by rupturing vesicles (6, 10). However, using formation of Gal3-Rub puncta as a marker of vesicle integrity, we found no evidence that tau aggregates directly induce rupture. Neither did we observe FL tau-Clo puncta formation in close proximity to Gal3-stained vesicles. Finally, based on live-cell imaging of hundreds of cells, in the majority of cases (~65–80%), we observed no evidence of vesicle rupture in association with seeding events. However, when we specifically tested the relationship of Gal3-Rub puncta to subsequent cell seeding, we observed a slight increase in the percentage of cells that exhibited pre-existing evidence of Gal3-Rub binding (~16%), relative to cells that did not have evidence of seeding (~12% of the total overall). Notably, 82% of seeded cells showed no evidence of preceding vesicle rupture. Pre-existing vesicle
instability might slightly predispose toward tau seeding, but is clearly not a major determinant. Genes associated with the endolysosomal system have been linked to AD (14). When we treated cells with LLOMe to rupture lysosomes following loading with tau, we only saw a 5× increase in seeding, from 1% to 5%. In contrast, treatment with Lipofectamine 2000 increased seeding 35×. This implies that the route by which tau enters the cytosol may impact its seeding efficiency and argues against simple vesicle rupture as a primary mode. The contrast of our findings to those of others (6, 10) may reflect our use of different systems and purified recombinant tau fibrils. Importantly, a recent study using Human embryonic kidney 293 cells and primary neurons also concluded that tau does not damage vesicles (15). We are continuing to investigate the molecular mechanism by which assemblies might cross the vesicle membrane in the absence of rupture, potentially via direct translocation.

**Cytoplasmic tau seeds are degraded by the proteasome**

Our data suggest trafficking of tau seeds into two compartments: the endolysosome and the cytoplasm. The relative amount of tau protein in both compartments was too low to measure via Western blot, ELISA, or even mass spectrometry,
but we readily quantified tau seeding activity using biosensor cells. We determined that tau seeds within the vesicle compartment are rapidly cleared. This was blocked by bafilomycin, consistent with lysosomal degradation. By contrast, seeds within the cytosol appeared to have a rapid and slow phase of degradation. The rapid phase, with a half-time of ~4 h, was blocked by proteasome inhibition with MG132. The persistence of seeds in the cytosol for up to 48 h after cell exposure suggests that a small subset may be protected from degradation, with a longer half-life. The role of autophagy in aggregate degradation has been proposed by many studies (16, 17). Our data suggest a more nuanced interpretation, with the proteasome rapidly degrading exogenous seeds that enter the cytosol. But we cannot exclude a role for autophagy for slower clearance. Since the proteasome must digest single unfolded proteins, we propose that disassembly factors could be
important. One candidate is valosin-containing protein (p97/VCP), which has been directly implicated in dominantly inherited tauopathy (18).

**Tau traffics to distinct pools**

Our data indicate that two populations of intracellular tau result from macropinocytosis. Most tau enters the endolysosomal system for rapid degradation, resulting in relatively inefficient seeding overall. We observed a steady increase of tau in the cytosol over time both by imaging and fractionation studies. Cytosol seeding thus appears to be derived from tau leakage out of the endolysosomal system, whether by vesicle rupture or other mechanisms such as membrane translocation (15, 19, 20). Our conclusions that there exist two paths for tau into the cell, with one leading to intracellular seed amplification, may help clarify the molecular mechanisms of tauopathy and may explain the identification of endolysomal genes as AD risk factors (14).

**Experimental procedures**

**Tau fibrillization, labeling, and imaging**

Recombinant FL (2N4R) wildtype tau was purified and fibrillized as described previously (3). To label tau, 8 μM purified tau fibrils were incubated with 0.025 mg AF647 succinimidyl ester dye (Invitrogen) for 1 h at room temperature and quenched with 100 mM glycine for 1 h at room temperature. The solution was dialyzed overnight into PBS using dialysis cassettes (Thermo Fisher Scientific) to remove unbound dye. Labeled fibrils were stored at 4 °C for short term (days to weeks) or at ~80 °C for the longer term. For live-cell imaging assays, labeled fibrils were added to the U2OS cells and imaged using an IN Cell Analyzer 6000 (GE).

**Cell lines**

Lentiviral delivery vectors were used to create U2OS cells that stably overexpressed proteins of interest. To generate the cell lines, cells were plated at 30,000 cells/well in a 12-well plate. After 24 h, ~200 μl conditioned media containing lentivirus were added to the wells. Cells were grown for 3 days and then replated in a 10 cm dish, grown to confluency, and stored in liquid nitrogen until use. Protein expression was confirmed by epifluorescence microscopy. The stable U2OS cell lines generated for this study are listed: Tet off tau FL(P301S)-mCerulean3/mClover3: FL 2N4R human tau with P301S mutation tagged to mCerulean3 and mClover3 expressed under Tet-regulated promoter Tet off tau FL(P301S)-Cerulean3/mClover3; Galectin3-mRuby3: FL 2N4R human tau with P301S mutation fused to mCerulean3 or mClover3, both expressed under Tet-regulated promoter; Galectin-3 is fused to mRuby3; Galectin-3-mRuby3: Galectin-3 is fused to mRuby3.

**Measuring nonvesicular cytoplasmic tau signal**

To segment cells within an image, we calculated the image gradient of the FITC channel using the Sobel method and normalized this output by the gradient magnitude. We then subtracted the resulting normalized image gradient from the original image to enhance cell edges. To remove the remaining image noise, we applied a Gaussian filter with sigma value of 5. Last, we calculated the Rosin threshold from the filtered image and used this threshold value to segment cell boundaries. To segment vesicle boundaries from the Cy5 channel, we applied a lower and upper intensity threshold. For the lower, we calculated the Rosin threshold and ignored all pixels below this value. For the upper (to avoid artifacts from dying cells), we calculated the top 1% intensity and ignored pixels above this.
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value. To compare tau signal inside and outside the vesicles, we calculated tau signal from the Cy5 channel from inside cell and inside vesicle boundaries or signal inside the cell but outside vesicle boundaries. We then calculated the median intensity for all pixels within these regions. After calculating the median intensity outside the vesicles from the first time point, we background subtracted this value from all subsequent time points.

Seeding assay

V2L Biosensor cells (9) expressing tau RD containing a disease-associated mutation (P301S) fused to mCerulean3 (Cer) or mClover3 (Clo) (tau RD-Clo/Cer) were plated at a density of 10,000 cells/well in a 96-well plate. Recombinant tau fibrils were sonicated for 30 s at a setting of 65 (Qsonica Sonicator) and applied to cells at 50 μl per well. Cells were then incubated 48 h. Tau (80 nM) was added directly to the cells after sonication (for naked seeding). Alternatively, Lipofectamine 2000 was used to transduce tau (5 nM). After 48 h, cells were harvested with 0.05% trypsin, fixed in 2% paraformaldehyde for 10 min, and then resuspended in flow cytometry buffer (Hank’s balanced salt solution plus 1% fetal bovine serum and 1 mM EDTA). We quantified FRET as described previously using an LSRFortessa flow cytometer (21). For each dataset, three technical replicates were used. For each experiment, a minimum of ~5000 single cells per replicate were analyzed. Data analysis was performed using FlowJo (BD Biosciences), version 10, software and GraphPad Prism, version 8 (GraphPad Software, Inc).

Cell culture and treatments

Human U2OS (American Type Culture Collection) and human embryonic kidney 293T cells were cultured in McCoy’s 5A and Dulbecco’s modified Eagle’s medium (Gibco) supplemented with GlutaMAX and 10% fetal bovine serum. Cells were incubated in humidified air with 5% CO2 at 37 °C and were subcultured every 3 to 4 days. For vesicle permeabilization, 1 mM LLOMe was added to the media for 6 h. Proteasome inhibitor MG132 (Sigma) dissolved in dimethylsulfoxide was used at 10 μM final concentration, and the autophagy inhibitor, bafilomycin, dissolved in dimethylsulfoxide was used at 200 nM final concentration.

Western blot

Lysates were mixed with 4× SDS buffer and run on a NuPAGE 10% Bis–Tris Gel at 100 V. The gel was then transferred onto Immobilon P membrane for 1 h at 20 V using a semidry transfer apparatus (Bio-Rad). The membrane was blocked with 5% Blotto (Bio-Rad) in Tris-buffered saline with Tween-20 (TBST) for 1.5 h before primary (GAPDH: 1:5000 dilution [R&D Systems], lamin B1: 1:1000 dilution [Cell Signaling Technology], LAMP1: 1:1000 dilution [Thermo Fisher Scientific], voltage-dependent anion channel: 1:500 dilution [Thermo Fisher Scientific]) antibody was added at specific dilutions and incubated on a shaker overnight at 4 °C. The membrane was then washed three times with TBST at 10 min intervals. It was reprobed with (goat anti-rabbit/mice) secondary antibody for 1.5 h at room temperature. The membrane was washed four times with TBST and exposed to Enhanced Chemiluminescence Prime Western blot detection kit (GE Lifesciences) for 2 min. Blots were imaged with a Syngene digital imager.

Cellular fractionation

Fractionation was guided by published protocols (22, 23). Cells were trypsinized and resuspended in 5 ml of culture medium. They were centrifuged at 500g for 10 min at 4 °C, the supernatant was discarded, and the pellet was washed with 500 μl of ice-cold PBS. About ~250 μl (depending on pellet size) of ice-cold lysis buffer (150 mM NaCl, 50 mM Hepes [pH 7.4], 25 μg/ml digitonin, and 1 M hexylene glycol) was added to the pellet and then incubated on end-over-end rotator for 10 to 15 min at 4 °C. The sample was centrifuged at 2000g for 10 min at 4 °C. The supernatant was collected, and the pellet was processed for the next step. The supernatant was clarified by centrifugation at 18,000g for 20 min. Supernatant was collected as the cytosol fraction.

The pellet was washed with wash buffer (150 mM NaCl, 50 mM Hepes [pH 7.4]) and then 250 μl of ice-cold buffer (150 mM NaCl, 50 mM Hepes [pH 7.4], 1% IGEPAL [v/v], 1 M hexylene glycol) and resuspended by vortexing. It was incubated on ice for 30 min and then centrifuged at 7000g for 10 min at 4 °C. The supernatant was collected. This fraction contains the proteins from all membrane-bound organelles (endosomes, mitochondria, endoplasmic reticulum, Golgi, etc) except nuclei. The pellet was washed with wash buffer and then 250 μl of ice-cold buffer (150 mM NaCl, 50 mM Hepes [pH 7.4], 0.5% sodium deoxycholate [w/v], 0.1% sodium dodecyl sulfate [w/v], 1 M hexylene glycol, and 7 μl of benzamase [25,000 units/ml]) was added. It was incubated on an end-over-end rotator for 30 min at 4 °C to allow complete solubilization of nuclei and digestion of genomic DNA. It was then centrifuged at 7800g for 10 min at 4 °C. The supernatant was collected. This fraction contains the nuclear proteins. 1× Roche EDTA-free cOmplete protease inhibitor was added to all buffers.

Seed degradation assay

The U2OS cells were incubated with tau fibrils for 16 h. The next day, cells were washed with PBS for 2 min, and a 30 s 0.05% trypsin wash was used to digest attached tau, followed by quenching with a 3 min wash with McCoy’s 5A media. Cells were then incubated for the defined amount of time in media without tau, trypsinized, and fractionated to collect the cytosolic or organelle fractions, which were used for seeding assays.

Immunoprecipitation

About 75 μl of Dynabeads Protein A (Thermo Fisher Scientific) were washed according to the manufacturer’s protocol and incubated with 15 μg of tau polyclonal antibody raised against tau RD TauA (9), for 1 h at room temperature. The beads were washed with PBS with Tween-20. The beads were
added to 75 μg of lysate (organellar fraction) and incubated with rotation overnight at 4 °C. The next day, beads were washed with PBS with Tween-20 and protein was eluted in low pH elution buffer (Pierce). The reaction was neutralized with washing with PBS with Tween-20 and protein was eluted in low

**Data availability**

All data generated and analyzed during this study are included in this article.

**Supporting information**—This article contains supporting information.

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**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: AD, Alzheimer’s disease; AF647, Alexa Fluor 647; FL, full-length; Gal3-Rub, galectin-3 β-galactoside–binding protein fused to mRuby3; LAMP1, lysosomal-associated membrane protein 1; LLOMe, i-leucyl-i-leucine O-methyl ester; TBST, Tris-buffered saline with Tween-20.

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