The extracellular chaperone Clusterin enhances Tau aggregate seeding in a cellular model

Patricia Yuste-Checa, Victoria A. Trinkaus, Irene Riera-Tur, Rahmi Imamoglu, Theresa F. Schaller, Huping Wang, Irina Dudanova, Mark S. Hipp, Andreas Bracher & F. Ulrich Hartl

Spreading of aggregate pathology across brain regions acts as a driver of disease progression in Tau-related neurodegeneration, including Alzheimer’s disease (AD) and frontotemporal dementia. Aggregate seeds released from affected cells are internalized by naïve cells and induce the prion-like templating of soluble Tau into neurotoxic aggregates. Here we show in a cellular model system and in neurons that Clusterin, an abundant extracellular chaperone, strongly enhances Tau aggregate seeding. Upon interaction with Tau aggregates, Clusterin stabilizes highly potent, soluble seed species. Tau/Clusterin complexes enter recipient cells via endocytosis and compromise the endolysosomal compartment, allowing transfer to the cytosol where they propagate aggregation of endogenous Tau. Thus, upregulation of Clusterin, as observed in AD patients, may enhance Tau seeding and possibly accelerate the spreading of Tau pathology.
Progression of several neurodegenerative diseases, prominently including tauopathies such as frontotemporal dementia (FTD) and Alzheimer’s disease (AD), is driven by the spreading of aggregate pathology across brain regions in a prion-like seeding mechanism. Tau aggregate spreading involves the exposure of aggregate seeds to the extracellular milieu, suggesting that extracellular protein quality control factors may modulate disease progression. Clusterin (Clu; apolipoprotein J) is a ∼70 kDa glycoprotein with chaperone-like properties found abundantly in plasma and extracellular fluid. During its passage through the secretory pathway, immature Clu is extensively N-glycosylated and cleaved into α and β-chains, which remain linked by disulfide bonds (Supplementary Fig. 1a). Although mainly a secreted chaperone, Clu is also found intracellularly under stress conditions. Clu stabilizes unfolded proteins against aggregation and can inhibit fibril formation of amyloid β (Aβ) and other amyloidogenic proteins in vitro, consistent with the function of an ATP-independent “holdase” chaperone.

The Clu gene ranks third among genetic risk factors for late-onset AD. However, the mechanism by which Clu modulates AD pathology remains unclear, as Clu has been associated with both neuroprotective and neurotoxic effects in AD. Clu protein expression is upregulated in AD patient brain and cerebrospinal fluid, localizing with Aβ deposits in senile plaques. Evidence has been presented that Clu can mediate uptake of Aβ by microglia via the endosomal pathway and is involved in clearance of Aβ via the blood-brain barrier. On the other hand, enhancement of Aβ toxicity by Clu has also been reported and elevated plasma levels of Clu were found to be associated with rapid progression of AD, suggesting that Clu could be a driver of pathology. Little is known about the possible role of Clu in the progression of Tau pathology, which strongly correlates with the severity of AD. Interestingly, Clu was identified as an interactor of soluble Tau in AD brain. More recently, it was shown that Clu also colocalizes with intracellular Tau aggregates and may provide a protective function by inhibiting fibril formation. Given the predominant role of extracellular Clu as the chaperone active form, it remains to be understood whether Clu modulates transcellular Tau seeding and influences overall pathology.

Here we analyzed the effect of Clu on the seeding competence of Tau aggregates formed in vitro and in cells. Our results show that Clu can strongly enhance Tau aggregate propagation by binding and stabilizing seeding active Tau species for cellular uptake. Thus, upregulation of Clu in AD has the potential to accelerate disease progression by enhancing the seeding competence of Tau aggregates.

Results

Clusterin potentiates seeding of Tau aggregates. To test whether Clu interferes with aggregate seeding of Tau, we purified chaperone-active Clu upon recombinant expression in HEK293-EBNA cells (Supplementary Fig. 1b, c). We measured aggregate seeding with TauRD-YT cells, a HEK293T cell line stably co-expressing the repeat domain of Tau (TauRD; residues 244-372 with FTD mutations P301L/V373M) fused to YFP or mTurquoise2, whose co-aggregation during fibril formation results in fluorescence resonance energy transfer (FRET) (Fig. 1a). Seed aggregates were generated with recombinant, cysteine-free TauRD (Tau residues 244-371, C291A/P301L/C222A/V337M) to avoid the use of reducing agents that might interfere with Clu function. Mutation of the two cysteines in TauRD avoids the formation of intramolecular disulfide bonds that slows fibril formation. Lipofectamine was used to render seed uptake independent of cellular machinery for internalization. The TauRD is critical for aggregation and forms the core of Tau fibrils.

TauRD rapidly formed thioflavin T (ThT)-positive fibrillar aggregates in vitro, induced by heparin (Fig. 1b, Supplementary Fig. 2a). The addition of Clu at an equimolar ratio to TauRD extended the lag phase and slowed fibril elongation but did not prevent fibril formation (Fig. 1b, Supplementary Fig. 2a). To observe seeding, we next transferred small quantities of TauRD (0.05 ng to 14 ng with lipofectamine) after different times of intravivo aggregation to TauRD-YT cells, followed by an analysis of endogenous aggregate formation by flow cytometry of FRET-positive cells and fluorescence microscopy (Fig. 1a, c, d, Supplementary Fig. 2b). Seeding competent TauRD accumulated with kinetics similar to the formation of ThT-positive aggregates (Fig. 1b, c). The presence of Clu in the aggregation reaction delayed the appearance of seed material (0% FRET positive cells after 1 h aggregation time, Fig. 1c, d). However, once ThT-positive aggregates formed (from 2 h on, Fig. 1b), the Clu-containing aggregation reaction surprisingly presented a markedly increased seeding potency resulting in ∼80% FRET-positive cells (exceeding the linear range of the assay) compared to ∼30% FRET-positive cells with TauRD aggregates alone (Fig. 1c, d, Supplementary Fig. 2b). When aggregation reactions were diluted 280-fold, seeding without Clu was virtually abolished, but was still measurable in the presence of Clu, resulting in ∼25% of aggregate containing cells (Fig. 1c). Again, the kinetics of seed formation correlated with the delayed formation of ThT-positive aggregates (Fig. 1b, c). The FRET-positive inclusions formed in cells with and without Clu were morphologically similar and stained with the amyloid dye X34 (Fig. 1d, Supplementary Fig. 2c). Clu alone neither formed ThT-positive species nor induced Tau aggregation when added to cells (Supplementary Fig. 2d). Titration experiments using seed material from the plateau phase of aggregation (Fig. 1b) showed that Clu increased seeding potency ~25-fold (defined as % FRET-positive cells/ng TauRD) (Fig. 1e, Supplementary Fig. 2e). To test the effect of Clu on seeding in cells with the unperturbed plasma membrane, we omitted the transfection reagent. Under these conditions, Clu still increased the seeding potency of TauRD aggregates approximately eightfold (Supplementary Fig. 3). However, as expected, higher amounts of TauRD and TauRD/Clu aggregates were necessary to observe aggregate seeding.

The effect of Clu on TauRD aggregation and seeding is concentration-dependent, since increasing the ratio of Clu to TauRD further delayed amyloid formation in vitro and increased seeding potency (Supplementary Fig. 4a, b). However, the effect on seeding potency is saturated at a 1:1 molar ratio of TauRD:Clu (Supplementary Fig. 4b).

As TauRD is a highly charged protein (21 positively and 10 negatively charged amino acids), we tested whether the effect of Clu on TauRD seeding is dependent on electrostatic interactions. TauRD and TauRD/Clu aggregates (Fig. 1a) were incubated with PBS or high salt buffer (PBS/500 mM NaCl) prior to addition to TauRD-YT cells. Incubation with high salt buffer resulted in a general increase in seeding (Supplementary Fig. 4c), suggesting that high ionic strength may stabilize seeding competent TauRD species. Importantly, Clu increased seeding potency both in PBS and in the high salt buffer (Supplementary Fig. 4c), consistent with hydrophobic forces playing a role in the Clu-TauRD interaction.

In order to test the effect of Clu on preformed Tau aggregates, Clu was added to the aggregation reaction once the ThT plateau was reached (at 1 h or 24 h after initiating aggregation, Supplementary Fig. 4d). When added at an equimolar ratio to TauRD aggregates, Clu amplified seeding competence ~3-4-fold.
The use of heparin in seed production results in a non-
physiological conformation of Tau fibrils. To exclude that the observed effect on TauRD seeding is dependent on heparin, we, therefore, investigated the effect of Clu on seeding by TauRD aggregates produced in cells. Cells were lysed under mild conditions in the presence of non-ionic detergent (0.05% Triton X-100) without sonication to preserve the structural properties of the aggregates. Lysates from TauRD-YT cells containing aggregates were incubated with increasing concentrations of Clu (Supplementary Fig. 4d), similar to when present during aggregation (Fig. 1c). Thus, Clu may act on preexistent fibrils or on aggregate species present in equilibrium.

The use of heparin in seed production results in a non-
physiological conformation of Tau fibrils. To exclude that the observed effect on TauRD seeding is dependent on heparin, we, therefore, investigated the effect of Clu on seeding by TauRD aggregates produced in cells. Cells were lysed under mild conditions in the presence of non-ionic detergent (0.05% Triton X-100) without sonication to preserve the structural properties of the aggregates. Lysates from TauRD-YT cells containing aggregates were incubated with increasing concentrations of Clu (Supplementary Fig. 4d), similar to when present during aggregation (Fig. 1c). Thus, Clu may act on preexistent fibrils or on aggregate species present in equilibrium.

The use of heparin in seed production results in a non-
physiological conformation of Tau fibrils. To exclude that the observed effect on TauRD seeding is dependent on heparin, we, therefore, investigated the effect of Clu on seeding by TauRD aggregates produced in cells. Cells were lysed under mild conditions in the presence of non-ionic detergent (0.05% Triton X-100) without sonication to preserve the structural properties of the aggregates. Lysates from TauRD-YT cells containing aggregates were incubated with increasing concentrations of Clu (Supplementary Fig. 4d), similar to when present during aggregation (Fig. 1c). Thus, Clu may act on preexistent fibrils or on aggregate species present in equilibrium.
Clusterin enhances the seeding potency of FL Tau aggregates. a Effect of Clu on the kinetics of aggregation of full-length (FL) Tau (10 μM) monitored by ThT fluorescence. FL Tau/Clu molar ratio was 1:1. Averages ± SEM (n = 5 independent experiments). arb.units, arbitrary units. b Formation of seeds in FL Tau aggregation reactions without (black) or with Clu (10 μM, red) as described in (a). Reporter cells were transfected with aggregation reactions containing 183 ng (solid lines) or 4.6 ng (dashed lines) FL Tau and 205 ng (solid lines) or 5.1 ng (dashed lines) Clu, respectively (molar ratio Clu: FL Tau 1:1). Averages ± SEM (n = 3 independent experiments). c Representative fluorescence microscopy images of TauRD-YT cells seeded with FL Tau aggregation reactions (183 ng FL Tau) after reaching the plateau of aggregation (10 days, (a)). TauRD-YFP and DAPI nuclear staining signals are shown in yellow and red, respectively. Scale bar, 20 μm. d Fold change of seeding potency of FL Tau aggregation reactions containing Clu (red) compared to control reactions without Clu (gray). Bar graphs represent the average slope ± SEM (n = 3 independent experiments) from the linear regression analyses described in Supplementary Fig. 5a. *p < 0.05 (p = 0.0106) by two-tailed Student’s t-test. Lipofectamine was used as a transfection reagent. e, f Seeded aggregates of FL Tau-YT contain phospho-Tau. (n = 3 independent experiments) e Fluorescence microscopy images of FL Tau-YT cells seeded with TauRD aggregates. FL Tau-YFP and immunostaining of phospho-Tau (p Tau, AT8 antibody) are shown in yellow and red, respectively. The AT8 antibody recognizes Tau phosphorylation at both serine 202 and threonine 205 and is widely used to detect Tau paired helical filaments. DAPI nuclear staining signal is additionally shown in blue in the merge. Arrowheads indicate aggregates. The small arrow indicates a cell without aggregates. Scale bar, 10 μm. f Representative immunoblot analysis showing Tau (Tau/Repeat Domain antibody) and phospho-Tau (p Tau, AT8 antibody) in FL Tau-YT cell lysates from cells treated with or without TauRD seeds. Molecular weight (MW) standards are indicated. g Clu enhances the seeding potency of FL Tau aggregates formed in FL Tau-YT cells. Whole-cell lysates of FRET-positive (pos.) FL Tau-YT cells were incubated without or with Clu (molar ratios FL Tau-YT:Clu 1:1 and 1:10). Bar graphs represent the average slope ± SEM from the linear regression analyses described in Supplementary Fig. 5e. Data represent the mean ± SEM (n = 3 independent experiments). **p < 0.01 (−Clu vs. FL Tau-YT:Clu 1:1 p = 0.0058); ***p < 0.001 (−Clu vs. FL Tau-YT:Clu 1:10 p = 7.9 × 10−4) by one-way ANOVA with Bonferroni post hoc test. Lipofectamine was used as a transfection reagent.

(Fig. 1f). A strong increase in seeding potency (up to ~60-fold) was observed upon Clu addition (Fig. 1f and Supplementary Fig. 4e). In this case, higher amounts of Clu relative to TauRD (up to ~20-fold excess) were effective, presumably due to lysate proteins competing for Clu binding with the aggregates.

To exclude the possibility that our findings are limited to the isolated repeat domain of Tau, we next performed experiments with full-length Tau (FL Tau 2N4R) aggregates as seeds in cells expressing either TauRD or FL Tau FRET constructs. As expected, in vitro amyloid formation of FL Tau was slow42 (t1/2 ~ 4.6 days, Fig. 2a) and was further delayed in the presence of Clu (t1/2 ~ 6.6 days, Fig. 2a). Clu dramatically enhanced (~55-fold) the potency of FL Tau aggregates to seed TauRD-YT aggregates (Fig. 2b–d and Supplementary Fig. 5a). An even greater potentiation of FL Tau seeds (~100-fold) was observed with cells stably co-expressing FL Tau (P301L/V337M) fusion proteins with YFP or mTurquoise2 (FL Tau-YT cells), forming FL Tau aggregates (Supplementary Fig. 5b–d).

As Tau aggregates in the patient brain typically contain highly phosphorylated Tau48, we also tested the effect of Clu using cell lysates containing phosphorylated FL Tau aggregates as seeds36,49 (Fig. 2e–g and Supplementary Fig. 5e). Phosphorylated FL Tau-YT aggregates were obtained by seeding naïve FL Tau-YT cells with Tau RD aggregates formed in vitro (Fig. 1b). Phosphorylation of the resulting aggregates was confirmed by the AT8 antibody36,49 (Fig. 2e, f). Clu enhanced the seeding competence of these phospho-Tau aggregates up to threefold (Fig. 2g and Supplementary Fig. 5e). The lower effect of Clu on the seeding potency of cellular FL Tau aggregates compared to Tau RD aggregates (Figs. 1f and 2g) may be due to the “fuzzy coat” around the core of FL Tau fibrils30, which may limit Clu binding, or to differential posttranslational modifications, including phosphorylation.
Physical interaction between Clu and phospho-FLTau-containing aggregates was confirmed by co-immunoprecipitation using the AT8 antibody (Supplementary Fig. 5f). However, the AT8 antibody also precipitated unmodified, apparently co-aggregated FL-Tau and thus a direct interaction of Clu with phospho-Tau remains to be demonstrated.

In summary, Clu robustly enhances the potency of TauRD and FL.Tau aggregates to seed aggregation in cells expressing TauRD or FL.Tau. This effect is independent of whether Clu is present during initial aggregation or added to preformed aggregates produced in vitro or in cells.

Clusterin stabilizes oligomeric Tau seeds. To biochemically characterize the seeding competent TauRD species, we fractionated the in vitro aggregation reaction by centrifugation. In the absence of Clu, aggregated TauRD was apparently insoluble after 1 h of aggregation (Fig. 3a, upper panel). When increasing amounts of the supernatant fraction (∼x10, ∼x20, ∼x30 the amount loaded in the upper panel (Fig. 3a, lower panel)) were analyzed, we found ~1.5% of total TauRD to be soluble. The amount of soluble TauRD increased to ~14% of the total in the presence of equimolar Clu (Fig. 3a, lower panel). Approximately 13% of Clu was recovered in the pellet fraction (Fig. 3a, upper panel), suggesting a weak association with insoluble fibrils. The seeding competence of the soluble and insoluble TauRD was compared by measuring the fraction of FRET positive cells per ng of TauRD in the seed material as determined by quantitative immunoblotting (Fig. 3a). The soluble fraction of the aggregation reaction had a much higher specific seeding capacity in the cellular assay than the resuspended pellet (Fig. 3b), indicating that soluble TauRD species are more seeding competent51,52. Notably, the specific seeding activity of the Clu-containing supernatant was ~40-fold higher than that in the absence of Clu (Fig. 3b), suggesting that Clu not only increased the amount of soluble TauRD aggregates but also enhanced their intrinsic seeding activity. To test this possibility directly, we added Clu to the seeding-active, soluble fraction of a TauRD aggregation reaction generated in the absence of Clu. This resulted in an approximately fivefold increase in seeding capacity (Fig. 3c). Thus, Clu not only increases the amount of soluble TauRD seeds when present during aggregation but also enhances the intrinsic seeding activity of pre-existing, soluble TauRD species.

The addition of Clu 6 h after initiating TauRD aggregation still produced a small, but detectable amount of soluble TauRD (Supplementary Fig. 6a), in line with enhanced seeding when Clu is added to preformed aggregates (Fig. 1f, Fig. 2g, and Supplementary Fig. 4d). Surprisingly, a small amount of soluble TauRD was also generated when Clu was added to the resuspended pellet fraction (Supplementary Fig. 6b). In both cases, ~4–5% of total Clu is associated with the insoluble TauRD aggregates (Supplementary Fig. 6a–c). Thus, Clu binding to preexistent, insoluble TauRD aggregates generates seeding competent, soluble TauRD.

To determine when during the aggregation reaction Clu interacts with TauRD, we utilized dual-color fluorescence cross-correlation spectroscopy (dcFCCS). To this end, we generated the cysteine mutant I260C in otherwise cysteine-free TauRD and labeled the protein with Alexa Fluor 532 (TauRD-A532). Residue 260 is situated outside the fibril core44,53. Both Clu chains were labeled N-terminally with Alexa Fluor 647 (Clu-A647). Labeled TauRD at 1 µM was added to an aggregation reaction of unlabeled TauRD (9 µM). Labeling did not substantially affect aggregation kinetics or seeding properties of the resulting aggregates (Supplementary Fig. 7a, b, labeled TauRD; Fig. 1b, c, unlabeled TauRD) and labeled Clu (1.25 µM) enhanced seeding to a similar extent as observed with the unlabeled proteins (Supplementary Fig. 7b, labeled proteins; Supplementary Fig. 4b, unlabeled proteins; TauRD:Clu 8:1). A clear fluorescence cross-correlation signal between TauRD-A532 and Clu-A647 was only detectable at 1.5–2 h after initiation of aggregation (Fig. 3d), when ThT-positive and seeding competent TauRD species had formed (Fig. 3e). Apparently, Clu does not bind to monomeric TauRD but interacts with TauRD aggregates. Based on their diffusion time (~16.5 ± 1.3 ms; Supplementary Fig. 7c), the TauRD/Clu complexes formed are on average ~5000 kDa in size, equivalent to Clu bound to TauRD fibrils comprising ~300 TauRD units.

To characterize the interaction of Clu with the soluble, highly seeding competent TauRD, we next measured the FCCS signal between Clu-A647 and TauRD-A532 in the soluble fraction of aggregation reactions. Clu did not interact with monomeric TauRD (0 h; Fig. 3f), but strong interaction with soluble TauRD was detected after aggregation had reached the plateau phase (6 h; Fig. 3f, Supplementary Fig. 7a). The average size of the soluble TauRD/Clu complexes was ~320 kDa (diffusion time ~4.95 ± 19.19 ms; Supplementary Fig. 7c), consistent with one or more Clu stabilizing small TauRD oligomers. The interaction between Clu and TauRD is dynamic, as the FCCS signal was rapidly reduced upon the addition of excess unlabeled Clu (Supplementary Fig. 7d, e). Together these data show that Clu binds and stabilizes soluble Tau oligomers, either when present during ongoing aggregation or when added to preexistent aggregates. These soluble species are highly competent for cellular uptake and seeding of endogenous aggregation.

Seeding competent Tau/Clu species enter cells by endocytosis. To test whether cells incorporate TauRD/Clu seeds by endocytosis, as described for Tau alone54–58, we incubated TauRD-YT cells during seeding with Bafilomycin, an inhibitor of the lysosomal H+ ATPase59, or leucyl-leucyl-O-methyl ester (LLOME), an agent that accumulates in acidic membrane compartments inducing their rupture60. A reduced amount of Tau/Clu aggregates compared to TauRD aggregates was applied to the TauRD-YT cells in order to obtain comparable seeding efficiencies. Both compounds increased seeding by TauRD and TauRD/Clu aggregates (in the absence of transfection reagent) to a similar extent and in a concentration-dependent manner (Fig. 4a, b). Thus, seed internalization occurs via endocytosis, with a fraction of seed material presumably undergoing lysosomal degradation in the absence of Bafilomycin or LLOME.

Heparan sulfate proteoglycans (HSPGs) are involved in cell surface binding and internalization of Tau seeds61, as well as in the clearance of aberrant extracellular proteins mediated by Clu51. To test the role of this internalization mechanism in Tau/Clu seed uptake, TauRD-YT cells were incubated with increasing concentrations of heparin, an HSPG blocker, and treated with Tau aggregates formed in the presence or absence of Clu. In both cases the number of FRET positive cells strongly decreased in a manner dependent on heparin concentration (Fig. 4c), suggesting that TauRD and TauRD/Clu aggregates are internalized via HSPGs. We next investigated whether Clu and TauRD seeds enter cells together. To this end, we incubated HEK293T cells stably expressing TauRD (P301L/V337M)-mTurqoise2 (TauRD-T cells) with fluorescence-labeled soluble TauRD-A532/Clu-A647 seeds (Supplementary Fig. 7a, b) for 24 h. Clu-A647 and TauRD-A532 entered the cells as a complex (Fig. 4d–g and Supplementary Fig. 8) and in several cases co-localized with CHMP2a (Fig. 4d and Supplementary Fig. 8a), a subunit of the endosomal sorting complexes required for transport (ESCRT) machinery62. Some seed material also co-localized with the...
danger receptor galectine-8 (GAL-8) (Fig. 4e and Supplementary Fig. 8b), a marker of ruptured endomembranes53. After incubation for a further 24 h, we observed incorporation of TauRD-A532 and Clu-A647 into aggregates formed by endogenous TauRD-mTurquoise2 (TauRD-mTurq) (Fig. 4f, g and Supplementary Fig. 8c–f). The level of colocalization of the three fluorophores in seeded TauRD-mTurq aggregates was quantiﬁed by plotting their relative intensity proﬁle, extracted from lines manually drawn in mid focal planes (Fig. 4g and Supplementary Fig. 8d, f). Co-localization of Tau-A532/Clu-A647 seeds with
danger receptor galectine-8 (GAL-8) (Fig. 4e and Supplementary Fig. 8b), a marker of ruptured endomembranes53. After incubation for a further 24 h, we observed incorporation of TauRD-A532 and Clu-A647 into aggregates formed by endogenous TauRD-mTurquoise2 (TauRD-mTurq) (Fig. 4f, g and Supplementary Fig. 8c–f). The level of colocalization of the three fluorophores in seeded TauRD-mTurq aggregates was quantiﬁed by plotting their relative intensity proﬁle, extracted from lines manually drawn in mid focal planes (Fig. 4g and Supplementary Fig. 8d, f). Co-localization of Tau-A532/Clu-A647 seeds with

Clusterin interferes with seeding of α-Synuclein aggregates. To test whether the effect of Clu on aggregate seeding is Tau speciﬁc, we next performed experiments with α-Synuclein (α-Syn), which
undergoes prion-like aggregate propagation in Parkinson’s disease (PD)\(^1\). Clu also delayed the formation of Th\(\beta\)-positive aggregates of \(\alpha\)-Syn with early-onset-\(\alpha\)-Syn mutation A53T), even at very low molar ratios to \(\alpha\)-Syn\(^1\)\(^4\)\(^6\)\(^4\) (Fig. 5a). However, as in the case of Tau, \(\alpha\)-Syn fibrils were nevertheless assembled (Supplementary Fig. 9a, b) and similar amounts of aggregates were generated in the absence of Clu, as demonstrated by filtration assay (Supplementary Fig. 9b). Seeding competent \(\alpha\)-Syn species formed slightly before the accumulation of Th\(\beta\)-positive aggregates, as analyzed in HEK293T cells stably expressing GFP-\(\alpha\)-Syn(A53T) (Fig. 5a–c). Lipofectamine was used to render seed uptake independent of cellular machinery for internalization. In the absence of Clu, seeding with \(\alpha\)-Syn(A53T) resulted in ~60% of cells with aggregates. Seeding was markedly suppressed by sub-stoichiometric amounts of Clu at ratios of Clu-\(\alpha\)-Syn of 1:1,000 to 1:100 (Fig. 5b, c). This effect was also confirmed with

**Fig. 4 Uptake of Clusterin-associated, seeding competent Tau aggregates by endocytosis.**

a, b Effect of inhibition of lysosomal H\(^+\) ATPase by Bafilomycin A1 (a) and permeabilization of acidic membrane compartments with LLOME (b) on seeding potency of TauRD aggregates formed with or without Clu. TauRD-YT cells were treated with increasing concentrations of the inhibitors in combination with 500 ng TauRD seeds or 200 ng TauRD/Clu seeds without a transfection reagent. FRET positive (pos.) cells were analyzed after 48 h. Data represent the mean ± SEM (n = 5 independent experiments). Significance is represented relative to control cells treated with the vehicle without Bafilomycin A1 or LLOME. **p < 0.01, ***p < 0.001 by two-way ANOVA with Sidak post hoc test. (TauRD vehicle vs. TauRD 10 nM Bafilomycin A1 p = 2.9 × 10\(^{-5}\); TauRD/Cliu vehicle vs. TauRD/Cliu 10 nM Bafilomycin A1 p = 3.9 × 10\(^{-4}\); TauRD vehicle vs. TauRD 500 µM LLOME p = 4 × 10\(^{-10}\); TauRD/Cliu vehicle vs. TauRD/Cliu 250 µM LLOME p = 0.002; TauRD/Cliu vehicle vs. TauRD/Cliu 500 µM LLOME p < 1 × 10\(^{-15}\)). c Heparan sulfate proteoglycan (HSPG) mediated internalization of TauRD and TauRD/Clu aggregates. TauRD-YT cells were treated with increasing concentrations of heparin in combination with 1 µg TauRD seeds or TauRD/Clu seeds without a transfection reagent. FRET positive (pos.) cells were analyzed after 48 h. Data represent the mean ± SEM (n = 4 independent experiments). Significance is represented relative to control cells treated with the vehicle without heparin. **p < 0.01, ***p < 0.001 by two-way ANOVA with Sidak post hoc test. (TauRD vehicle vs. TauRD 2 µg/ml heparin p = 0.0017; TauRD vehicle vs. TauRD 20 µg/ml heparin p = 3 × 10\(^{-4}\); TauRD vehicle vs. TauRD 200 µg/ml heparin p = 2.5 × 10\(^{-4}\); TauRD/Cliu vehicle vs. TauRD/Cliu 0.2 µg/ml heparin p = 4.4 × 10\(^{-6}\); TauRD/Cliu vehicle vs. TauRD/Cliu 2 µg/ml heparin, TauRD/Cliu vehicle vs. TauRD/Cliu 20 µg/ml heparin and TauRD/Cliu vehicle vs. TauRD/Cliu 200 µg/ml heparin p < 1 × 10\(^{-15}\)]. d, e Colocalization of TauRD/Cliu seeds (TauRD-A532 in green, Cliu-A647 in red) (arrowheads) with the endocytosis marker CHMP2a (magenta) (d) and the marker of ruptured endomembranes, galectin-8 (GAL8; magenta) (e) in TauRD-T cells. A representative result of confocal imaging is shown. The cell outline is indicated by a white dashed line. Scale bar, 10 µm. (n = 3 independent experiments). f, g Colocalization of TauRD/Cliu seeds with endogenous TauRD-mTurquoise2 (TauRD-mTurq) aggregates. f A representative slice from a confocal stack is shown (scale bar, 10 µm) where cells are outlined with a white dashed line. One aggregate region, marked with a square in the slice, is represented by volume rendering (1 µm scale bars indicated by arrows). Channels are also displayed separately. TauRD-A532 seed in green, Cliu-A647 in red, endogenous TauRD aggregate in turquoise. (n = 3 independent experiments). g Quantification of relative fluorescence intensity in the aggregate shown in the inset. TauRD-mTurq (blue), TauRD-A532 (green) and Cliu-A647 (red). The colocalization line profile on a mid focal plane (inset image) along the white arrow is shown. Scale bar, 1 µm.
neuroblastoma SH-SY5Y cells stably expressing GFP-α-Syn (A53T) (Supplementary Fig. 9c, d), however higher amounts of seeding material (10 µg α-Syn for SH-SY5Y cells, 200 ng α-Syn for HEK293T) were necessary to obtain comparable seeding efficiencies. The observed decrease in α-Syn seeding in the presence of Clu does not appear to be due to reduced formation of insoluble fibrils (Supplementary Fig. 9b), but rather to changes in the effective concentration of other seeding competent species. Note that Clu increased the seeding potency of TauRD aggregates approximately twofold even when used at a low Clu:TauRD ratio of 1:100 (Supplementary Fig. 9e). Thus, while having similar effects on aggregation kinetics in vitro, Clu has opposite effects on the seeding activity of Tau and α-Syn aggregates in the cellular assay, enhancing the former and suppressing the latter.

To test the effect of Clu on α-Syn seeding in cells with the unperturbed plasma membrane, we omitted the transfection reagent. Under these conditions, Clu still clearly suppressed α-Syn seeding (Fig. 5d). Consistent with the notion that HSPGs are also involved in α-Syn internalization, aggregate seeding by α-Syn and α-Syn/Clu was completely suppressed in the presence of heparin (200 µg/ml, Fig. 5d). Thus, HSPGs participate in the internalization of both α-Syn and TauRD seeds, independent of the presence of Clu.

**Clusterin enhances Tau seeding and toxicity in neurons.** To extend our findings to neuronal cells, we used primary mouse neurons transduced with TauRD (residues 244-372, P301L/V337M) fused to YFP (TauRD-Y). We incubated neurons with either aggregates of TauRD, TauRD/Clu, or buffer control without transfection reagent and after 4 days monitored the formation of aggregates of endogenous TauRD-Y by fluorescence microscopy (Fig. 6a–c). Incubation with TauRD seeds alone induced the formation of TauRD-Y inclusions in ~12% of neurons, while TauRD/Clu induced aggregation in ~45% of neurons (Fig. 6b, c). While there was no difference in neuronal viability 4 days after seeding, treatment with TauRD/Clu resulted in a ~30% decrease in viability 7 days after seeding compared to cells seeded with
The structural properties of these Tau species seeding aggregation of endogenous Tau upon uptake by recipient and stabilizes soluble Tau species in a state highly competent in Clu can modulate the Tau component of AD by accelerating Tau aggregation and toxicity, our results suggest the possibility that previous investigations focused on the effects of Clu on Aβ aggregation and toxicity, data represent the mean ± SEM (n = at least 800 cells examined over 3 independent experiments). *p < 0.01 (p = 0.0038) by two-tailed Student’s t-test. d Viability of neurons expressing TauRD-YFP at 4 and 7 days after incubation with TauRD (black) or TauRD/Clu (red) seeds (11 ng TauRD). Data from MTT assays are normalized to the control sample incubated with PBS (100%) and represent the mean ± SEM (n = 5 independent experiments). *p < 0.05 (p = 0.034); n.s., not significant (p = 0.99) by two-way ANOVA with Sidak post hoc test. e Hypothetical model for the role of Clu (red) in amyloid seeding of Tau (brown) and α-Synuclein (α-Syn) (blue). Tau and α-Syn seeding competent species, partially produced by chaperone-mediated disaggregation, are released to the extracellular space from cells containing amyloid aggregates (gray). Clu (red) interacts with these species increasing seeding competence for Tau upon uptake by neighboring cells via the endosomal pathway. In contrast, Clu inhibits seeding for α-Syn. Tau/Clu seeds efficiently template aggregation of endogenous Tau, resulting in cytotoxicity, while α-Syn/Clu seeds are unable to template aggregation of endogenous α-Syn.

Discussion
Extensive evidence links Clusterin with AD, and both neuroprotective and pathology-enhancing functions have been ascribed to this abundant extracellular chaperone15–27,31–33. While these previous investigations focused on the effects of Clu on Aβ aggregation and toxicity, our results suggest the possibility that Clu can modulate the Tau component of AD by accelerating Tau aggregate seeding. Using cellular models, we show that Clu binds and stabilizes soluble Tau species in a state highly competent in seeding aggregation of endogenous Tau upon uptake by recipient cells (Fig. 6e). The structural properties of these Tau species remain to be defined. Within the cell, clearance of aggregates by intracellular chaperones such as the Hsp70 system65 may generate seeding competent Tau species, which could be a substrate for stabilization by Clu after release into the extracellular space (Fig. 6e). Although the exact mechanism by which Tau species are released from cells remains unclear7, seeding competent, high-molecular-weight Tau that could be acted on by Clu, has been detected in the cerebrospinal fluid of AD patients56. TauRD enters target cells in complex with Clu, apparently by endocytosis (Fig. 6e). However, TauRD/Clu seeds induce vesicle rupture and escape from endosomes to the cytosol, as described for Tau alone and other amyloidogenic proteins54,58 (Fig. 6e), enabling them to induce aggregation of endogenous Tau. Thus, Clu fails to mediate efficient lysosomal degradation of Tau, in contrast to other Clu-bound misfolded proteins31,32. Seed uptake and permeation of endolysosomal membranes may be facilitated by the relatively small size of the Tau species stabilized by Clu.

In contrast to a possible role of extracellular Clu in promoting Tau pathology, intracellular Clu, accumulating under stress conditions9, may be protective by interfering with de novo Tau aggregation. The latter activity would be consistent with a recent study reporting colocalization of Clu with intracellular Tau tangles in the brain of mice overexpressing human Tau (P301L) and exacerbated Tau pathology in Clu knock-out animals40. However, aggregate spreading was not explicitly assessed. It also remains to be investigated whether other apolipoproteins including ApoE, which also influences Tau pathology67–70 may compensate for the loss of extracellular Clu1–74. Whether Clu ultimately delays or promotes Tau pathology may depend on the stage of the disease and multiple other factors.

The seeding-enhancing effect of Clu appears to be Tau specific. In the case of α-Syn, another amyloidogenic protein that undergoes prion-like aggregate spreading4, Clu blocked both aggregation and seeding (Fig. 6e), consistent with recent observations64,75,76. Indeed, Clu is upregulated in PD and other synucleinopathies77. Interestingly, progressive PD in older patients is often associated with mixed brain pathologies,
including Tau aggregation, raising the possibility of a tug of war between protective functions of Clus and collateral damage incurred. Thus, the ability of Clus to modulate transcellular Tau aggregate seeding may be broadly relevant in understanding the progressive nature of neurodegenerative pathologies.

**Methods**

**Plasmids.** The N1-TauRD (P301L/V337M)-EYFP construct and the mTurquoise2 plasmids were gifts from Marc Diamond and Michael Davidson, respectively (Addgene #54843). The N1-TauRD (P301L/V337M)-mTurquoise2 plasmid for TauRD-mTurquoise2 expression in HEK293T cells was constructed by cloning the TauRD sequence (Tau amino acids 244-371, containing the disease-related mutations P301L and V337M) from N1-TauRD (P301L/V337M)-EYFP into the N1-TauRD-mTurquoise2N1 plasmid using the EcoRI and NbH II restriction sites. The N1-FLTau (0N4R, P301L/V337M)-EYFP and N1-FLTau (0N4R, P301L/V337M)-mTurquoise2 plasmids were constructed by substitution of the TauRD (P301L/V337M) sequence from N1-TauRD (P301L/V337M)-EYFP and N1-TauRD (P301L/V337M)-mTurquoise2 plasmids by the FLTau (0N4R, P301L/V337M) sequence from the pFR5-EYFP-Tau P301L plasmid (a gift from Karen Asher, Addgene #46098) after introducing the mutation V337M, by Gibson assembly using the Gibson Assembly Master Mix (New England Biolabs). The linker between Tau and the fluorescent protein was composed of 21 amino acids in all cases (YPGQLGQSTVRDPPAPVAT channels for fluorescent protein with fibril formation. The Flp-in N1-TauRD (P301L/V337M)-EYFP construct was made by inserting the TauRD sequence (Tau amino acids 244-371) in pently by cloning the pHUE-TauRD (C291A/P301L/C322A/V337M) containing cysteine-free TauRD and pHUE-TauRD (C291A/P301L/C322A/V337M) containing TauRD-L260C for fluorescent labeling was constructed by mutagenesis of the pHUE-TauRD (P301L/V337M) plasmid using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs).

The Tau/PTEN29 plasmid was used for wild-type FLTau (2N4R) expression and purification was a gift from Peter Klein (Addgene plasmid #16316). pFhSynW2 and the pVsVg packing plasmids used for lentivirus production was a gift from Hilal Lashuel (Addgene plasmid #105727).

The pB-T-PAF vector was a gift from James Rini. The pB-T-PAF-CliStrep plasmid, which was used for Clus (Clu) (Clu followed by a strep tag, WSHPPQEQK) expression and purification, was constructed by cloning the Clu cDNA sequence amplified from human embryonic kidney 293T (HEK293T) cells into the pB-T-PAF vector. RNA was extracted from the cell pellet using the RNasey Mini kit (Qiagen). CDNA was then synthesized using the Quantitect Reverse Transcription Kit (Qiagen) and the Clu cDNA amplified by PCR. The PCR product was then digested and cloned into the pB-T-PAF vector.

Cloning pFhSynW2 TauRD (P301L/V337M)-EYFP for the expression and purification of recombinant α-Syn was a gift from Halil Lashuel (Addgene plasmid #105722) and EGFp-α-SynA53T plasmid for α-Syn expression in HEK293T and SH-SY5Y cells was a gift from David Rubinshtein (Addgene plasmid #40823).

**Cell lines.** A HEK293-EBNA suspension cell line stably expressing the recombinant protein Clus/Strep-tag (HEK293 Clintag) was generated by using a piggyBac transposon-based expression system employing the pB-T-PAF-CliStrep plasmid.

The monoclonal HEK293T cell line stably expressing two different constructs, TauRD N-terminally fused to either EYFP or mTurquoise2 (TauRD-NT cell line) was generated by transfecting HEK293T cells with N1-TauRD (P301L/V337M)-EYFP and N1-TauRD (P301L/V337M)-mTurquoise2 plasmids using Lipofectamine 3000 (Thermo Fisher Scientific). Cells expressing the constructs were selected by 1 mg/ml G418 antibiotic (Gibco) selection, and monoclonal cell lines were generated by isolating cells expressing both TauRD fusion proteins in a 96 well-plate by cell sorting with a BD FACS Aria III (BD Biosciences) flow cytometry. FRET signal detection with high efficiency was selected. In a similar way, a monoclonal HEK293T cell line stably expressing FLTau (0N4R, P301L/V337M)-EYFP was used for immunoblotting. A cell line expressing both TauRD and mTurquoise2 was generated by transfecting HEK293T cells with the pHUE-TauRD (C291A/P301L/C322A/V337M) construct, which was used for immunoblotting. Neurons in 4-well plates were treated with 20 ng/ml G418 for 10 days. Neurons in 96-well plates were treated with 0.25% trypsin containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 15 µl 0.1% DNase I for 20 min at 37 °C. The enzymatic digestion was stopped by removing the supernatant and washing the tissue twice with a Neurobasal medium (B27 containing 5% FBS). The tissue was resuspended in resuspension solution and triturated to achieve a single cell suspension. Cells were spun at 150 x g, the supernatant was removed, and the cell pellet was resuspended in a Neurobasal medium with 2% B27 (Invitrogen), 1% L-Glutamine (Invitrogen), and 1% Penicillin/Streptomycin (Invitrogen). For immunofluorescence, neurons were cultured in 24-well plates on 13 mm coverslips coated with 1 mg/ml poly-D-Lysine (Sigma) and 1 µg/ml 1-laminin (Thermo Fisher Scientific) (100,000 neurons per well). For viability measurements, neurons were cultured in 96-well plates coated in the same (19,000 neurons per well). Lentiviral transduction was performed at DIV 10. Viruses were thawed and immediately added to a freshly prepared neurobasal medium to form a 1:1 mixture. Neurobasal medium was removed and replaced by fresh medium 24 h later.

**Lentivirus production.** HEK293T cells for lentiviral packaging (Lenti-X 293T cell line, Takara) were expanded to 70–85% confluency in DMEM Glutamax (4.9 g/l D-Glucose, - Pyruvate) supplemented with 10% FBS (Sigma), 1% G418 (Gibco), 1% NEAA (Thermo Fisher Scientific), and 1% HEPES (Biomol). Only low passage cells were used. For lentiviral production, a three-layered TS252 cell flask (Falcon) was seeded and cells were grown in a 2D culture medium. NEAAs were removed and the equivalent volume of virus-containing medium was added.

**Chemicals and cell treatments.** Baclofen (A1 was purchased from Invivogen. L-Leucyl-L-Leucine methyl ester (hydrochloride) (LLOME) was purchased from Cayman chemicals. Both compounds were dissolved in DMSO and small aliquots were stored at −20 °C until further use. For non-treated samples, DMSO alone was used as a control. Alexa Fluor 532 C5 maleimide and Alexa Fluor 647 N-hydroxysuccinimide (NHS) ester were purchased from Thermo Fischer Scientific and freshly dissolved in DMSO before protein labeling. Heparin sodium salt from porcine intestinal mucosa was purchased from Merck (H3393).

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.** Protein samples were boiled in SDS-PAGE sample buffer for 5 min. Protein samples were separated by electrophoresis on NuPAGE 4–12% Bis-Tris gel using MOPS buffer in MES running buffer (Thermo Fisher Scientific) at 180 V. Coomassie blue staining was performed with InstantBlue (Merck). For immunoblotting, proteins were transferred at 70 V for 2 h onto a nitrocellulose membrane (GE Healthcare) using a wet electrophoresis system (Bio-RAD). Membranes were blocked for at least 1 h with 0.05% TBS-Tween and 5% milk in Tris buffered saline to block nonspecific bindings. Immunodetected bands were visualized using mouse anti-Tau (Clone 8057) and Tau Repeat Domain antibody (TECAN, 2B11), anti-Tau-1 antibody (non-phosphorylated Tau, clone PC1iC6, Merck, MAB3420), Tau monoclonal antibody (TAU-5, NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-021-25060-1 | www.nature.com/naturecommunications
Total Tau, Thermo Fisher Scientific, MA-12808), phospho-Tau antibody (Ser202, Thr205, AT8, Thermo Fisher Scientific, MN1020), mouse monoclonal C19-antibody (Babraham Institute, CR-C) against recombinant anti-Tau antibody (EPBR291, abcamb, ab92548) and anti-alpha-Synuclein antibody (LR509, abcamb, ab27766). Conjugated goat-anti mouse immunoglobulin G (IgG)-horse radish peroxidase (HRP) (Merck, A4416) or anti-rabbit IgG (whole molecule)-peroxidase antibody produced in goat (Merck, A9169) were used as secondary antibody. Western Blotting Classic Wash Buffer or HRP substrate or Immobilon ECL Ultra Western HRP Substrate (Merck) were used for detection. Quantification by densitometry was performed with AIDA Image Analyzer v.4.27 (Elysia Raytest) or Image J (Rasband, W.S., National Institutes of Health, USA). Full scan sheets are provided in the Source Data file.

Protein purification. Seed aggregates for addition to cells were generated with purified recombinant cysteine-free TauRD (Tau residues 244-371, C291A/P301L) expressed in BL21(DE3) cells with an N-terminal His6-ubiquitin-fusion. The cell pellet from 6 l of culture was resuspended in 180 ml lysis buffer (50 mM MES-NaOH pH 6.5, 250 mM NaCl, 10 mM imidazole, 2 mM β-mercaptoethanol (βME)) supplemented with 1 mg ml⁻¹ lysozyme, Complete EDTA-free protease inhibitor cocktail (MERK) and benzamidine, and incubated while shaking at 4 °C for 30 min. Cells were lysed by ultrasonication, and the lysate was cleared by centrifugation (1 h, 40,000 × g at 4 °C). The supernatant was loaded onto a Ni-NTA column equilibrated with lysis buffer. The TauRD protein was further purified by size exclusion chromatography (SEC) on Superdex-75 in phosphate-buffered saline (PBS). For TauRD-I260C, the buffer used for SEC contained 1 mM tris(2-carboxyethyl) aminodithiol (TCEP) by SEC using a Nap5 (GE Healthcare) column pre-equilibrated with PBS. The column was washed with high salt buffer (50 mM Pipes-NaOH pH 6.5, 500 mM NaCl, 10 mM imidazole, 2 mM β-ME) and wash buffer (50 mM Pipes-NaOH pH 6.5, 250 mM NaCl, 50 mM imidazole, 2 mM β-ME), and His6-ubiquitin-TauRD was eluted with elution buffer (50 mM Pipes-NaOH pH 6.5, 50 mM NaCl, 250 mM imidazole, 2 mM β-ME). The eluted fractions were collected and the salt concentration was reduced by diluting the sample (1:5) with Pipes buffer (50 mM Pipes-NaOH pH 6.5, 2 mM β-ME), followed by incubation with Usp2 ubiquitin protease (0.5 mg overnight at 4 °C) in order to cleave the His-tag ubiquitin tag. The cleavage mixture was applied to a Source30S cation exchange column and the TauRD protein was eluted with a 0–500 mM NaCl gradient in 40 mM Pipes-NaOH pH 6.8, 20 mM NaCl, 10 mM imidazole, 2 mM β-ME. The eluted TauRD fractions were collected, and the salt concentration was reduced by diluting the sample (1:5) with Pipes buffer (50 mM Pipes-NaOH pH 6.5, 2 mM β-ME), followed by boiling. The lysate was cleared by centrifugation (1 h, 40,000 × g at 4 °C). The supernatant was loaded onto a Source30S cation exchange column and the TauRD protein was eluted with a 0–500 mM NaCl gradient. The TauRD protein was further purified by size exclusion chromatography (SEC) on Superdex-75 in phosphate-buffered saline (PBS). For TauRD-I260C, the buffer used for SEC contained 1 mM tris(2-carboxyethyl) phosphine (TCEP) in order to prevent the formation of disulfide bonds. Fractions containing pure protein were combined, aliquoted, and flash-frozen in liquid nitrogen for storage at −80 °C. A more detailed protocol can be found here. 

Fluorescence cross-correlation spectroscopy (dcFCCS). dcFCCS measurements were performed on a Micro Time 200 microscope (PicoQuant) as described previously. Samples were diluted 100-fold in PBS (from 10 μM to 10 nM each labeled protein) immediately before each measurement. A autocorrelation was recorded for 30 min. The diffusion time was obtained by fitting the curves with the triplet diffusion equation using Symptomatic 64. The confocal volume (V₀) was calibrated daily using Atto655 maleimide dye.

Estimation of the average molecular weight of Tau/Clu complexes. To estimate the average size of Tau/Clu complexes, we performed PCS measurements to determine the diffusion coefficient (D). Diffusion coefficients (D) were converted into hydrodynamic radii (R) via the Stokes–Einstein equation (Eq. (1)), where k is the Boltzmann constant, T is the temperature (in K) and η is the solvent viscosity. Second, the correlation between R and the chain length (in amino acid residues, N) (Eq. (3)) for elongated proteins was applied, and this chain length was converted to molecular weight by averaging a molar mass of amino acids of mₐ = 113 g/mol (relative to amino acid abundance in eukaryotic proteins).

Protein aggregation reactions and thiolaflavin-T (TTh) fluorescence measurements. Tau aggregation: 100 μM of Tau (Tauref or FLTau), 2.5 μM heparin, 2 mM MgCl₂ were incubated in the presence or absence of Clu at 37 °C with constant agitation (1000 rpm). Aliquots were removed at the indicated time points, flash-frozen in liquid nitrogen, and stored at −80 °C until measurement of TTh fluorescence and seeding activity. α-Syn aggregation: purified α-Syn (5 mg ml⁻¹, 330 μM) was centrifuged at 100,000 × g for 1 h. The supernatant was transferred into a new reaction tube and incubated in the presence or absence of Clu with constant agitation (1000 rpm) at 37 °C in a thermomixer. Aliquots were removed at the indicated time points and stored at −80 °C until measurement of TTh fluorescence and seeding activity. For monitoring amyloid formation by TTh fluorescence, aliquots from aggregation reactions of Tau or α-Syn were diluted 50 or 100-fold, respectively, into 20 μM TTh in PBS. Excitation and emission wavelengths were 440 nm and 480 nm, respectively. Measurements were performed with a FluoroMax-4 Spectrophotometer (HORIBA) using FluorEssence V3.9 (HORIBA). The emission signal was corrected with the reference signal of the lamp (S1/R1 wavelength, S: emission signal, R: reference signal) and by subtraction of the minimum data point. TTh kinetics were fitted using Sigma plot 14.0 (Sigmoidal dynamic fitting, sigmoid 3 parameter model). A more detailed protocol can be found here. 

Filter retardation assay. Different amounts of a-Syn aggregation reaction were diluted in PBS and subsequently applied onto a pre-wetted 0.2 μm pore size cellulose acetate membrane in a Hoefer slot-blot apparatus. The membrane was subsequently washed twice with 0.1% Triton-X 100. Immunodetection was performed as described above (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.)
Fractionation of in vitro aggregation reactions. TauRD and TauRD/Clu aggregation reactions were centrifuged at 16,100 x g and 4°C for 1 h. The supernatant of 2 µl centrifuged with 1 µl of 4°C for 30 min, and the resulting pellet was resuspended in the initial volume of PBS. The Tau and Clu content was quantified by immunoblotting.

Cell-based seeding assays. Seeding of HEK293T cells: 100,000 cells per well of the HEK293T reporter cell line (TauRD-YT or FLtau-YT) were dispensed into a 12-well plate. For subsequent fluorescence microscopy imaging, a coverslip was placed on the well. In total, 16–24 h later, Tau aggregates were transfected with lipofectamine 3000 (Thermo Fisher Scientific). Specifically, aggregate samples were mixed with 50 µl Opti-MEM (Gibco) and 1.6 µl lipofectamine 3000 reagents (Thermo Fisher Scientific) and incubated for 20 min at RT. The mixtures were added to the cells with 0.5 ml of fresh medium. When lipofectamine was not used, the aggregates were mixed with 0.5 ml of fresh medium and added directly to the cells replacing the medium. After 16–20 h (when using lipofectamine) or 48 h (without lipofectamine or when using the FLtau-YT reporter cells), cells were processed for FRET signal analysis by flow cytometry or fluorescence microscopy imaging. When heparin was used to block HSPGs receptors, 1 µg of TauRD or TauRD/Clu aggregates were pre-incubated with different concentrations of heparin in 0.5 ml medium for 16 h at 4°C. After incubation, mixtures were added to the cells by exchanging the medium and 48 h later the FRET signal of seeder aggregates was analyzed by flow cytometry.

Quantification of FRET positive cells by flow cytometry: cells were harvested with Trypsin Express Enzyme (Gibco), washed with PBS once, and resuspended in PBS for analysis with an Attune NxT Flow Cytometer (Thermo Fisher Scientific). To measure FRET and FRET fluorescence before and after medium exchange, cells incubated with 405 nm laser light and fluorescence was determined using 440/50 and 530/30 filters, respectively. To measure the YFP fluorescence signal, cells were excited at 488 nm and emission was recorded using a 530/30 filter. For each sample, 50,000 single cells were analyzed. Data processing was performed using FlowJo V9 and FlowJo V10 software (FlowJo LLC). After gating single cells, an additional gate was introduced to exclude YFP-only cells that show a false-positive signal in the FRET channel due to excitation at 405 nm.93 The FRET positive gate was set by plotting the FRET fluorescence signal versus the mTurquoise2 fluorescence signal using as reference non-seeded cells (Supplementary Fig. 10). A more detailed protocol and the original file can be found here, https://edmond.mpdl.mpg.de/nuclj/collection/3spV1M1Q0fmYeuYK50.

Fluorescence microscopy imaging: cells were fixed with 4% paraformaldehyde (PFA) in PBS for 10 min, washed with PBS, and permeabilized with 0.1% Triton-X100/PBS for 5 min. Nuclei were stained with NucBlue Fixed Cell ReadyProbes Reagent (Thermo Fisher Scientific) following the manufacturer’s instructions, and the coverslips were mounted with Dako fluorescence mounting medium (Agilent). Confocal imaging was performed as described below (immunofluorescence microscopy).

When lysates from cells containing aggregates were used as seed material, cell pellets were fixed with 1 µl Triton X-100/PBS, Complete EDTA-free protease inhibitor cocktail (MERK) and benzonase for 20 min on ice, total protein was quantified by Bio-Rad Protein Assay (Bio-Rad) and the amount of Tau protein was quantified by SDS-PAGE and immunoblotting using purified Tau as standard.

Seeding of primary neurons: 70 ng of TauRD aggregates mixed with fresh medium were added in the medium in the wells that were directly added to human primary neuronal cultures in 24-well plates at DIV 13. After 4 days of incubation (DIV 17), coverslips were processed as described below (immunofluorescence microscopy).

Seeding of α-Syn aggregation: HEK293T and SH-SY5Y cells expressing EGFP-α-Syn(A53T) were seeded in a 24-well plate containing a coverslip and α-Syn seeding experiments, HEK293T and SH-SY5Y cells were imaged at a Coherent microscopy (Thermo Fisher) in spinning disc mode with a 63x oil immersion objective. Images were acquired with MAPS software (Thermo Fisher) and afterward analyzed by Image J. Cells were counted manually and α-Syn accumulations with high fluorescence intensity and a diameter larger than 500 nm were considered aggregates. Fractions containing aggregates were calculated by using Origin Pro 2019b.

Immunofluorescence microscopy. Cells were fixed with 4% PFA/PBS for 10 min, washed with PBS, and permeabilized with 0.1% Triton-X100/PBS for 5 min. Blocking solution (8% RSA/PBS) was added for 30 min. Coverslips were transferred to a humid chamber and incubated overnight with the primary antibody diluted in 1% BSA/PBS (anti-phospho-Tau AT8, MN1020, Thermo Fisher Scientific, 1:100 dilution; anti-CHMP2, 10477-1-AP, Proteintech, 1:50 dilution; anti-Galectin 8, BAF174, Abcam, 1:50 dilution). Cells were then washed with the secondary antibody (Fab/α-Gold 5 nm, Mouse IgG Alexa Fluor 633 (A-21053); goat-anti Rabbit IgG Alexa Fluor 405 (A-31556), Thermo Fisher Scientific) diluted in 1% BSA/PBS (1:500) for 1 h, washed with 0.1% Tween-20/PBS and stained with NucBlue Fixed Cell ReadyProbes Reagent (Thermo Fisher Scientific) in the case of using anti-phosphorylated tau (AT8) antibody. Coverslips were mounted with Dako fluorescence mounting medium (Agilent). The confocal imaging was performed at the Imaging Facility of Max Planck Institute of Biochemistry, Martinsried, on a LEICA TCS SP8 AOS confocal laser scanning microscope (Wetzlar, Germany) equipped with a LEICA HCX PL APO 63×/NA1.4 oil immersion objective. Images were analyzed with Image J (Research W. S., National Institutes of Health, USA).

To detect colocalization of TauRD-A532/Cu-A647 with endogenous TauRD-

mturq aggregates, a series of z-stack images were acquired and then deconvolved using Huygens Essentials 19.10 (Scientific Volume Imaging). Three-dimensional volume renderings were generated using Velocity V6.3 (Quorum Technologies).

Aggregate formation in primary neurons: neurons were fixed at DIV 17 with 4% PFA/PBS for 20 min; remaining free groups of PFA were blocked with 50 mM ammonium chloride in PBS for 10 min at RT. Cells were rinsed once with PBS and permeabilized with 0.25% Triton X-100/PBS for 5 min. After washing with PBS, a blocking solution consisting of 2% BSA (w/v) (Roth) and 4% donkey serum (w/v) (Jackson ImmunoResearch Laboratories) in PBS was added for 30 min at RT. Coverslips were transferred to a light-protected humid chamber and incubated in anti-MAP2 (NB300-213, Novus Biologicals) primary antibody diluted in blocking solution (1:500) for 1 h. Cells were washed with PBS and incubated with the secondary antibody Alexa Fluor 647 AffiniPure Donkey Anti-Chicken IgG (Jackson ImmunoResearch Laboratories) for 1 h at RT. Coverslips were washed 3 times with washing buffer, transferred to a clean tube, and elution was performed by addition of 40 µl sample buffer/100 mM DTT to the beads and incubation at 70 °C for 10 min. 5 µl of 1.5 M Tris pH 8.8 was added to the elution after removal of the magnetic beads. Samples (13 µl of eluate and 2 µl of lysate as input) were subsequently analyzed by SDS-PAGE and immunoblotting as described above (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting).

Immunofluorescence staining: Cells were incubated with a CorreSight microscopy (Thermo Fisher) in spinning disc mode with a 63x oil immersion objective. Images were acquired with MAPS software (Thermo Fisher) and afterward analyzed by Image J. Cells were counted manually and α-Syn accumulations with high fluorescence intensity and a diameter larger than 500 nm were considered aggregates. Fractions containing aggregates were calculated by using Origin Pro 2019b.

Neuronal viability measurements. At DIV 13, 11 ng of TauRD aggregates mixed with fresh medium (one-tenth of the medium in the well) were directly added to the neuronal cultures in 96-well plates. Viability was determined with the MIT assay using 2-(4-methylthiazolyl-5)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma-Aldrich). The cell medium was exchanged for 100 µl of fresh medium. Subsequently, 20 µl of 5 mg ml⁻¹ MTT in PBS was added and incubated for 2–4 h at 37°C, 5% CO₂. Subsequently, 100 µl solubilizer solution (10% SDS, 45% dimethylformamide in water, pH 4.5) was added, and on the following day, absorbance was measured at 570 nm. Each condition was measured in six replicates per experiment and absorbance values were averaged for each experiment.

Electron microscopy. For negative stain analysis, continuous carbon grids (Quantifoil) were glow discharged using a plasma cleaner (DPC-XG, Harrick) for 30 s. Grids were incubated for 5 min with Tau samples, blotted and stained with...
0.5% uranyl acetate solution, dried, and imaged in a Titan Halo (FEI) transmission electron microscope using SerialEM.

In the case of α-Syn grids, were incubated for 1 min with the samples, blotted, and subsequently washed two times with water for 10 s. The blotted grids were stained with 0.5% uranyl acetate solution, dried, and imaged in a Polara cryo-electron microscope (FEI) at 300 kV using SerialEM.

Statistical analysis. Statistical analysis was performed with Sigma Plot 14.0 or GraphPrism6. Sample size (n) given in figure legends describe measurements taken from distinct, independent samples. Normality was assessed in all cases. Log-transformation was applied on Batfomycin treatment data to conform to normal distribution prior to statistical analysis. A two-tailed Student’s t-test was used for simple comparisons. One-way ANOVA with Bonferroni post hoc test or Two-way ANOVA with Sidak post hoc test was used for multiple comparisons.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
All data supporting the findings of this study are available within the manuscript. Other data are available from the corresponding author upon reasonable request. Source data are provided with this paper.

Received: 26 January 2021; Accepted: 16 July 2021;
Published online: 11 August 2021

References
1. Peng, C., Trojanowski, J. Q. & Lee, V. M. Protein transmission in neurodegenerative disease. Nat. Rev. Neuro. 16, 199–212 (2020).
2. Jacker, M. & Walker, L. C. Propagation and spread of pathogenic protein assemblies in neurodegenerative diseases. Nat. Neurosci. 21, 1341–1349 (2018).
3. Vaquer-Alicea, J. & Diamond, M. I. Propagation of protein aggregation in neurodegenerative diseases. Annu. Rev. Biochem. 88, 785–810 (2019).
4. Iadanza, M. G., Jackson, M. P., Hewitt, E. W., Ranson, N. A. & Radford, S. E. A new era for understanding amyloid structures and disease. Nat. Rev. Mol. Cell Biol. 19, 755–773 (2018).
5. Sibilla, C. & Bertolotti, A. Prion properties of SOD1 in amyotrophic lateral sclerosis and potential therapy. J Cell Biol 219, https://doi.org/10.1083/jcb.201911126 (2020).
6. Yeh, P. L., Wang, Y., Tom, L., Gonzalez, L. C. & Sheng, M. TREM2 binds to apolipoproteins, including APOE and CLU/APOJ, and thereby facilitates uptake of amyloid-beta by microglia. Neuron 91, 328–340 (2016).
7. Bell, R. D. et al. Transport pathways for clearance of human Alzheimer’s amyloid beta-peptide and apolipoproteins E and J in the mouse central nervous system. J. Cereb. Blood Flow Metab. 27, 909–918 (2007).
8. Chapman, K. & Albergoni, A. Association of plasma clusterin concentration with severity, pathology, and progression in Alzheimer disease. Arch. Gen. Psychiatry 67, 739–748 (2010).
9. Schrijvers, E. M., Koudstaal, P. J., Hofman, A. & Breteler, M. M. Plasma factors that protect against tau aggregation. Acta Neuropathol. 129, 643–652 (2020).
10. Braak, H., Thal, D. R., Ghebremedhin, E. & Del Tredici, K. Stages of the pathologic process in Alzheimer disease: age categories from 1 to 100 years. J. Neuropathol. Exp. Neurol. 70, 960–969 (2011).
11. Arriagada, P. V., Growdon, J. H., Hedley-Whyte, E. T. & Hyman, B. T. Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer’s disease. Neurology 42, 631–639 (1992).
12. Nelson, P. T. et al. Correlation of Alzheimer disease neuropathologic changes with cognitive status: a review of the literature. J. Neuropathol. Exp. Neurol. 71, 362–381 (2012).
13. Haieh, Y. C. et al. Tau-mediated disruption of the spliceosome triggers cryptic RNA splicing and neurodegeneration in Alzheimer’s disease. Cell Rep. 29, 301–316 e310 (2019).
14. Wotjas, A. M. et al. Clusterin ameliorates tauopathy in vivo by inhibiting fibril formation. Acta Neuropathol. Commun. 8, 210 (2020).
15. Holmes, B. B. et al. Proteopathic tau seeding predicts tauopathy in vivo. Proc. Natl Acad. Sci. USA 111, E4376–E4385 (2014).
16. Barghorn, S. & Mandelkow, E. Toward a unified scheme for the aggregation of tau into Alzheimer paired helical filaments. Biochemistry 41, 14885–14896 (2002).
17. von Bergen, M., Barghorn, S., Biernt, J., Mandelkow, E. & Mandelkow, E. Tau aggregation is driven by a transition from random coil to beta sheet structure. Biochim. Biophys. Acta 1739, 158–166 (2005).
18. Scheres, S. H., Zhang, W., Falcon, B. & Goedert, M. Cryo-EM structures of tau filaments. Curr. Opin. Struct. Biol. 64, 17–25 (2020).
19. Goedert, M. et al. Assembly of microtubule-associated protein tau into Alzheimer-like filaments induced by sulphonated glycosaminoglycans. Nature 383, 550–553 (1996).
20. Arosio, P., Knowles, T. P. & Linse, S. On the lag phase in amyloid fibril formation. Phys. Chem. Chem. Phys. 17, 7966–7818 (2015).
21. Mikel, D. A. et al. Mapping interactions with the chaperone network reveals factors that protect against tau aggregation. Nat. Struct. Mol. Biol. 25, 384–393 (2018).
48. Iqbal, K., Liu, F. & Gong, C. X. Tau and neurodegenerative disease: the story so far. Nat. Rev. Neuro. 12, 15–27 (2016).
49. Goedert, M., Jakes, R. & Vanmechelen, E. Monoclonal antibody AT8 recognises tau protein phosphorylated at both serine 202 and threonine 205. Neurosci. Lett. 189, 167–169 (1995).
50. Wischik, C. M. et al. Structural characterization of the core of the paired helical filament of Alzheimer disease. Proc. Natl Acad. Sci. USA 85, 4884–4888 (1988).
51. Dujardin, S. et al. Tau molecular diversity contributes to clinical heterogeneity in Alzheimer’s disease. Nat. Med. 26, 1256–1263 (2020).
52. Dujardin, S. & Hyman, B. T. Tau prion-like propagation: state of the art and current challenges. Adv. Exp. Med. Biol. 1184, 305–325 (2019).
53. Kumar, S. et al. Stages and conformations of the Tau repeat domain during aggregation and its effect on neuronal toxicity. J. Biol. Chem. 289, 20318–20332 (2014).
54. Chen, J. et al. Compromised function of the ESCRT pathway promotes endolysosomal escape of tau seeds and propagation of tau aggregation. J. Biol. Chem. 294, 18952–18966 (2019).
55. Falcon, B., Noad, J., McMahan, H., Randow, F. & Goedert, M. Galectin-8-mediated selective autophagy protects against seeded tau aggregation. J. Biol. Chem. 293, 2438–2451 (2018).
56. Rauch, J. N. et al. Tau internalization is regulated by 6-O sulfation on heparan sulfate proteoglycans (HSPGs). Sci. Rep. 8, 6382 (2018).
57. Holmes, B. B. et al. Heparan sulfate proteoglycans mediate internalization and propagation of tau neuropathic seeds. Proc. Natl Acad. Sci. USA 110, E1318–E1317 (2013).
58. Flavin, W. P. et al. Endocytic vesicle rupture is a conserved mechanism of cellular invasion by amyloid proteins. Acta Neuropathol. 134, 629–653 (2017).
59. Yoshimori, T., Yamamoto, A., Moriyama, Y., Futai, M. & Tashiro, Y. Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. J. Biol. Chem. 266, 17707–17712 (1991).
60. Thiele, D. L. & Lipsky, P. E. Mechanism of L-leucyl-L-leucine methyl ester-mediated killing of cytotoxic lymphocytes: dependence on a lysosomal thiol protease, dipetidyl peptidase I, that is enriched in these cells. Proc. Natl Acad. Sci. USA 87, 83–87 (1990).
61. Alavi Naini, S. M. & Soussi-Yanicostas, N. Heparan sulfate as a therapeutic target in tauopathies: insights from zebrafish. Front Cell Dev. Biol. 6, 163 (2018).
62. Hurley, J. H. ESCRTs are everywhere. EMBO J. 34, 2398–2407 (2015).
63. Thurston, T. L., Wandel, M. P., von Mellehin, N., Foegein, A. & Randow, F. Galectin 8 targets damaged vesicles for autophagy to defend cells against bacterial invasion. Nature 482, 414–418 (2012).
64. Whiten, D. R. et al. Single-molecule characterization of the interactions between extracellular chaperones and toxic alpha-synuclein oligomers. Cell Rep. 23, 3492–3500 (2018).
65. Nachman, E. et al. Disassembly of Tau fibrils by the human Hsp70 disaggregation machinery generates small seed-containing species. J. Biol. Chem. 295, 9676–9690 (2020).
66. Takeda, S. et al. Seed-dependent high-molecular weight tau species accumulates in the cerebrospinal fluid of Alzheimer’s disease mouse model and human patients. Ann. Neurol. 80, 355–367 (2016).
67. Wang, C. et al. Selective removal of astrocytic APOE4 strongly protects against tau-mediated neuronal degeneration and decreases synaptic phagocytosis by microglia. Neuroin. https://doi.org/10.1016/j.neuroni.2021.03.024 (2021).
68. Livitinschuk, A. et al. Apolipoprotein E reduction with antisense oligonucleotides decreases neurodegeneration in a tauopathy model. Ann. Neurol. 89, 952–966 (2021).
69. Shi, Y. et al. ApoE4 markedly exacerbates tau-mediated neurodegeneration in a mouse model of tauopathy. Nature 549, 523–527 (2017).
70. Zhao, N. et al. APOE epsilon2 is associated with increased tau pathology in primary tauopathy. Nat. Commun. 9, 4388 (2018).
71. Kwon, M. J. et al. Deficiency of clusterin exacerbates high-fat diet-induced insulin resistance in male mice. Endocrinology 155, 2089–2101 (2014).
72. Prettts, R. B. and his department for microscope support; Martin Spitaler, Markus Oster, and Gio-Gropp for the pHUE-TauRD (P301L/V337M) plasmid; Saurabh Gautam for FLTau production. Nat. Commun. 78. Buchman, A. S. et al. Progressive parkinsonism in older adults is related to the burden of mixed brain pathologies. Neurology 92, E1821–E1830 (2019).
73. Zhang, D. J., Lee, V. M. Y. & Trojanowski, J. Q. Parkinson’s disease dementia: convergence of alpha-synuclein, tau and amyloid-beta pathologies. Nat. Rev. Neurol. 14, 626–636 (2013).
74. Goedhart, J. et al. Structure-guided evolution of the Wnt signaling pathway: a molecular mechanism for lithium action. Dev. Biol. 185, 82–91 (1997).
75. Rospigliosi, C. C. et al. E6K Parkinson’s-linked mutation enhances C-terminal-to-N-terminal contacts in alpha-synuclein. J. Mol. Biol. 388, 1022–1032 (2009).
76. Filipini, A. et al. Extracellular clusterin limits the uptake of alpha-synuclein fibrils by murine and human astrocytes. Glu. https://doi.org/10.1002/glia.23920 (2020).
77. Vranova, H. P. et al. CSF markers of neurodegeneration in Parkinson’s disease. J. Neural Transm. 117, 1177–1181 (2010).
78. Buchman, A. S. et al. Progressive parkinsonism in older adults is related to the burden of mixed brain pathologies. Neurology 92, E1821–E1830 (2019).
79. Hedgepeth, C. M. et al. Activation of the Wnt signaling pathway: a molecular mechanism of chaperonin-assisted protein folding demonstrated at single-molecule level. J. Mol. Biol. 426, 2739–2754 (2014).
80. Wilkins, D. K. et al. Hydrodynamic radii of native and denatured proteins measured by pulse field gradient NMR techniques. Biochemistry 38, 16243–16431 (1999).
81. Banning, C. et al. A flow cytometry-based FRET assay to identify and analyse protein-protein interactions in living cells. PLoS ONE 5, e9344 (2010).
microscopy. I.D. supervised experiments with primary neurons. M.S.H. initially co-supervised the project and commented on the manuscript. A.B. co-supervised the project and contributed to experimental design. P.Y., A.B., and F.U.H. wrote the manuscript with input from the other authors.

**Funding**
Open Access funding enabled and organized by Projekt DEAL.

**Competing interests**
The authors declare no competing interests.

**Additional information**
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-25060-1.

Correspondence and requests for materials should be addressed to F.U.H.

Peer review information Nature Communications thanks Lukasz Joachimiak and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021