Distinct differences in prion-like seeding and aggregation between Tau protein variants provide mechanistic insights into tauopathies

Received for publication, August 30, 2017. Published, Papers in Press, December 19, 2017

Kevin H. Strang 1§, Cara L. Croft 1‡, Zachary A. Sorrentino 1‡, Paramita Chakrabarty 1‡§, Todd E. Golde 1§, and Benoit I. Giasson 1§

From the 1 Department of Neuroscience, the 2 Center for Translational Research in Neurodegenerative Disease, and the 3 McKnight Brain Institute, College of Medicine University of Florida, Gainesville, Florida 32610

Edited by Paul E. Fraser

The accumulation of aberrantly aggregated MAPT (microtubule-associated protein Tau) defines a spectrum of tauopathies, including Alzheimer’s disease. Mutations in the MAPT gene cause frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), characterized by neuronal pathological Tau inclusions in the form of neurofibrillary tangles and Pick bodies and in some cases glial Tau pathology. Increasing evidence points to the importance of prion-like seeding as a mechanism for the pathological spread in tauopathy and other neurodegenerative diseases. Herein, using a cell culture model, we examined a multitude of genetic FTDP-17 Tau variants for their ability to be seeded by exogenous Tau fibrils. Our findings revealed stark differences between FTDP-17 Tau variants in their ability to be seeded, with variants at Pro301 and Ser320 showing robust aggregation with seeding. Similarly, we elucidated the importance of certain Tau protein regions and unique residues, including the role of Pro301 in inhibiting Tau aggregation. We also revealed potential barriers in cross-seeding between three-repeat and four-repeat Tau isoforms. Overall, these differences alluded to potential mechanistic differences between wildtype and FTDP-17 Tau variants, as well as different Tau isoforms, in influencing Tau aggregation. Furthermore, by combining two FTDP-17 Tau variants (either P301L or P301S with S320F), we generated aggressive models of tauopathy that do not require exogenous seeding. These models will allow for rapid screening of potential therapeutics to alleviate Tau aggregation without the need for exogenous Tau fibrils. Together, these studies provide novel insights in the molecular determinants that modulate Tau aggregation.

Tauopathies are a spectrum of neurodegenerative diseases characterized by the presence of pathological inclusions composed of aberrantly aggregated and hyperphosphorylated MAPT (microtubule-associated protein Tau). Tauopathies are pathologically and phenotypically diverse, and include Alzheimer’s disease, progressive supranuclear palsy, corticobasal degeneration, chronic traumatic encephalopathy, Pick’s disease, and frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17)2 (1–4). Tau is abundant in neurons and expressed at lower levels in glia (1, 6, 7), and primarily stabilizes microtubules (MTs) among other diverse physiological functions (1, 5, 8, 9). Six different isoforms of Tau, ranging from 352 to 441 amino acids, are expressed in the adult human brain as a result of alternative splicing (10, 11). Differential splicing results in the inclusion or exclusion of the R2 MT-binding repeat, producing Tau isoforms with either three (3R) or four (4R) repeats, respectively, and one or two N-terminal inserts (see Fig. 1).

Over 50 mutations have been identified in the MAPT gene in families with FTDP-17 (1, 2, 4, 12). These mutations cause autosomal dominant disease, and patients typically experience disease onset at ~49 years of age with an average disease duration of 8.5 years (13). Certain intronic and some exonic mutations can affect splicing and alter the ratio of 4R Tau to 3R Tau, potentially increasing unbound Tau and leading to aggregation (1, 2, 4). Additionally, some Tau missense mutants can negatively affect MT assembly and binding affinity (1, 14–16), alter the interaction of Tau with other protein complexes (17), and influence post-translational modifications (18). Furthermore, in vitro Tau filament assembly studies have shown that some mutations cause Tau to more readily self-aggregate compared with WT Tau (15, 19, 20).

Although many functional consequences of Tau mutations have been documented, differences between Tau mutants regarding aggregation with seeding, however, are not well characterized. Tau aggregation is thought to occur in a nucleation-dependent manner, with an initial lag phase followed by a more rapid elongation phase, as protein subunits are added to the growing Tau fibril (21). Proteinaceous seeding as a concept stems from prion disease, in which an exogenous pathogenic agent, or prion, can act as a template to induce conformational change in native prion proteins, causing them to misfold and aggregate and leading to neurodegeneration (22–24). Mount-

---

**Footnotes:**

1 To whom correspondence should be addressed: BMS J483/CTRND, 1275 Center Dr., Gainesville, FL 32610. Tel.: 352-273-9363; E-mail: bgiasson@ufl.edu.

This is an open access article under the CC BY license.

---

**References:**

2. The abbreviations used are: FTDP-17, frontotemporal dementia with Parkinsonism linked to chromosome 17; FTD, frontotemporal dementia; ANOVA, analysis of variance; BCA, bicinchoninic acid assay; FBS, fetal bovine serum; MT, microtubule; 3R, three repeat; 4R, four repeat; RD, repeat domain. TBS, Tris-buffered saline.
Seeding propensity differs significantly among FTDP-17 Tau mutants

The ability of exogenously added preformed fibrils to seed Tau intracellular inclusions in cell culture has been demonstrated in a number of different cell lines and models (29–31, 46, 48). Using a human embryonic kidney 293T (HEK293T) cell Tau seeding model, we initially sought to assess and compare the propensity of 15 different FTDP-17 Tau mutants (Figs. 1 and 2) to be seeded to form inclusions. HEK293T cells were transfected with plasmids encoding WT full-length Tau (0N/4R) protein and FTDP-17 mutants thereof and inoculated with WT K18 Tau fibrils. Cells were harvested at 48 h post-transfection and fractionated into Triton-soluble and Triton-insoluble fractions. The accumulation of Tau in the Triton-insoluble pellets indicates the intracellular seeding and induction of intracellular Tau inclusion formation (48). Furthermore, we observed similar results using sarkosyl solubility fractionation (Fig. S1). Sharp differences among the first series of mutants analyzed was observed, with only P301L, P301S, and S320F Tau showing significant induced aggregation (Fig. 2, A and B; p < 0.01, p < 0.05, respectively). P301L and P301S Tau showed similar amounts of aggregation, whereas S320F Tau aggregated to a lesser extent. Furthermore, in the absence of WT K18 fibrils, S320F Tau transfected cells displayed a small amount of self-aggregation (p < 0.01), which was not observed for the other mutants or WT Tau (Fig. 2, A and C).

Next, because there is evidence to suggest that Tau pathologies could transmit in a “strain-dependent manner” (4, 33, 41, 42) and that Tau mutants can cause Tau to form conformationally distinct fibrils (43, 49), we sought to assess whether homo-
typic seeding may be more robust or necessary for some of the Tau mutants to aggregate. The previous experiment was thus repeated, this time comparing heterotypic seeding with WT K18 Tau fibrils to homotypic seeding with mutant K18 Tau fibrils. For the mutants that previously showed aggregation with seeding (P301L, P301S, and S320F), there was no difference in the level of Tau aggregation when WT or homotypic mutant K18 Tau fibrils were added (Fig. 3, A and B). Furthermore, the mutantsthat previously did not aggregate with WT K18 seeds also were not induced to aggregate with their respective mutant K18 fibrils (Fig. 3 C; data not shown). To further compare the ability of these different Tau mutant fibrils to induce aggregation, P301L 0N/4R Tau-transfected cells were seeded with various mutant K18 fibrils and analyzed for their levels of aggregation. All mutant K18 fibrils were able to induce a similar level of aggregation of P301L Tau, with Δ280K fibrils causing slightly less aggregation (Fig. S2).

Next, we compared WT and P301 mutant Tau seeding in either the 0N/4R or 2N/4R Tau isoform, which can be resolved as discrete bands on Western blots because of their distinct molecular masses. Similar to 0N/4R Tau, WT 2N/4R Tau did not show a propensity to aggregate with seeding, whereas P301L 2N/4R Tau seeding–induced aggregation was observed (Fig. 4). Mutant P301L 2N/4R Tau was co-expressed with WT 0N/4R Tau and seeded with WT K18 fibrils to ascertain whether intracellular WT Tau can be recruited into the insoluble portion by intracellular P301L Tau aggregates, which can be discerned based on their different molecular masses. Even in the presence of intracellular P301L 2N/4R Tau aggregates, WT Tau was not recruited into the inclusions (Fig. 4).

To further investigate the role and specificity of P301 in modulating the aggregation propensity of Tau in this cell model, proline residues located in each of the four microtubule binding repeats in Tau were mutated to leucine. Each proline amino acid is the first residue in a conserved PGGG motif located near the end of each MT-binding repeat and is thought to play an important role in tubulin binding, with mutations at these sites altering the binding ability of Tau (13–16, 50, 51). It is noteworthy that mutants P332S and P364S, which are relatively identical mutations to P301S within these PGGG motifs, did not influence the propensity of Tau to aggregate (Fig. 2). However, to specifically compare the effects of P to L mutations at each PGGG motif, P270L, P301L, P332L, and P364L 2N/4R Tau mutants were expressed with the addition of K18 seeds (Fig. 5 A). Only the P301L Tau mutant demonstrated a propensity to be seeded in cells (Fig. 5 B).

To further investigate the influence of Pro301 and its local surrounding molecular environment, we extended our studies to other FTDP-17 Tau mutants in this region. P301T also demonstrated a similar propensity to aggregate in cells under seeding conditions, but not the G303V, G304S, or S305N mutants (Fig. 6).

Given that all three pathogenic mutations at the Pro301 site uniquely enhance aggregation of Tau, we decided to assess whether the loss of the proline residue in this region was sufficient to replicate this finding. Indeed, the deletion of Pro301 (ΔP301) was sufficient to promote the aggregation of Tau under
seeding conditions (Fig. 7A). Furthermore, mutating the neighboring residue, Gly302, to a proline in P301L Tau (P301L/G302P double mutant) was sufficient to impair the propensity of P301L Tau to aggregate (Fig. 7B). Pro301 is in close proximity to an established sequence of Tau termed “PHF6” (Fig. 1) that is important for both aggregation and, specifically, β-pleated sheet formation (37–40). Located in the beginning of the third microtubule binding repeat, PHF6 is adjacent to the Pro301 site, and only nine residues away from the Ser320 site, which, when mutated, also showed robust aggregation with seeding. We assessed another proline residue near the PHF6 site, Pro312, to determine whether it affected Tau in a manner similar to Pro301. However, the P312L mutation did not increase the propensity of Tau to aggregate in the seeded cell assay (Fig. 7C). We previously showed that P270L within 4R Tau did not promote Tau aggregation (Fig. 5). However, in 3R Tau, this Pro270 residue is located in a similar position to PHF6 as the Pro301 residue in 4R Tau, because of the absence of the R2 MT-binding repeat (Figs. 1 and 7D). Thus Pro270 was changed to a leucine in 0N/3R Tau, and the seeding experiment was performed, but P270L 0N/3R Tau also did not demonstrate a propensity to be seeded by WT K18 fibrils (Fig. 7D). Because it is suggested that certain cross-seeding barriers between 3R and 4R Tau exist (52), this P270L 0N/3R Tau mutant was also seeded with K19 Tau fibrils (i.e. truncated Tau containing three rather than four MT-binding repeats) (Fig. 1). A small amount of Tau aggregation was observed upon treatment with K19 fibrils, compared with no aggregation when treated with K18 fibrils (Fig. 8, A and B). Interestingly, treatment with K18 fibrils was much more sufficient at seeding P301L 0N/4R Tau compared with K19 fibrils (Fig. 8, C and D).

The P301L/S320F double mutant results in robust aggregation even in the absence of exogenous Tau fibrils

To investigate whether the P301L and S320F mutations could have an additive or synergistic effect, the propensity of P301L/S320F Tau double mutant on Tau aggregation with or without exogenous WT K18 fibrils was assessed. Of note, both
Tau mutants and seeding

**Figure 4.** P301L Tau does not recruit WT Tau in aggregate formation following induced seeding in HEK293T cells. All cells were biochemically fractionated into Triton-soluble and Triton-insoluble fractions. Western blot analysis of total Tau (antibody H150) was performed. The results are shown in triplicate. The mobilities of molecular mass markers in kDa are indicated on the right. A, HEK293T cells were transfected with plasmids expressing either WT 0N/4R Tau or 2N/4R Tau and treated with fibrillar WT K18 Tau seeds as indicated above each lane (+). B, HEK293T cells were transfected with plasmids expressing P301L 0N/4R Tau or P301L 2N/4R Tau and treated with fibrillar WT K18 Tau seeds as indicated above each lane (+). C, HEK293T cells were co-transfected with plasmids expressing P301L 2N/4R and WT 0N/4R Tau and treated with WT K18 Tau fibrillar seeds as indicated above each lane (+).

of these sites flank the PHF6 site. Expression of P301L/S320F Tau demonstrated robust aggregation without need for exogenous WT K18 seeds (Fig. 9, A–D). In fact, there was no discernible difference between the amount of Triton-insoluble Tau with or without WT K18 Tau fibril treatment. Amyloid inclusion formation by P301L/S320F Tau was further confirmed with thioflavin S staining that was not observed with WT Tau or the single Tau mutants (Fig. 10). To ensure that this effect was due to this specific combination of mutations, we also created the double mutants P301L/A152T and P301L/L266V. Unlike P301L/S320F, P301L/A152T and P301L/L266V Tau showed no aggregation without seeding (Fig. 9E). The double mutant P301S/S320F was also tested and aggregated without seeding at levels similar to P301L/S320F Tau (Fig. 9, F and G).

To assess whether the P301L/S320F double mutant could fibrillize in vitro without the use of a polyanionic inducer such as heparin (53), WT, P301L, S320F, and P301L/S320F 0N/4R Tau proteins were incubated with or without heparin with shaking at 37 °C for 8 days. Although thioflavin T analysis showed no significant amyloid formation for any of the groups without heparin at 8 days (Fig. 11A), electron microscopy showed sparsely populated Tau fibrils for the P301L and S320F single mutants and the P301L/S320F double mutant (Fig. 11B). However, these were rare compared with Tau proteins incubated with heparin (Fig. 11B).

**Discussion**

The current work illuminates marked differences between seeded aggregation properties among a large series of different FTDP-17 Tau mutants. Pathogenic mutants at the Pro301 site (P301L, P301S, and P301T) and to a lesser extent at the Ser320 site (S320F) showed marked aggregation when cells were treated with exogenous preformed fibrillar Tau seeds, whereas WT and the remaining FTDP-17 associated mutant Tau displayed relatively limited to no aggregation, consistent with and expanding upon previous direct comparisons (30, 54). Furthermore, studies of other FTDP-17 mutations around the PHF6 site of Tau as well as additional artificial mutants near this site or at other proline residues in the three other PGGG motifs within Tau further demonstrated the unique properties of the Pro301 residue and mutants thereof in regulating seeding–induced Tau aggregation.

These studies were performed in the context of mounting evidence that points to prion-like transmission involving intracellular seeding as an important mechanism for the spread of tauopathy (4, 25–27, 55), with far reaching mechanistic and therapeutic implications. Seeding properties of many FTDP-17 mutants had yet to be examined, and current cultured cell Tau seeding models tend to utilize either mutated truncated RD Tau (29, 44, 47), which can intrinsically aggregate in these models (56, 57) or full-length Tau mutants at the Pro301 site (30). WT Tau has been reported to aggregate with seeding in some cell culture studies, albeit only at low levels, especially when compared with P301L Tau or RD Tau (29, 30, 48). Our data reveal that specific Tau mutants, i.e. those at Pro301 and Ser320, are uniquely able to robustly aggregate with seeding when compared with WT Tau and other FTDP-17 Tau mutants, and thus pathogenicity by these other FTDP-17 mutants might be driven by different disease mechanisms. For example, many Tau missense mutations reduce Tau MT assembly and/or the ability of Tau to stabilize MTs (1, 13–16), and this might be the major pathological mechanism for some of these mutations. It is also known that many intronic and even some exonic FTDP-17 Tau mutants appear to significantly alter the ratio of exon 10 splicing, resulting in an imbalance in the normal proportion of Tau isoforms with 3 or 4 MT-binding repeats (1–3, 58, 59).

The findings observed here have important implications for some of the most ubiquitous models of tauopathies. Indeed, many commonly used Tau transgenic mouse lines express either P301L and P301S full-length or truncated Tau (45, 60). *In vivo* studies of prion-like induction of tauopathy have utilized intracerebral injection of brain lysate from Tau transgenic mice or human tauopathy into P301L or P301S human Tau transgenic mouse models (61–63), as well as the ALZ17 WT human Tau mouse line, which has been reported to show inductions following brain lysate from tauopathy mice or human cases, but this is a slower process (32, 33). Studies using recombinant fibrillar Tau for direct intracerebral seeding demonstrated the induction of Tau pathology in transgenic mice expressing either P301L or P301S human Tau, but not in transgenic mice expressing WT human Tau (34, 35, 64, 65). However, it is likely that Tau aggregates within brain lysate have higher seeding potencies than recombinant Tau fibrils (54). Indeed the cerebral injection of human tauopathy brain lysates can induce local Tau pathology or pathology that spreads along connected brain regions, in nontransgenic mice (33, 66).

We showed that aggregated P301L Tau failed to sequester soluble WT Tau into insoluble aggregates when co-expressed in the HEK293T seeding model, demonstrating that even when...
intracellular Tau aggregates are generated, WT Tau is resilient to recruitment into inclusions. Given that there seemed to be less aggregation of P301L Tau in the presence of WT Tau expression, it is plausible that WT Tau may reduce templated conformation of P301L Tau. The lack of intracellular cross-seeding between P301L Tau and WT Tau is consistent with findings that in patients with the P301L mutation, pathological inclusions are comprised predominantly of P301L Tau and not WT protein, even though both are expressed (67, 68). In addition, in vitro studies showed that P301L Tau preformed fibrils can induce the aggregation of P301L Tau, but not WT Tau, whereas WT Tau preformed fibrils can simulate the aggregation of both WT and P301L Tau (69). Collectively, these data could support the notion that aggregated P301L is a different conformer or “strain” than WT Tau.

The distinct property of Pro301 in dramatically influencing the ability of Tau to aggregate when cells are exposed to exogenous Tau amyloid seeds was demonstrated by a series of complementary studies. The effects of mutating the individual proline residues within the functionally conserved PGGG motif present in each of the four MT-binding domain to leucine residue on seed-induced aggregation was investigated. Only the P301L mutant showed a propensity to aggregate. Although these other proline-to-leucine mutants in addition to P301L are not found in FTD patients, P332S and P364S mutants located in the PGGG motif of MT repeats 3 and 4, respectively, have been
**Tau mutants and seeding**

![Diagram](image)

**Figure 7. Differential importance of P residues near the PHF6 site in inhibiting K18-seeded Tau aggregation.** A, the sequence of the R2 MT-binding repeat showing a deletion at the Pro301 residue. HEK293T cells were transfected with plasmids expressing ΔP301 ON/4R Tau and treated with WT K18 Tau fibrillar seeds when indicated above each lane (+). B, the sequence of the R2 MT-binding repeat showing the P301L/G302P double mutant. HEK293T cells were transfected with plasmids expressing ON/4R P301L Tau or P301L/G302P ON/4R Tau and treated with WT K18 Tau fibrillar seeds as indicated above each lane (+). C, the sequence of the R3 MT-binding repeat showing the P312L mutant. HEK293T cells were transfected with plasmids expressing P312L ON/4R Tau and treated with WT K18 Tau fibrillar seeds when indicated above each lane (+). D, R1 and R3 MT-binding repeats are shown, because in ON/3R, the R2 MT-binding repeat is absent. The P270L mutant is highlighted and shown in relation to the PHF6 site, with numbering of the residues conforming to the longest isoform of Tau. HEK293T cells were transfected with plasmids expressing ON/3R P270L Tau and treated with WT K18 Tau fibrillar seeds as indicated above each lane (+). The cells were biochemically fractionated into Triton-soluble and -insoluble fractions. Immunoblot analysis of total Tau (antibody H150). All results are in triplicate. The mobilities of molecular mass markers in kDa are indicated on the left.

Identified in FTDP-17 patients (51, 70). However, again only P301S and not P332S and P364S Tau demonstrated increased propensity to aggregate in the HEK293T seeded model.

The proximity of Pro301 to PHF6, a hexapeptide motif that is necessary and sufficient for β-pleated sheet formation, may explain its structural importance (37–40). Indeed, recent cryo-electron microscopy data on fibrillar Tau isolated from a patient with Alzheimer’s disease (71) and previous structural NMR findings of Tau (72) indicate that the PHF6 site is within a critical β-sheet stretch for amyloid folding needed to generate the core of paired helical filaments and straight filaments. Therefore, the impact of additional FTDP-17–associated mutations located near the PHF6 site—P301T, G303V, G304S, and S305N—were investigated. Again, the mutant at the Pro301 site, in this case P301T, was the only one that showed a propensity to aggregate with seeding. These striking differences in the propensity of FTDP-17 Tau mutants to be seeded further raises the possibility that Tau missense mutations may have differing pathogenic mechanisms. For example, although G303V, G304S, and S305N are in close proximity to Pro301, the primary pathological etiology of these mutations may be to affect Tau RNA splicing (59, 73), similar to many intronic Tau mutations (1, 2, 13), resulting in an altered ratio of Tau isoforms.

Because of the unique characteristics of prolines as inhibitors of β-sheet formation (74, 75), we tested whether other proline residues near this amyloidogenic region of Tau served a similar purpose. We first demonstrated the importance of Pro301 in regulating Tau aggregation by deleting this residue, resulting in the permissive seed-induced aggregation of Tau. We then sought to find out whether reinsertion of a proline adjacent to the P301L Tau mutant would block aggregation. Expressing the P301L/G302P double mutant in our seeding model resulted in inhibition of aggregation with seeding to levels similar to WT Tau. In striking comparison to P301L, mutating Pro312 to a leucine, a position immediately downstream to the PHF6 site, did not promote Tau aggregation. These findings demonstrate the importance of specific residues such as Pro301 as well as the surrounding molecular milieu in significantly influencing the permissiveness of Tau to aggregate.

Another proline residue, Pro270, is in the same position preceding PHF6 in 3R Tau as Pro301 in 4R Tau, because of the absence of the R2 MT-binding repeat. When this residue was mutated to a leucine in ON/3R Tau and treated with WT K18 fibrils, it was not driven to aggregate. However, previous in vitro data have indicated that a seeding barrier between the 3R and 4R Tau isoforms exists, particularly when 4R Tau acts as a conformational template for 3R Tau (52). Thus treatment of P270L ON/3R Tau with K19 fibrils was performed, and a small but significant amount of aggregation (p < 0.05) was observed. This barrier to cross-seeding existed for 4R Tau as well; P301L ON/4R Tau was treated with either K19 or K18 WT fibrils, resulting in significantly different rates of aggregation, with K19 fibrils seeding significantly less than K18. These data suggest that the presence or absence of the R2 MT-binding repeat can change the global conformation of Tau, affecting its propensity to form amyloid inclusions, at least in this model. Furthermore, this isoform difference seems to create a conformational barrier that can affect the spread of pathological Tau.

Of all the FTDP-17 or experimentally designed Tau missense mutations investigated, only mutations at the Pro301 and Ser320 residues readily aggregated when seeded, with the Ser320 mutant resulting in aggregation, albeit at lower levels, even without the addition of exogenous Tau fibrils. Because of these unique properties, the ability of double mutant P301L/S320F, as well as P301S/S320F, to aggregate was investigated and shown to be a robust model of Tau amyloid formation in vivo,
even without exogenous seeding. In vitro, aggregation without heparin was not significant, as shown by thioflavin T fluorometry assay, although scant P301L/S320F fibrils were observed by electron microscopy. However, these were rare compared with incubation with heparin. These fibrils results indicate that even for this double mutant Tau, the recombinant protein is not readily folded in a structure that is permissive for amyloid formation. In addition, these findings suggest that certain in vivo factors, perhaps chaperone activities, may actively promote Tau aggregation, at least in some contexts.

Despite being an intrinsically disordered protein, Tau can adopt a global fold resembling a paperclip-like structure, in which the MT-binding repeat, C terminus, and N terminus all approach each other (1, 76). In this structure, Tau would be unable to polymerize, and it is possible that the S320F mutation disrupts this conformation, allowing for Tau to locally initiate amyloid conversion. Alternatively, cryo-electron microscopy data (71) indicate that in Tau fibrils, Ser320 is folded within a hydrophobic pocket, and thus the S320F mutation would strongly stabilize Tau amyloid fold and subsequent fibril polymerization. Coupled with the paucity of the proline at residue 301 that would normally suppress β-pleated sheet formation, this combination of Tau mutations increases the propensity for both secondary and tertiary structure needed for amyloid formation, thus allowing this double mutant to readily polymerize to form inclusions.

In all, a large series of FTDP-17 Tau missense mutations were studied for their propensity to be induced to aggregate by exogenous preformed Tau fibrils in the HEK293T cellular model. It is demonstrated that the relative proneness to be seeded and to form inclusions varies greatly between Tau mutants, with the three pathogenic mutations at residue Pro301 as well as S320F being permissive, whereas the other mutants tested were refractory. This finding suggests that Pro301 within 4R Tau acts as a major gatekeeper in Tau amyloid conversion, within a unique molecular context that renders it critical in regulating Tau aggregation. These findings have important implications for the use of models that focus on particular mutants, because different mutants may have different primary disease mechanisms. Furthermore, by combining two mutations that are more prone to readily aggregate, P301L and S320F, we have identified a novel and aggressive model of Tau inclusion formation, which could allow for more rapid analysis of pathogenic mechanisms and screening of therapeutics.

**Experimental procedures**

**Tau mammalian expression plasmids**

The cDNAs encoding the full-length 0N/4R or 2N/4R human Tau isoforms were cloned in the mammalian expression vector pcDNA3.1 (+). The different missense MAPT mutations were created through QuikChange site-directed mutagenesis (Agilent Technologies, Santa Clara, CA) using mutant-specific oligonucleotides. All mutations and the absence of errors throughout the entire length of the Tau cDNA was confirmed by Sanger sequencing performed as a service by the Interdisci-
Tau mutants and seeding

A

|                | Triton Soluble | Triton Insoluble |
|----------------|----------------|------------------|
| P301L/320F    | *             | *                |
| WT            | -             | -                |

B

P301L/S320F Insoluble Tau with and without Seeding

C

Total Tau (3026)  AT8  DAPI Merge

WT

P301L

P301S

S320F

P301L/S320F

D

AT8/Total Tau

WT  P301L  P301S  S320F  P301L/S320F

E

Double Mutant Tau Aggregation without Seeding

| Triton Soluble | Triton Insoluble |
|----------------|------------------|
| P301L/A152T    | P301L/320F       | P301L/S320F |
| P301L/L266V    |                   |            |

F

|                | Triton Soluble | Triton Insoluble |
|----------------|----------------|------------------|
| P301L/S320F    |                |                  |
| P301S/S320F    |                |                  |

G

ON/4R P301L/S320F vs P301S/S320F

% Aggregation
plinary Center for Biotechnology Research at the University of Florida.

Expression and purification of recombinant Tau

The cDNA corresponding to the human Tau K18 fragment (residues Gln244–Glu372 in 2N/4R human Tau) with an added methionine residue at the N terminus cloned in the bacterial expression plasmid pRK172 was used for expression in BL21 (DE3)/RIL *Escherichia coli* (Agilent Technologies). The K19

Figure 9. The P301L/S320F Tau double mutant endogenously aggregates in HEK293T cells. A, HEK293T cells were transfected with the plasmid expressing 0N/4R P301L/S320F Tau and treated with WT K18 Tau fibrillar seeds as indicated above each lane (+). The cells were biochemically fractionated into Triton-soluble and Triton-insoluble fractions. Western blot analysis of total Tau (antibody H150) was performed. The results are in triplicate. The mobilities of molecular mass markers in kDa are indicated on the left. B, quantification of Triton-insoluble Tau expressed as a percentage of total Tau (n = 3), C, representative immunofluorescence images of HEK293T cells transfected with plasmids expressing WT, P301L, P301S, S320F, or P301L/S320F 0N/4R Tau. The cells were stained with antibodies 3026 (total Tau; green) and AT8 (phospho-Tau; red). Scale bar, 200 μm. D, AT8-positive cells expressed as a percentage of total cells expressing Tau and analyzed by one-way ANOVA with post hoc multiple comparisons comparing each group to WT (n = 3). *, p < 0.05; **, p < 0.01. E, HEK293T cells were transfected with plasmids expressing the double mutants P301L/A152T, P301L/L266V, and P301L/S320F in 0N/4R Tau. The cells were biochemically fractionated into Triton-soluble and -insoluble fractions. Western blot analysis of total Tau (antibody H150) was performed. The results are in duplicate. The mobilities of molecular mass markers in kDa are indicated on the left. F, HEK293T cells were transfected with plasmids expressing either P301L/S320F or P301S/S320F 0N/4R human Tau. The cells were biochemically fractionated into Triton-soluble and -insoluble fractions, and the Western blots shown depict Triton-soluble and -insoluble total Tau (antibody H150) in HEK293T cells. The mobilities of molecular mass markers in kDa are indicated on the left. G, quantification of Triton-insoluble Tau expressed as a percentage of total Tau. Statistical analysis was done using Student’s t test (n = 3). **, p < 0.01.

Figure 10. P301L/S320F Tau double mutant aggregates are thioflavin S-positive. Shown are representative immunofluorescence images of HEK293T cells transfected with plasmids expressing WT, P301L, P301S, S320F, or P301L/S320F 0N/4R Tau. The cells were stained with thioflavin S (green) as described under “Experimental procedures” and with antibody 3026 (total Tau; red). Scale bar, 200 μm.

Figure 11. P301L, S320F, and P301L/S320F Tau only modestly fibrillize in vitro without a chemical chaperone. WT, P301L, S320F, or P301L/S320F 0N/4R Tau proteins (1 mg/ml) were incubated with agitation at 37 °C with or without heparin for 8 days. A, quantification of thioflavin T fluorometry. The results are in triplicate. The presence of heparin in the samples is indicated as H in the x-axis. B, representative electron microscopy images of these 0N/4R Tau protein after incubation in vitro. The presence or absence of heparin is indicated above the images. Scale bar, 250 nm.

Figure 12. P301L, S320F, and P301L/S320F Tau only modestly fibrillize in vitro without a chemical chaperone. WT, P301L, S320F, or P301L/S320F 0N/4R Tau proteins (1 mg/ml) were incubated with agitation at 37 °C with or without heparin for 8 days. A, quantification of thioflavin T fluorometry. The results are in triplicate. The presence of heparin in the samples is indicated as H in the x-axis. B, representative electron microscopy images of these 0N/4R Tau protein after incubation in vitro. The presence or absence of heparin is indicated above the images. Scale bar, 250 nm.

Expression and purification of recombinant Tau

The cDNA corresponding to the human Tau K18 fragment (residues Gln244–Glu372 in 2N/4R human Tau) with an added methionine residue at the N terminus cloned in the bacterial expression plasmid pRK172 was used for expression in BL21 (DE3)/RIL *Escherichia coli* (Agilent Technologies). The K19

Figure 9. The P301L/S320F Tau double mutant endogenously aggregates in HEK293T cells. A, HEK293T cells were transfected with the plasmid expressing 0N/4R P301L/S320F Tau and treated with WT K18 Tau fibrillar seeds as indicated above each lane (+). The cells were biochemically fractionated into Triton-soluble and Triton-insoluble fractions. Western blot analysis of total Tau (antibody H150) was performed. The results are in triplicate. The mobilities of molecular mass markers in kDa are indicated on the left. B, quantification of Triton-insoluble Tau expressed as a percentage of total Tau (n = 3), C, representative immunofluorescence images of HEK293T cells transfected with plasmids expressing WT, P301L, P301S, S320F, or P301L/S320F 0N/4R Tau. The cells were stained with antibodies 3026 (total Tau; green) and AT8 (phospho-Tau; red). Scale bar, 200 μm. D, AT8-positive cells expressed as a percentage of total cells expressing Tau and analyzed by one-way ANOVA with post hoc multiple comparisons comparing each group to WT (n = 3). *, p < 0.05; **, p < 0.01. E, HEK293T cells were transfected with plasmids expressing the double mutants P301L/A152T, P301L/L266V, and P301L/S320F in 0N/4R Tau. The cells were biochemically fractionated into Triton-soluble and -insoluble fractions. Western blot analysis of total Tau (antibody H150) was performed. The results are in duplicate. The mobilities of molecular mass markers in kDa are indicated on the left. F, HEK293T cells were transfected with plasmids expressing either P301L/S320F or P301S/S320F 0N/4R human Tau. The cells were biochemically fractionated into Triton-soluble and -insoluble fractions, and the Western blots shown depict Triton-soluble and -insoluble total Tau (antibody H150) in HEK293T cells. The mobilities of molecular mass markers in kDa are indicated on the left. G, quantification of Triton-insoluble Tau expressed as a percentage of total Tau. Statistical analysis was done using Student’s t test (n = 3). **, p < 0.01.
Tau fragment and seeding

Tau fragment, which does not code the R2 repeat (Fig. 1), was similarly generated. pRK172 plasmids expressing the various missense Tau mutations within the K18 fragment were created by site-directed mutagenesis as described above. K18 Tau proteins were purified as previously described for Tau proteins (77). Protein concentrations were determined using the bicinchoninic acid assay (Pierce) and BSA as the standard.

Assembly of recombinant Tau fibrils

Recombinant K18, K19, or 0N/4R Tau proteins (1 mg/ml) were assembled into filaments by incubation at 37 °C in sterile PBS with 50 μM heparin (53, 78) while shaking at 1050 rpm with an Eppendorf Thermomixer R for at least 48 h. Tau amyloid fibril formation was confirmed by K114 or thioflavin T fluorescence as previously described (48, 79, 80). For the thioflavin T assays, 5 μL of each sample were diluted into a 50 μM working concentration of thioflavin T in 0.1 M glycerol, pH 8.6. To remove the heparin, Tau fibrils were centrifuged at 100,000 × g and resuspended in sterile PBS, and the resulting protein concentrations were determined using bicinchoninic acid assay. Tau filaments were fragmented into shorter Tau “seeds” by bath sonication for 60 min as previously described (48).

Cell culture and transfection

HEK293T cells were maintained with Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 units penicillin/ml, and 100 μg streptomycin/ml, at 37 °C and 5% CO2. The cells were plated on polystyrene 6- or 12-well plates and transfected with the various plasmids expressing 0N/4R or 2N/4R human Tau using calcium phosphate precipitation at ~30–50% confluency. For transfection per 2 mL of cell culture media, 3 μg of plasmid DNA was diluted into 37.5 μL of 1 M CaCl2 and stepwise added to an equal volume of 50 mM N,N-bis-(2-hydroxymethyl)-2-aminoethanesulfonic acid, 280 mM NaCl, 1.5 mM Na2HPO4, pH 6.96. This mixture was incubated at room temperature for 15–20 min before adding dropwise to the media in each well. For cellular Tau seeding studies, K18 or K19 Tau fibrils (final concentration, 1 μM) were added 1 h following the beginning of the transfection (48). The cells were either harvested for biochemical fractionation or fixed for immunofluorescence staining 48 h thereafter.

Biochemical cellular fractionation

The cells were washed with PBS and harvested in 400 μL for 6-well plates and 200 μL for 12-well plates of CSK buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 20 mM NaF) and a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 1 mg/ml each of pepstatin, leupeptin, N-tosyl-l-phenylalanyl chloromethyl ketone, N-tosyllysine chloromethyl ketone, and soybean trypsin inhibitor). The samples were sedimented at 100,000 × g for 30 min at 4 °C, and the supernatants were collected. To ensure complete removal of the supernatant, the pellets were washed with 400 μL (6-well plates) or 200 μL (12-well plates) of CSK buffer and underwent 100,000 × g centrifugation. The supernatants were completely removed, and the pellets were resuspended in 400 μL (6-well plates) or 200 μL (12-well plates) of CSK buffer. 5 × SDS sample buffer (final concentration of 10 mM Tris, pH 6.8, 1 mM EDTA, 40 mM DTT, 0.005% bromphenol blue, 0.0025% pyronin yellow, 1% SDS, 10% sucrose) was then added to both the CSK buffer–soluble and –insoluble fractions (referred to as the Triton-soluble and –insoluble fractions), and the samples were heated at 100 °C for 10 min and probe-sonicated for the Triton-insoluble samples. For sarkosyl fractionation, the cells were washed with PBS and harvested from 6-well plates in 400 μL of lysis buffer containing 1% sarkosyl, 10 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, and 5 mM EDTA, pH 7.4, with protease inhibitor as previously described (81). The cell suspensions were syringe-sheared with a 27-gauge needle, followed by incubation on ice for 15 min, bath sonication two times for 2 min, and incubation at room temperature for 20 min. The samples were sedimented at 100,000 × g for 1 h. The supernatants were saved as the sarkosyl-soluble fractions, whereas the sarkosyl-insoluble pellets were resuspended in 1% sarkosyl with 5 × SDS sample buffer, heated for 10 min at 100 °C, and probe-sonicated.

Antibodies

Total Tau antibodies used in these experiments were H150 (Santa Cruz, Dallas, TX), a rabbit polyclonal antibody raised against amino acids 1–150 of human Tau, and rabbit polyclonal antibody 3026 raised against amino acids 1–150 of human Tau (82). Additionally, AT8 (Thermo Fisher) is a mouse monoclonal antibody that reacts with Tau phosphorylated at Ser202 and Thr205 (83).

Western blot analysis

Equal volumes of Triton-soluble and Triton-insoluble protein fractions or sarkosyl-soluble and sarkosyl-insoluble protein fractions were loaded onto 10% polyacrylamide gels and resolved by SDS-PAGE, followed by electrophoretic transfer onto nitrocellulose membranes. The membranes were blocked in 5% dry milk/TBS (50 mM Tris, pH 7.5, 150 mM NaCl) and incubated overnight at 4 °C with primary antibody diluted in 5% dry milk/TBS or 5% BSA/TBS for the phosho-specific AT8 antibody. After washing, membranes were incubated in goat anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories) diluted in 5% dry milk/TBS for 1 h, and immunoreactivity was detected using Western Lightning-Plus ECL reagents (PerkinElmer) followed by chemiluminescence imaging (PXi, Syngene, Frederick, MD). ImageJ software was then used to quantify the signal of the resulting protein bands, and the results are represented as the percentages of Triton-insoluble Tau over total Tau, as previously described (48). Statistical analysis was done in GraphPad Prism using either a Student’s t test or one-way analysis of variance (ANOVA), with post hoc analysis using Dunnett’s test to compare each “group” (i.e. mutant) to our WT control.

Immunofluorescence analyses

For immunofluorescence staining, the cells were plated onto poly-d-lysine–coated glass coverslips. The cells were rinsed with PBS and fixed with 4% paraformaldehyde for 10 min, followed by PBS washes. The cells were blocked with PBS, 2% FBS,
0.1% Triton for 30 min, followed by the application of primary antibody diluted in PBS, 2% PBS for 1 h at room temperature. The cells were washed with PBS before adding the secondary antibodies, conjugated to Alexa fluorophores 488 or 594 (Thermo Fischer Scientific, Waltham, MA). The cells were washed with PBS, stained with 4',6'-diamidino-2-phenylindole, and mounted using Fluoromount-G (Southern Biotech, Birmingham, AL). For thioflavin S staining, a stock solution of 1% thioflavin S (Sigma–Aldrich) in 50% ethanol in PBS was filtered through a 0.2-μm filter and kept foil-wrapped. The cells were rinsed with PBS and fixed with 4% paraformaldehyde for 10 min. Millipore auto-fluorescence reagent (Millipore, Billerica, MA) was applied to cells for 5 min, followed by washing with 40% ethanol. The cells were then blocked, and the primary and secondary antibodies were sequentially applied as described above, keeping the samples in the dark when possible. The coverslips were similarly stained with 4',6'-diamidino-2-phenylindole and mounted using Fluoromount G (Southern Biotech). The images were captured using an Olympus BX51 fluorescence microscope mounted with a DP71 digital camera (Olympus, Center Valley, PA). Cell count analysis was performed with ImageJ software.

Electron microscopy imaging

The samples were adsorbed onto 300-mesh carbon coated copper grids (Electron Microscopy Sciences, Hatfield, PA), negatively stained with 1% uranyl acetate, and visualized with a Hitachi 7600 transmission electron microscope at 50,000× magnification.

Acknowledgment—We thank Michael Strickland for helping with the cell count studies.

References

1. Wang, Y., and Mandellkow, E. (2016) Tau in physiology and pathology. Nat. Rev. Neurosci. 17, 5–21 CrossRef Medline

2. Iqbal, K., Liu, F., and Gong, C.-X. (2016) Tau and neurodegenerative disease: the story so far. Nat. Rev. Neuro. 12, 15–27 CrossRef Medline

3. Lee, V. M., Goedert, M., and Trojanowski, J. Q. (2001) Neurodegenerative tauopathies. Annu. Rev. Neurosci. 24, 1121–1159 CrossRef Medline

4. Goedert, M., Eisenberg, D. S., and Crowther, R. A. (2017) Propagation of Tau aggregates and neurodegeneration. Annu. Rev. Neurosci. 40, 189–210 CrossRef Medline

5. Cleveland, D. W., Hwo, S. Y., and Kirschner, M. W. (1977) Physical and chemical properties of purified Tau factor and the role of Tau in microtubule assembly. J. Mol. Biol. 116, 227–247 CrossRef Medline

6. LoPresti, P., Szuheit, S., Papasozomenos, S. C., Zinkowski, R. P., and Binder, L. I. (1995) Functional implications for the microtubule-associated protein Tau: localization in oligodendrocytes. Proc. Natl. Acad. Sci. U.S.A. 92, 10369–10373 CrossRef Medline

7. Binder, L. I., Frankfurter, A., and Rehblun, L. I. (1985) The distribution of Tau in the mammalian central nervous system. J. Cell Biol. 101, 1371–1378 CrossRef Medline

8. Weingarten, M. D., Lockwood, A. H., Hwo, S. Y., and Kirschner, M. W. (1975) A protein factor essential for microtubule assembly. Proc. Natl. Acad. Sci. U.S.A. 72, 1858–1862 CrossRef Medline

9. Guo, T., Noble, W., and Hanger, D. P. (2017) Roles of Tau protein in health and disease. Acta Neuropathol. 133, 665–704 CrossRef Medline

10. Goedert, M., Spillantini, M. G., Potier, M. C., Ulrich, J., and Crowther, R. A. (1989) Cloning and sequencing of the cDNA encoding an isoform of microtubule-associated protein Tau containing four tandem repeats: differential expression of Tau protein mRNAs in human brain. EMBO J. 8, 393–399 Medline

11. Goedert, M., Spillantini, M. G., Jakes, R., Rutherford, D., and Crowther, R. A. (1989) Multiple isoforms of human microtubule-associated protein Tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease. Neuron. 3, 519–526 CrossRef Medline

12. Hutton, M., Lendon, C. L., Rizzu, P., Baker, M., Frolich, S., Houlden, H., Pickering-Brown, S., Chakraverty, S., Isaacs, A., Grover, A., Hackett, J., Adamson, J., Lincoln, S., Dickson, D., Davies, P., et al. (1998) Association of missense and 5′-splice-site mutations in Tau with the inherited dementia FTDP-17. Nature 393, 702–705 CrossRef Medline

13. Ghetti, B., Oblak, A. L., Boeve, B. F., Johnson, K. A., Dickerson, B. C., and Goedert, M. (2015) Frontotemporal dementia caused by microtubule-associated protein Tau gene (MAPT) mutations: a chameleon for neuro-pathology and neuroimaging. Neuropathol. Appl. Neurobiol. 41, 24–46 CrossRef Medline

14. Hasegawa, M., Smith, M. J., and Goedert, M. (1998) Tau proteins with FTD-17 mutations have a reduced ability to promote microtubule assembly. FEBS Lett. 437, 207–210 CrossRef Medline

15. Barghorn, S., Zheng-Fischhöfer, Q., Ackmann, M., Biernat, J., von Bergen, M., Mandellkow, E. M., and Mandellkow, E. (2000) Structure, microtubule interactions, and paired helical filament aggregation by Tau mutants of frontotemporal dementias. Biochemistry 39, 11714–11721 CrossRef Medline

16. Hong, M., Zhukareva, V., Vogelsberg-Ragaglia, V., Wszolek, Z., Reed, L., Miller, B. L., Geschwind, D. H., Bird, T. D., McKeel, D., Goate, A., Morris, J., C., Wilhelmsen, K. C., Schellenberg, G. D., Trojanowski, J. Q., and Lee, V. M. (1998) Mutation-specific functional impairments in distinct Tau isoforms of hereditary FTDP-17. Science 282, 1914–1917 CrossRef Medline

17. Magnani, E., Fan, J., Gasparini, L., Golding, M., Williams, M., Schiavo, G., Goedert, M., Amos, L. A., and Spillantini, M. G. (2007) Interaction of Tau protein with the dynactin complex. EMBO J. 26, 4546–4554 CrossRef Medline

18. Alonso Adel, C., Mederlyova, A., Novak, M., Grundke-Iqbal, I., and Iqbal, K. (2004) Promotion of hyperphosphorylation by frontotemporal dementia Tau mutations. J. Biol. Chem. 279, 34873–34881 CrossRef Medline

19. Nacharaju, P., Lewis, J., Easson, C., Yen, S., Hackett, J., Hutton, M., and Yen, S. H. (1999) Accelerated filament formation from Tau protein with specific FTD-17 missense mutations. FEBS Lett. 447, 195–199 CrossRef Medline

20. Goedert, M., Jakes, R., and Crowther, R. A. (1999) Effects of frontotemporal dementia FTD-17 mutations on heparin-induced assembly of Tau filaments. FEBS Lett. 450, 306–311 CrossRef Medline

21. Friedhoff, P., von Bergen, M., Mandelkow, E. M., Davies, P., and Mandellkow, E. (1998) A nuclelated assembly mechanism of Alzheimer paired helical filaments. Proc. Natl. Acad. Sci. U.S.A. 95, 15712–15717 CrossRef Medline

22. Prusiner, S. B. (1998) Nobel lecture: prions. Proc. Natl. Acad. Sci. 95, 13363–13383 CrossRef Medline

23. Eisenberg, D., and Jucker, M. (2012) The amyloid state of proteins in human diseases. Cell 148, 1188–1203 CrossRef Medline

24. Aouzzi, A., Heikenwalder, M., and Polymenidou, M. (2007) Insights into prion strains and neurotoxicity. Nat. Rev. Mol. Cell Biol. 8, 552–561 CrossRef Medline

25. Lewis, J., and Dickson, D. W. (2016) Propagation of Tau pathology: hypotheses, discoveries, and yet unresolved questions from experimental
and human brain studies. *Acta Neuropathol.* **131**, 27–48 CrossRef Medline

26. Ayers, J. I., Glasson, B. I., and Borchert, D. R. (2018) Prion-like spreading in tauopathies. *Biop. Psychiatry** **83**, 337–346 CrossRef Medline

27. Goedert, M., Masuda-Suzukake, M., and Falcon, B. (2017) Like prions: the propagation of aggregated Tau and α-synuclein in neurodegeneration. *Brain** **140**, 266–278 CrossRef Medline

28. Wu, J. W., Herman, M., Liu, L., Simoes, S., Acker, C. M., Figueroa, H., Steinberg, J. I., Margittai, M., Kayed, R., Zurzolo, C., Di Paolo, G., and Duff, K. E. (2013) Small misfolded Tau species are internalized via bulk endocytosis and anterogradely and retrogradely transported in neurons. *J. Biol. Chem.* **288**, 1856–1870 CrossRef Medline

29. Frost, B., Jacks, R. L., and Diamond, M. I. (2009) Propagation of Tau misfolding from the outside to the inside of a cell. *J. Biol. Chem.* **284**, 12845–12852 CrossRef Medline

30. Guo, J. L., and Lee, V. M. (2011) Seeding of normal Tau by pathological Tau conformers drives pathogenesis of Alzheimer-like tangles. *J. Biol. Chem.* **286**, 15317–15331 CrossRef Medline

31. Nonaka, T., Watanabe, S. T., Iwatsubo, T., and Hasegawa, M. (2010) Seeded aggregation and toxicity of α-synuclein and Tau: cellular models of neurodegenerative diseases. *J. Biol. Chem.* **285**, 34885–34898 CrossRef Medline

32. Clavaguera, F., Bolmont, T., Crowther, R. A., Abramowski, D., Frank, S., Probst, A., Fraser, G., Stalder, A. K., Beibel, M., Staufenbiel, M., Jucker, M., Goedert, M., and Tolnay, M. (2009) Transmission and spreading of tauopathy in transgenic mouse brain. *Nat. Cell Biol.* **11**, 909–913 CrossRef Medline

33. Clavaguera, F., Akatsu, H., Fraser, G., Crowther, R. A., Frank, S., Hench, J., Probst, A., Winkler, D. T., Reichwald, J., Staufenbiel, M., Ghetti, B., Goedert, M., and Tolnay, M. (2013) Brain homogenates from human tauopathies induce Tau inclusions in mouse brain. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 9535–9540 CrossRef Medline

34. Iba, M., Guo, J. L., McBride, J. D., Zhang, B., Trojanowski, J. Q., and Lee, V. M. (2013) Synthetic Tau fibrils mediate transmission of neurofibrillary tangles in a transgenic mouse model of Alzheimer’s-like tauopathy. *J. Neurosci.* **33**, 1024–1037 CrossRef Medline

35. Peeraer, E., Bottelbergs, A., Van Kolen, K., Stancu, I. C., Vasconcelos, B., Guo, J. L., and Lee, V. M. (2011) Seeding of normal Tau by pathological Tau aggregates and to toxic to cells but can be reversed by inhibitor drugs. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 20452–20457 CrossRef Medline

36. Berhe, M., Toth, L., Rizentin, M. T., and Shea, J. E. (2015) Tau assembly: theominated Tau repeats and the protofibril. *Neurobiol. Dis.* **60**, 41–51 CrossRef Medline

37. Frost, B., Jacks, R. L., and Diamond, M. I. (2009) Conformational diversity of wild-type Tau fibrils specified by templated conformation change. *J. Biol. Chem.* **284**, 3546–3551 CrossRef Medline

38. Elbaum-Garfinkle, S., Cobb, G., Compton, J. T., Li, X. H., and Rhodes, E. (2014) Tau mutants bind tubulin heterodimers with enhanced affinity. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 6311–6316 CrossRef Medline

39. Deramcoret, V., Lebert, F., Maurage, C. A., Fernandez-Gomez, F. J., Dujardin, S., Colin, M., Sergeant, N., Bué-Scherrer, V., Clot, F., Ber, I. L., Brice, A., Pasquier, F., and Bué, L. (2012) Clinical, neuropathological, and biochemical characterization of the novel Tau mutation P332S. *J. Alzheimer Dis.* **31**, 741–749 CrossRef Medline

40. Yu, X., Luo, Y., Dinkel, P., Zheng, J., Wei, G., Margittai, M., Nussinov, R., and Ma, B. (2012) Cross-seeding and conformational selection between three- and four-repeat human Tau proteins. *J. Biol. Chem.* **287**, 14950–14959 CrossRef Medline

41. Goedert, M., Jakes, R., Spillantini, M. G., Hasegawa, M., Smith, M. J., and Crowther, R. A. (1996) Assembly of microtubule-associated protein Tau into Alzheimer-like filaments induced by sulphated glycosaminoglycans. *Nature* **383**, 550–553 CrossRef Medline

42. Falcon, B., Cavallini, A., Angers, R., Glover, S., Murray, T. K., Barnham, L., Jackson, S., O’Neill, M. J., Isacs, A. M., Hutton, M. L., Szekeres, P. G., Goedert, M., and Bse, S. (2015) Conformation determines the seeding potencies of native and recombinant Tau aggregates. *J. Biol. Chem.* **290**, 1049–1065 CrossRef Medline

43. Jucker, M., and Walker, L. C. (2013) Self-propagation of pathogenic aggregates in neurodegenerative diseases. *Nature** **501**, 45–51 CrossRef Medline

44. Khlistunova, I., Biernat, J., Jucker, M., von Bergen, M. T., and Shea, J. E. (2015) Tau assembly: theominated Tau repeats and the protofibril. *Neurobiol. Dis.* **60**, 41–51 CrossRef Medline

45. Meyer, V., Dinkel, P. D., Luo, Y., Yu, X., Wei, G., Zheng, J., Eaton, G. R., Ma, B., Nussinov, R., Eaton, S. S., and Margittai, M. (2014) Single mutations in Tau modulate the populations of fibril conformers through seed selection. *Angew. Chem. Int. Ed. Engl.* **53**, 1590–1593 CrossRef Medline

46. Kfouri, N., Holmes, B. B., Jiang, H., Holtzman, D. M., and Diamond, M. I. (2012) Trans-cellular propagation of Tau aggregation by fibrillar species. *J. Biol. Chem.* **287**, 19440–19451 CrossRef Medline

47. Roberson, E. D. (2012) Mouse models of frontotemporal dementia. *Annu. Rev. Neurosci.* **35**, 837–849 CrossRef Medline

48. Li, A., Barker, S., and Wade-Martins, R. (2009) Knock-out and transgenic models of tauopathies. *Neurobiol. Aging* **30**, 1–13 CrossRef Medline

49. Sherrington, D., Colwill, R. M., and Wade-Martins, R. (2009) Knock-out and transgenic models of tauopathies. *Neurobiol. Aging* **30**, 1–13 CrossRef Medline
61. Ahmed, Z., Cooper, J., Murray, T. K., Garn, K., McNaughton, E., Clarke, H., Parhizkar, S., Ward, M. A., Cavallini, A., Jackson, S., Bose, S., Clavaguera, F., Tolnay, M., Lavenir, I., Goedert, M., et al. (2014) A novel in vivo model of Tau propagation with rapid and progressive neurofibrillary tangle pathology: The pattern of spread is determined by connectivity, not proximity. Acta Neuropathol. 127, 667–683 CrossRef Medline

62. Boluda, S., Iba, M., Zhang, B., Raiblé, K. M., Lee, V. M., and Trojanowski, J. Q. (2015) Differential induction and spread of Tau pathology in young PS19 Tau transgenic mice following intracerebral injections of pathological Tau from Alzheimer’s disease or corticobasal degeneration brains. Acta Neuropathol. 129, 221–237 CrossRef Medline

63. Jackson, S. J., Kerridge, C., Cooper, J., Cavallini, A., Falcon, B., Cella, C. V., Landi, A., Szekeress, P. G., Murray, T. K., Ahmed, Z., Goedert, M., Hutton, M., O’Neill, M. J., and Bose, S. (2016) Short fibrils constitute the major species of seed-competent Tau in the brains of mice transgenic for human P301S Tau. J. Neurosci. 36, 762–772 CrossRef Medline

64. Iba, M., McBride, J. D., Guo, J. L., Zhang, B., Trojanowski, J. Q., and Lee, V. M. (2015) Tau pathology spread in PS19 Tau transgenic mice following locus coeruleus (LC) injections of synthetic Tau fibrils is determined by the LC’s afferent and efferent connections. Acta Neuropathol. 130, 349–362 CrossRef Medline

65. Stancu, I. C., Vasconcelos, B., Ris, L., Wang, P., Villers, A., Peerer, E., Buist, A., Terwel, D., Baatsen, P., Oyelami, T., Pierrot, N., Casteels, C., Bormans, G., Kienlen-Campard, P., Octave, J. N., et al. (2015) Templated misfolding of Tau by prion-like seeding along neuronal connections impairs neuronal network function and associated behavioral outcomes in Tau transgenic mice. Acta Neuropathol. 129, 875–894 CrossRef Medline

66. Guo, J. L., Narasimhan, S., Changolkar, L., He, Z., Stieber, A., Zhang, B., Gathagan, R. J., Iba, M., McBride, J. D., Trojanowski, J. Q., and Lee, V. M. (2016) Unique pathological Tau conformers from Alzheimer’s brains transmit Tau pathology in nontransgenic mice. J. Exp. Med. 213, 2635–2654 CrossRef Medline

67. Miyasaka, T., Morishima-Kawashima, M., Ravid, R., Kamphorst, W., Nagashima, K., and Ihara, Y. (2001) Selective deposition of mutant Tau in the FTDP-17 brain affected by the P301L mutation. J. Neuropathol. Exp. Neurol. 60, 872–884 CrossRef Medline

68. Spillantini, M. G., Crowther, R. A., Kamphorst, W., Heutink, P., and van Swieten, J. C. (1998) Tau pathology in two Dutch families with mutations in the microtubule-binding region of Tau. Am. J. Pathol. 153, 1359–1363 CrossRef Medline

69. Aoyagi, H., Hasegawa, M., and Tamaoka, A. (2007) Fibrilligenic nuclei composed of P301L mutant Tau induce elongation of P301L Tau but not wild-type Tau. J. Biol. Chem. 282, 20309–20318 CrossRef Medline

70. Rossi, G., Bastone, A., Piccoli, E., Mazzoleni, G., Morbin, M., Uggetti, A., Giacone, G., Sperber, S., Beeg, M., Salmona, M., and Tagliavini, F. (2012) New mutations in MAPT gene causing frontotemporal lobar degeneration: biochemical and structural characterization. Neurobiol. Aging 33, 834.e1–834.e6 CrossRef Medline

71. Fitzgerald, A. W. P., Falcon, B., He, S., Murzin, A. G., Murshudov, G., Garringer, H. J., Crowther, R. A., Ghetti, B., Goedert, M., and Scheres, S. H. (2017) Cryo-EM structures of Tau filaments from Alzheimer’s disease. Nature 547, 185–190 CrossRef Medline

72. Mukrasch, M. D., Bibow, S., Korukottu, J., Jeganathan, S., Biernat, J., Griesinger, C., Mandelkow, E., and Zweckstetter, M. (2009) Structural polymorphism of 441-residue Tau at single residue resolution. PLoS Biol. 7, e34 Medline

73. Hasegawa, M., Smith, M. J., Iijima, M., Tabira, T., and Goedert, M. (1999) FTDP-17 mutations N279K and S305N in Tau produce increased splicing of exon 10. FEBS Lett. 443, 93–96 CrossRef Medline

74. Steward, A., Adhya, S., and Clarke, J. (2002) Sequence conservation in Ig-like domains: the role of highly conserved proline residues in the fibronectin type III superfamily. J. Mol. Biol. 318, 935–940 CrossRef Medline

75. Monselier, E., and Chiti, F. (2007) Prevention of amyloid-like aggregation as a driving force of protein evolution. EMBO Rep. 8, 737–742 CrossRef Medline

76. Jeganathan, S., von Bergen, M., Brutlach, H., Steinhoff, H. J., and Mani, A. W. P., Falconer, S., Brierley, S., Jackson, S., Bose, S., Clavaguera, F., Tolnay, M., Lavenir, I., Goedert, M., et al. (2014) A novel monoclonal antibody GM6 targets the pathological conformer of Tau. FEBS Lett. 586, 754–760 CrossRef Medline