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

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 allowed 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 withparkinsonism linked to chromosome 17 (FTD-P17)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-
Involving the stacking of monomer to Tau “seed” must first take place. This transition templating and seeding, the spontaneous aggregation of Tau FTDP-17 vary significantly between mutants and even within (R1–R4). Also depicted in black letters are the mutations in Tau that can cause FTDP-17 were used in the studies, as well as mutations in red letters that were generated for study purposes only. The sequence of the microtubule-binding domain (R1–R4) is shown specifically to illustrate the mutants located in this region, as well as areas of interest within the repeats. The PGGG sequence present in each MT-binding repeat is shown in green letters, and the PHF6/PHF6* domains important for Tau aggregation are shown in purple letters. The numbers on the right of each isoform corresponds to their amino acid length.

Results
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 (ON/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 < 0.01, and 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-

Figure 1. Schematic diagram of the three human Tau protein isoforms and the FTD-associated missense mutations used in this study. The three human Tau isoforms depicted and used for the studies are normally expressed in human brains as a result of alternative splicing of exons 2, 3, and 10 of the MAPT gene, which correspond to the N-terminal inserts N1 and N2, as well as MT-binding repeat R2, respectively, resulting in isoforms with three or four MT-binding repeats (R1–R4). Also depicted in black letters are the mutations in Tau that can cause FTDP-17 were used in the studies, as well as mutations in red letters that were generated for study purposes only. The sequence of the microtubule-binding domain (R1–R4) is shown specifically to illustrate the mutants located in this region, as well as areas of interest within the repeats. The PGGG sequence present in each MT-binding repeat is shown in green letters, and the PHF6/PHF6* domains important for Tau aggregation are shown in purple letters. The numbers on the right of each isoform corresponds to their amino acid length.

Even within the proposed mechanism of conformational templating and seeding, the spontaneous aggregation of Tau monomer to Tau “seed” must first take place. This transition involves the stacking of β-sheeted Tau into fibrillar “amyloid” Tau, which are the basis of larger Tau inclusions (36). Two hexapeptide motifs, coined PHF6* and PHF6 (paired helical filament 6), within Tau have been shown to promote aggregation, with PHF6 being necessary and sufficient for this β-pleated sheet formation (37–40).

Moreover, there is evidence to suggest that seeding occurs in a “strain-dependent” manner (4, 33, 41, 42) and that individual mutations in Tau may confer the ability to aggregate into conformationally distinct templates (43). Despite this, there is a lack of data in the field comparing the seeding propensities of multiple Tau mutants, and much of the current data utilize specific mutants P301L and P301S or truncated repeat domain (RD) Tau (29, 30, 44–47). However, disease phenotypes in FTDP-17 vary significantly between mutants and even within the same family (13). In this study, we examined the intrinsic propensity of a large array of FTDP-17 Tau mutants to be induced to aggregate when exogenously seeded in cultured cells. Furthermore, we assessed the seeding outcomes of additional synthetic mutants in close proximity to regions of structural interest to inform on the importance of specific motifs in regulating Tau aggregation. Our data show sharp differences in the ability for WT and most FTDP-17 Tau mutants to be seeded ex vivo when compared with mutants at Pro301 or Ser326, indicating that specific residues/regions within Tau are critical in modulating aggregation and seeding, thus highlighting novel targets for Tau therapeutics.
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 mutants that previously did not aggregate with WT K18 seeds also were not induced to aggregate with their respective mutant K18 fibrils (Fig. 3C; 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. 5A). Only the P301L Tau mutant demonstrated a propensity to be seeded in cells (Fig. 5B).

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
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-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 ON/4R Tau or WT 2N/4R Tau and treated with fibrillar WT K18 seeds as indicated above each lane (+). B, HEK293T cells were transfected with plasmids expressing P301L ON/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 ON/4R Tau and treated with WT K18 Tau fibrillar seeds as indicated above each lane (+).

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 Pro$^{301}$ site (P301L, P301S, and P301T) and to a lesser extent at the Ser$^{320}$ 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 Pro$^{301}$ 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 intercellular 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 Pro$^{301}$ 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 Pro$^{301}$ and Ser$^{320}$, 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 inclusions 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

Figure 5. P301L is unique in its ability to be seeded compared with other Pro-to-Leu substitutions in the PGGG sequences of each MT-binding repeat. A, each Pro residue within the PGGG motifs of the MT-binding repeats was individually mutated to an Leu in full-length 2N/4R Tau. B, HEK293T cells were transfected with plasmids expressing P270L, P301L, P332L, or P364L 2N/4R Tau as indicated above each lane and treated with WT K18 Tau fibrillar seeds. The results are in duplicate. The mobilities of molecular mass markers in kDa are indicated on the left.

Figure 6. Mutations at the Pro301 site uniquely drive Tau aggregation with seeding in HEK293T cells compared with other FTD mutants near the PHF6 site. A, Tau residues Pro301, Gly303, Gly304, and Ser305, which immediately precede the PHF6 motif, were individually mutated to the FTDP-17 mutants P301T, G303V, G304S, or S305N in 0N/4R Tau. B, HEK293T cells were transfected with plasmids expressing P301T, G303V, G304S, or S305N 0N/4R Tau as indicated above each lane and treated with WT K18 Tau fibrillar seeds. The cells were biochemically fractionated into Triton-soluble and Triton-insoluble fractions. Immunoblots of total Tau (antibody H150) are shown for each fraction. The results are in triplicate. The mobilities of molecular mass markers in kDa are indicated on the left.
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, Pro379, is in the same position preceding PHF6 in 3R Tau as Pro301 is 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, Ser	extsuperscript{320} 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 Pro	extsuperscript{301} as well as S320F being permissive, whereas the other mutants tested were refractory. This finding suggests that Pro	extsuperscript{301} 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

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B

P301L/S320F Insoluble Tau with and without Seeding

C

Total Tau (3R26)  AT8  DAPI Merge

WT

P301L

P301S

S320F

P310L/S320F

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Triton Soluble  Triton Insoluble

AT8/Total Tau

E

Double Mutant Tau Aggregation without Seeding

F

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ON/4R P301L/S320F vs P301S/S320F
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 Tau double mutant endogenously aggregates in HEK293T cells.

**Figure 9.** The 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.

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**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.
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 ON/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 fluorometry 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 glycine, 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 ON/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-hydroxyethyl)-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-tosyl-lysine 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% bromophenol 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 sonications 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 full-length recombinant ON/3R 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 phospho-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 Fisher 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 autofluorescence reagent (Millipore, Billerica, MA) was applied to cells for 5 min, followed by washing with 40% ethanol. The cells were incubated with 0.0125% thioflavin S in 50% ethanol, PBS for 3 min in the dark, followed by sequential washing with 50% ethanol and PBS. 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.

**Author contributions**—K. H. S. conducted experiments and contributed to the experimental designs and writing of the manuscript; C. L. C. assisted in some cell culture studies and writing of the manuscript; Z. A. S. conducted experiments; P. C. and T. E. G. contributed to the experimental designs; and B. I. G. contributed to writing the manuscript and the experimental designs.

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**References**


