Cryo-EM structure of pro-aggregant P301L/S320F double-mutant tau filaments formed in mouse brains following peripheral AAV delivery

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Abstract

Tauopathies are characterized by the accumulation of abnormally phosphorylated tau filaments, with disease-specific folds revealed by cryo-electron microscopy (cryo-EM). Here, we delivered a recombinant human tau carrying the P301L/S320F double mutation into *App*$^{NL\text{-}G\text{-}F}$/*MAPT* double knock-in mice using a blood-brain-barrier-permeable AAV (AAV-PHP.eB), enabling rapid induction of tau aggregation *in vivo*. The mutant tau was systemically administered via retro-orbital injection, providing a minimally invasive approach for widespread neuronal transduction. Phosphorylated tau aggregation, as well as seeding activity, were observed without the need for exogenous seeds. Our cryo-EM analysis resolved a novel filament fold incorporating residues 279-329, in which the P301L and S320F mutations introduced stabilizing interactions that reinforced filament assembly. This conformation was distinct from previously reported folds, including Alzheimer and Pick tau filaments. Moreover, the filaments adopted a more compact architecture than those observed in patient-derived samples or other model mice. This finding demonstrates that P301L/S320F double-mutant tau adopts a structurally unique, highly aggregation-prone fold, and that this system provides a rapid platform for modeling tau filament formation.

Keywords

Alzheimer disease, cryo-EM, Pick disease tau, tauopathy

Introduction

Tauopathies comprise a spectrum of neurodegenerative disorders defined by intracellular inclusions of abnormally phosphorylated and aggregated microtubule-associated protein tau, which is encoded by the Microtubule-Associated Protein Tau *(MAPT*) gene. These disorders include Alzheimer disease (AD), frontotemporal dementia (FTD), Pick's disease (PiD), and corticobasal degeneration. In these conditions, the accumulation of tau filaments, known as neurofibrillary tangles (NFTs), is closely associated with progressive neurodegeneration and synaptic dysfunction [1,2].

Tau is an intrinsically disordered protein that stabilizes and dynamically regulates microtubules. The human *MAPT* gene generates six isoforms through alternative splicing, which differ in the number of microtubule-binding repeats (three-repeat (3R) or four-repeat (4R)) and the presence or absence of N-terminal inserts (Supplementary Fig. 1) [1]. In the adult human brain, 3R and 4R tau isoforms are expressed at approximately equal ratios, whereas murine brains predominantly express 4R tau [3]. The binding of tau to microtubules is tightly regulated by its phosphorylation state [4], and dysregulation of this balance is thought to underlie the onset of pathological aggregation. Notably, the discovery of pathogenic MAPT mutations in families with FTDP-17 provided strong genetic evidence that tau dysfunction is sufficient to cause neurodegeneration, and linked tau aggregation pathology to the pathogenesis of tauopathies [5,6].

In AD brains, aggregated tau forms paired helical filaments (PHFs) [7], which assemble into NFTs. This filament structure exhibits an AD-specific fold that reflects the disease's molecular identity [8–10]. These filaments propagate in a prion-like manner

between neurons, driving the stepwise regional spread of NFTs described by Braak staging [11]. The progression of NFT pathology correlates strongly with neuronal loss and cognitive decline [11,12], suggesting that tau filament accumulation directly drives neurodegeneration. Filamentous tau is not only hyperphosphorylated but also modified by ubiquitination [13–15], acetylation [16,17], and SUMOylation [18], among other post-translational modifications.

The advent of cryo-electron microscopy (cryo-EM) technology has enabled near-atomic structural determination of tau filaments isolated from tauopathy patient brains [8–10]. These structural studies have substantially advanced our understanding of the molecular mechanisms underlying tau aggregation and the pathological heterogeneity of tau-related disorders.

In parallel, *in vivo* modeling of tau pathology has advanced considerably. Transgenic mice expressing tauopathy-associated mutations [19] and models involving intracerebral inoculation of patient-derived [20] or recombinant tau filaments [21,22] have provided invaluable insights into the mechanisms of tau propagation and filament formation. More recently, expression of the P301L/S320F double-mutant tau—combining the frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17)–associated P301L mutation [23,24] and the PiD-associated S320F mutation [25,26]—via adeno-associated virus (AAV) injection into neonatal mouse ventricles has been shown to induce robust tau aggregation within a short period [27]. This approach has attracted attention as a rapid and reproducible model of tau filament formation.

In this study, we sought to develop a less invasive and efficient *in vivo* model of

92 AD-related tau pathology and to elucidate the structural determinants underlying the
93 marked aggregation propensity of tau by analyzing the filamentous structures formed in
94 this model. To this end, we utilized the AAV-PHP.eB system, a modified AAV9 variant
95 that enables highly efficient central nervous system transduction following systemic
96 administration, and expressed P301L/S320F mutant human tau *via* retro-orbital venous
97 injection [28,29]. The AAV-PHP.eB serotype is reported to cross the blood–brain barrier
98 following systemic administration efficiently and to robustly transduce cortical and
99 hippocampal neurons, offering a less invasive alternative to direct intracranial injection.
100 For the recipient mouse, we used humanized tau knock-in mice with an amyloid plaque–
101 forming background ($App^{NL\text{-}G\text{-}F}$/*MAPT* double knock-in mice), in which the murine *App*
102 and *Mapt* genes are replaced by the human *APP* exons carrying familial AD-linked
103 mutations as well as the *MAPT* locus, respectively [30]. This mouse model expresses both
104 3R and 4R human tau isoforms under endogenous control [31], with a comparable balance
105 of tau isoforms to that observed in the human brain and also accumulates amyloid-β (Aβ)
106 [30], thereby allowing investigation of tau aggregation within a genetic and pathological
107 milieu that closely resembles the human AD brain.

108 Following systemic administration of AAV-PHP.eB encoding P301L/S320F
109 mutant human tau, phosphorylated tau–positive aggregates were induced within
110 approximately 1.5 months in a minimally invasive manner. Biochemical and cryo-EM
111 analyses of sarkosyl-insoluble fractions revealed tau filaments with a near-atomic fold.
112 These findings provide mechanistic insight into how the P301L/S320F mutation enhances
113 tau aggregation propensity and support the emergence of a unique filament structure
114 formed in a human tau background.

115 **Material and methods**

116 **Mouse and cell culture**

117 *App*$^{NL\text{-}G\text{-}F}$/*hMAPT* double knock-in mice were maintained on a C57BL/6J
118 background [32]. HEK293A cell was a kind gift from Dr. Hidenori Ichijo (The University
119 of Tokyo)[33,34]. Biosensor (BS) cells, which provide a fluorescence resonance energy
120 transfer (FRET) signal by seeding-competent tau filaments, were purchased from ATCC
121 (CRL-3275). The cells were cultured as previously described [35,36].

122

123 **Extraction of the tau filaments from culture cells**

124 Cells were resuspended in 1 mL of phosphate-buffered saline (PBS) and
125 centrifuged at 4,500 × g for 5 min at 25 °C. The resulting pellets were resuspended in 300
126 μL of lysis buffer containing 1% sarkosyl in A68 buffer (10 mM Tris–HCl, pH 7.5, 10%
127 sucrose, 0.8 M NaCl, and 1 mM EGTA) [37], followed by sonication using HG-30
128 homogenizer (Hitachi). An additional 300 μL of lysis buffer was then added, and the
129 samples were centrifuged at 168,000 × g (S55A2-2698 roter, Himac) for 20 min at room
130 temperature. The supernatant (soluble fraction) and pellet (insoluble fraction) were
131 collected separately [38,39]. Protein concentrations in the supernatant were determined
132 using a BCA assay, and 5× SDS sample buffer was added. The pellet fractions were
133 resuspended in 50 μL of 2 × SDS sample buffer for western blotting, followed by brief
134 sonication. All samples were heated at 100 °C for 5 min prior to electrophoresis. Prepared
135 samples were separated by SDS–PAGE and transferred onto polyvinylidene difluoride
136 (PVDF) membranes (Millipore) as described[35]. The list of primary antibodies and their

137 dilution ratios is provided in Tables S1 and S2.

138

139 **Fluorescence resonance energy transfer (FRET)**

140 BS cells were seeded into 24-well plates, and 20 μg of mouse brain homogenate
141 was added to each well. The homogenate was mixed with Lipofectamine 2000 (Thermo
142 Fisher Scientific) at 1.47 μL per well, incubated for 15 min at room temperature, and then
143 applied to the cells. After treatment, the cells were cultured for 72 h. After incubation,
144 cells were washed with PBS, detached by trypsinization, and resuspended in PBS
145 containing 20% FBS. FRET signals were measured by flow cytometry using the MA900
146 cell sorter (SONY), with the increase in acceptor fluorescence upon donor excitation as
147 the readout. The percentage of FRET-positive cells was calculated as described [40].

148

149 **Electron microscopy**

150 For electron microscopy of cultured cell-derived tau filaments, pellet fractions
151 were resuspended in 50 μL of Tris buffer (10 mM Tris-HCl, pH 7.4, 1 mM DTT, 1 mM
152 EGTA). After sarkosyl fractionation of brain homogenates, the sarkosyl-insoluble pellet
153 was resuspended in 10 μL of physiological saline, and 1.5 μL was applied onto collodion-
154 coated 200 mesh copper grids (Nisshin EM Co). Negative staining was performed with
155 2% phosphotungstic acid. Samples were examined using a transmission electron
156 microscope (JEM-1200EXII, JEOL) operated at an acceleration voltage of 80 kV. Images
157 were acquired at magnifications ranging from 12,000× to 200,000× using a CCD camera
158 (Veleta, JEOL) controlled with iTEM software (Olympus Soft Imaging Solutions). For

159 immuno-electron microscopy, purified tau filaments were adsorbed onto collodion-coated
160 200 mesh copper grids (Nisshin EM Co.) for 30 s, followed by blocking with PBS
161 containing 1% gelatin for 10 min and incubation with primary antibodies for 1 h. The
162 grids were then washed with blocking buffer and incubated with 10 nm gold-conjugated
163 anti-rabbit IgG or 5 nm gold-conjugated anti-mouse IgG (1:50 in blocking buffer) for 1 h
164 (Table S1, 2). After washing with water, the samples were negatively stained with 2%
165 PTA for 90 s[41].

166

167 **Recombinant AAV system**

168 A recombinant AAV system of the PHP.eB [42] serotype was used to express
169 human 1N4R MAPT carrying the P301L and S320F mutations. The expression cassette
170 consists of the human MAPT cDNA with the P301L/S320F mutation under the control of
171 the human synapsin I promoter, followed by a woodchuck hepatitis virus post-
172 transcriptional regulatory element (WPRE) and a polyadenylation signal. The expression
173 cassette was inserted into the pAAV-hSyn1-eYFP backbone (Addgene plasmid #117382)
174 [29]. The eYFP coding sequence was removed by digestion with EcoRI and KpnI, and
175 the MAPT insert was introduced using HiFi DNA Assembly (New England Biolabs). The
176 recombinant AAV viruses used for mouse injections were produced and purified by the
177 Department of Neurodegenerative Medicine, Gunma University Graduate School of
178 Medicine. Recombinant virus production was carried out in HEK293T cells using the
179 triple plasmids; pAAV-hSyn1-tau-WPRE, pHelper (Agilent Technologies), pAAV-
180 PHP.eB [43,44]. AAV-PHP.eB were produced using the ultracentrifugation method
181 described in a previous paper[43]. For *in vivo* experiments, recombinant viruses were

182 delivered to mice via the retro-orbital sinus at a titer of 2–5 × 10^{11} vg per mouse.

183

184 **Immunohistochemistry and image quantification**

185 Immunohistochemical analyses were performed as previously described [45].
186 Stained sections were examined using fluorescence microscopy (BZ-X800, Keyence).
187 High-resolution tiled images of AT8, AT180, PHF-1, and pS396 were reconstructed by
188 stitching without compression, and exported using the Keyence analyzer package (BZ-X
189 series). For the quantitative analysis of protein associations with phospho-tau lesions, we
190 used a 20 × objective lens. Regions of interest were manually selected and cropped from
191 the acquired images based on anatomical landmarks. The percentage of areas stained by
192 AT8, AT180, PHF-1, and pS396 in selected brain regions was quantified using the BZ-
193 X800 Analyzer Hybrid Cell Count Module (Keyence). Parameters were set to a threshold
194 of control and applied automatically to all images within the proper comparisons. To
195 minimize the influence of background staining and potential artifacts, AT8- or pS396-
196 positive regions exceeding 10,000 μm^2 in area were excluded from the quantification.
197 This threshold was set to filter out abnormally large signals likely due to non-specific
198 staining. Statistical analyses were conducted using R software (version 4.5.1). Group
199 means were compared using one-way analysis of variance (ANOVA), followed by
200 Tukey's Honest Significant Difference (HSD) test for post-hoc analysis (*$p < 0.05$).

201

202 **Sampling of mouse brains for western blotting**

203 Fractionation of mouse brains for western blotting was performed as previously

204 described with some modifications [46]. Half brains were homogenized in 10 volumes of
205 homogenization buffer (10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.8 M
206 NaCl, 10% sucrose, supplemented with PhosSTOP (Sigma) and a protease inhibitor
207 cocktail (cOmplete™ ULTRA, 1 tablet/50 mL; Roche) using a HG-30 homogenizer. The
208 homogenates were ultracentrifuged at 168,000 × g for 20 min at 25 °C with an S55A2-
209 2698 rotor. Sarkosyl was then added to the supernatant at a final concentration of 2%,
210 followed by shaking at 37 °C for 30 min. After sonication, the samples were centrifuged
211 at 20,000 × g for 20 min at 25 °C with an S55A2-2698 rotor, and the resulting supernatant
212 was further ultracentrifuged at 168,000 × g for 20 min at 25 °C using an S55A2-2698
213 rotor. The pellet was resuspended in homogenization buffer containing 1% sarkosyl and
214 subjected to the same centrifugation cycle. The final pellet was resuspended in Tris buffer,
215 mixed with an equal volume of 2× SDS sample buffer, and boiled at 100 °C for 10 min
216 to obtain the sarkosyl-insoluble fraction. Supernatants obtained at each centrifugation
217 step were combined with sample buffer and used as sarkosyl-soluble fractions.

218

219 **Extraction of the tau filaments from mouse brains**

220 Mouse brains were weighed and homogenized in 10 volumes (w/v) of
221 homogenization buffer. Homogenization was performed using the HG-30 homogenizer
222 (Hitachi). The homogenates were centrifuged at 11,400 × g (SW41Ti rotor, Beckman
223 Coulter) for 10 min at room temperature, and the supernatants were collected. The
224 supernatants were then centrifuged at 183,000 × g (SW41Ti rotor) for 4 h at 25 °C. The
225 resulting pellets were resuspended in 1% sarkosyl solution prepared with half the volume
226 of the original tissue buffer and incubated at 37 °C for 60 min with shaking. After

227 incubation, the suspensions were centrifuged at 12,100 × g for 20 min at 25 °C (S55A2-
228 2698 rotor), and the supernatants were collected. These supernatants were further
229 centrifuged at 168,000 × g for 20 min at 25 °C (S55A2-2698 rotor), and the resulting
230 pellets were resuspended in 10 mL of tissue buffer. To remove contaminating components,
231 the suspension was treated with 0.2 mg/mL collagenase (Sigma-Aldrich), 2 mM $MgCl_2$,
232 0.5 mM $CaCl_2$, and 200 U/mL DNase I (Takara), followed by shaking at 200 rpm at 37 °C
233 for 1 h. After enzymatic digestion, the final concentration of NaCl was adjusted to 1 M,
234 and Triton X-100 was added to 0.5% (v/v). The mixture was further incubated at 37 °C
235 for 30 min with shaking. To precipitate proteins, three volumes of pre-chilled methanol
236 at –30 °C were added, and the samples were incubated for 30 min at –30 °C, followed by
237 centrifugation at 20,000 × g for 30 min at 4 °C. The resulting pellets were resuspended in
238 10 mL of 5% sarkosyl solution. Subsequently, the following fractionation steps were
239 repeated: centrifugation at 11,400 × g for 20 min at 25 °C to remove debris, followed by
240 centrifugation of the supernatants at 168,000 × g for 30 min at 25 °C (S55A2-2698 rotor).
241 The resulting pellets were resuspended in 2 mL of 5% sarkosyl solution, homogenized by
242 passing through a 25-gauge needle (Terumo), and sequentially treated with 2% and 1%
243 sarkosyl solutions in the same manner. At each step, the suspensions were mixed
244 thoroughly by pipetting (100 times) before centrifugation. Finally, the pellets obtained
245 were resuspended in 500 μL of 1% sarkosyl solution, homogenized again by pipetting,
246 and centrifuged at 168,000 × g for 30 min (S55A2-2698 rotor). The final pellets,
247 containing the sarkosyl-insoluble tau filaments, were collected and stored at –80 °C until
248 use.

249

250 **Cryo-Electron Microscopy (cryo-EM)**

251 Samples were resuspended in cryo-EM buffer (50 mM Tris-HCl (pH 7.4), 1 mM
252 DTT, 0.04% sarkosyl [47]). A 2 µl aliquot of the sample was applied to each side of
253 freshly glow-discharged UltrAuFoil R1.2/1.3 300 mesh grids (Quantifoil Micro Tools
254 GmbH). The grids were blotted from both sides for 4 seconds at 12°C under 100%
255 humidity and subsequently plunge-frozen in liquid ethane using a Vitrobot Mark IV
256 (Thermo Fisher Scientific).

257 Images were recorded on a CRYO ARM 300 II (JEOL, Tokyo, Japan), operated
258 at 300 kV, equipped with an Omega filter whose slit width was set to 20 eV, and a Gatan
259 K3 direct electron detector in correlated-double sampling (CDS) mode. The nominal
260 magnification was set to 60,000×, yielding a physical pixel size of 0.878 Å/pixel. Movies
261 were acquired using the SerialEM software [48], with a target defocus range of -0.8~-1.8
262 µm. Each movie was recorded with a total electron dose of 50 e^{-}/ $Å^2$, divided into 50
263 frames.

264

265 **Data processing of Cyro-EM data**

266 Raw movie frames were motion-corrected, and the contrast transfer function
267 (CTF) was estimated using the Patch CTF function on cryoSPARC v4.7.1[49]. Fibrils
268 were automatically picked using the filament tracer tool, and segments were extracted
269 with a box size of 420 pixels scaled to 300 pixels and an inter-box distance set to 10% of
270 the box size. Several rounds of 2D classification were performed to exclude bad classes
271 that lacked discernible helical twists and non-amyloid contaminants. Initial helical

parameters were estimated from 2D class averages with a box size of 1000 pixels scaled to 750 pixels, and an initial reference was generated using the relion_helix_inimodel2d program [50]. The helical refinement was performed using local helical parameter searches in cryoSPARC. After further local and global CTF refinement, reference-based motion correction, and 3D classification, the particle coordinates were converted into Relion STAR format using UCSF pyem [51] with in-house modifications to convert metadata generated by the filament tracer tool to Relion's counterparts. Relion v5.0 [52,53] was used for further CTF refinement and the 3D auto-refine with local helical parameter searches.

Model building and refinement

An initial model was generated using ModelAngelo[53], and the model was refined using Coot v0.9.8.1 [54], Phenix v1.21.2-5419 [55], and Servalcat v0.4.105 [56]. The refined models were validated using Phenix. Image rendering was performed using UCSF ChimeraX [57] and MaskChains tool [58]. The schematic figures were produced using atom2svg.py [59].

289 **Result**

290 **P301L/S320F double-mutant tau induced tau aggregation in cultured cells.**

291 In the human brain, six tau isoforms are generated by combinations of the
292 number of microtubule-binding repeats,3R or 4R, and the presence of 0, 1, or 2 N-
293 terminal insertions (Fig. S1).To partially recapitulate this isoform expression of the 3R
294 and 4R pattern, we generated HEK293A-derived cell lines that stably express the
295 representative 3R and 4R tau isoforms, 1N3R and 1N4R human tau. We then transfected
296 these cells with a plasmid encoding the 1N4R P301L/S320F mutant tau (Fig. 1a). Three
297 days after transfection, cells were collected and separated into sarkosyl-soluble and -
298 insoluble fractions (Fig. 1b). Western blot analysis showed that P301L/S320F double-
299 mutant tau was prominently detected in the sarkosyl-insoluble fraction, exhibiting a
300 higher signal compared with wild-type (WT) tau. This finding indicates that the
301 P301L/S320F mutation promoted the tau aggregation in cells, as previously described
302 (Fig. 1c, d). Furthermore, electron microscopy of the sarkosyl-insoluble fraction revealed
303 fibrillar structures with a twisted morphology and a diameter of approximately 20 nm
304 (Fig. 1e). Taken together, these findings suggest that P301L/S320F tau can promote
305 highly intracellular aggregation and filament formation.

306

307 **AAV-PHP.eB–mediated delivery of P301L/S320F double mutant tau induces intracellular tau**
308 **aggregation in the mouse brain.**

309 In AD, tau aggregation is known to occur subsequent to the accumulation of Aβ.
310 To model this coexisting Aβ and tau pathology *in vivo*, we employed $App^{NL\text{-}G\text{-}F}$/*hMAPT*

double knock-in mice [30,32]. This mouse line carries a knock-in human *APP* allele harboring the Swedish (KM670/671NL), Iberian (I716F), and Arctic (E693G) mutations ($App^{NL\text{-}G\text{-}F}$), combined with a knock-in human *MAPT* (a.k.a., *hMAPT*) allele in which the endogenous mouse *Mapt* locus is replaced. In $App^{NL\text{-}G\text{-}F}$ mice, age-dependent deposition of human Aβ begins around 2 months of age and reaches saturation by approximately 8 months. To induce tau aggregation under an Aβ-rich background, 9–11-month-old $App^{NL\text{-}G\text{-}F}$/*hMAPT* mice were intravenously injected via the retro-orbital sinus with a recombinant AAV-PHP.eB expressing human P301L/S320F double-mutant tau under the human synapsin I promoter. Control groups included mice injected with WT human tau and non-injected mice (Fig. 2a). After two months, the brains were collected, hemisected, and used for immunohistochemical and biochemical analyses (Fig. 2a). AT8 immunoreactivity, a marker of pathological tau phosphorylation [60], revealed prominent AT8-positive signals in the CA2 region of the hippocampus and in layer II of the cerebral cortex in mice expressing P301L/S320F double-mutant tau (Fig. 2b). Quantification confirmed significant AT8 accumulation in the medial parietal association area (MPtA) (Fig. 2c, d) and hippocampal CA2 (Fig. 2e, f). Additionally, other antibodies detecting the phosphorylation-dependent tau pathology, such as AT180 [61], anti-pS396 [62], and PHF-1 [60], likewise detected exclusively in mice expressing P301L/S320F tau (Fig. S2–4). Collectively, these findings indicate that P301L/S320F tau robustly induces the formation of phosphorylated tau aggregates *in vivo* without exogenous seeding.

332 **Overexpressed P301L/S320F double-mutant tau in the mouse brain acts as a seed with strong**
333 **aggregation propensity.**

334 We then biochemically analyzed the right hemisphere of the brains from *App*$^{NL-}$
335 $^{G-F}$/*hMAPT* mice injected with AAV expressing P301L/S320F tau by sequential sarkosyl
336 fractionation and western blotting (Fig. 3b). Quantification of the amount of the
337 aggregated tau in the sarkosyl-insoluble fraction relative to the soluble fraction was
338 markedly increased in mice expressing P301L/S320F double-mutant tau compared with
339 those expressing WT tau (Fig. 3c). Notably, endogenous 3R as well as 4R tau were also
340 detected within the insoluble fraction. Furthermore, murine endogenous tau was also
341 detected in the sarkosyl-insoluble tau fraction in mice expressing P301L/S320F double-
342 mutant tau (Fig. S5a.b). Pellets from the sarkosyl-insoluble fractions were analyzed by
343 negative-stain electron microscopy using phosphotungstic acid and by immuno-electron
344 microscopy (Fig. 3d). Only in the P301L/S320F group, fibrillar structures were observed
345 that were immunoreactive for TauC4 [8], AT8, and anti-pS396 antibodies (Fig. 3e-g).
346 Finally, we assessed the seeding activity of brain lysates using BS cells [40]. After
347 transfection of the mouse brain homogenates, the proportion of FRET-positive cells was
348 measured by flow cytometry only in the P301L/S320F group. In contrast, WT tau and
349 uninjected controls showed no detectable FRET signal (Fig. S6). These data suggested
350 that the double-mutant tau acted as a seed for endogenous tau protein regardless of repeat
351 numbers or species.

352

353 **Structural analysis of tau filaments derived from mice expressing P301L/S320F mutant tau by**
354 **cryo-EM**

355 To characterize the ultrastructure of tau assemblies formed *in vivo*, we analyzed
356 the sarkosyl-insoluble tau filaments isolated from the mouse brain expressing the
357 P301L/S320F double-mutant tau (PDB: 22JY) by cryo-EM (Fig. S7). The predominant
358 filaments consisted of a single helical filament type (Fig. 4a, b), and the final
359 reconstructed map reached a resolution of 2.24 Å. The filaments exhibited an approximate
360 diameter of 7 nm and a helical pitch of ~35 nm (Fig. 4a, b). The ordered core comprised
361 residues 279–329 of tau, forming five β-strands connected by loops and turns (Fig. 4c, d).
362 These β-strands corresponded to β1 (N279–S285), β2 (H299–L301), β3 (S305–Y310),
363 β4 (V319–C322), and β5 (L325–H329). The five β-strands were stacked to form a layered
364 β-sheet architecture that contributed to filament stabilization. The β-strands were linked
365 by flexible loops, giving the overall structure a gently curved, folded conformation (Fig.
366 4c, d).

367 In addition, three prominent extra densities protruding from the filament surface
368 were observed near lysine residues; although consistently positioned, their molecular
369 identities remain unclear (Fig. 5a, b). The pathogenic substitutions, P301L and S320F,
370 were each located within the second and fourth β-strands of the core, respectively. P301L
371 formed hydrophobic interactions with I328 (Fig. 5c), whereas S320F was buried within a
372 loop region, where its aromatic ring engaged in Z-axis π–π stacking that further stabilized
373 the filament. The aromatic ring of S320F additionally established hydrophobic contacts
374 with V309, P312, L315, and V318, likely increasing the stability of the entire loop
375 structure. The Q307 side-chain amide groups (–$CONH_2$) were positioned to form

376 hydrogen bonds between adjacent layers aligned along the helical axis, further supporting
377 inter-layer stabilization (Fig. 5d).

378 These hydrophobic interactions are not present in WT tau, where P301 and S320
379 are normally solvent-exposed. Therefore, the incorporation of these residues into the
380 filament core represents a structural feature unique to the mutant filaments.

381

382 **The structure of P301L/S320F mutant tau filaments was distinct from previously reported**
383 **patient-derived tau filament structures.**

384 To compare the structure of the P301L/S320F mutant tau filaments obtained in
385 this study (magenta) with the structure of tau filaments derived from an FTDP-17 patient
386 carrying the P301L mutation (PDB 9GG0; cyan) [63], we performed structural alignment.
387 The results showed limited similarity, with an RMSD of 4.23 Å over 40 aligned residues
388 (Fig. 6a). In the patient-derived fold, the filament bends at the P301L position, whereas
389 in the P301L/S320F mutant, the filament bends at a different region. Moreover, in our
390 structure, a hydrophobic interaction between P301L and I328—absent in the patient
391 P301L fold—is observed. This is likely due to the S320F-induced folding of a unique
392 loop through extensive hydrophobic interactions, bringing P301L into proximity with
393 I328. The combined effects of P301L- and S320F-mediated interactions appear to rigidify
394 the L301–H329 region, enabling the formation of a second compact loop in which V306
395 and I308 interlock with L282, L284, and V287 (Fig. 5a, b).

396 In patients with FTDP-17 carrying the S320F mutation [25,26], Pick bodies
397 composed of 3R tau are observed as a characteristic pathology [64]. We therefore

398 compared our structure with the tau filament derived from PiD (PDB 6GX5; cyan)[65].
399 The residue numbering corresponds to the 441-aa human tau isoform, in which the R2
400 repeat (residues 275–305) is absent. The similarity was again low, with an RMSD of 3.87
401 Å over 42 residues (Fig. 6b).

402 Likewise, comparison with AD-derived PHFs (PDB 6HRE; cyan) [66] showed
403 similarity only in a short loop near S320 (RMSD = 2.09 Å over 16 residues), whereas the
404 overall fold was clearly distinct (Fig. 6c). Taken together, the fold obtained here is distinct
405 from previously reported tau filament structures, including the Pick fold, the three-lobed
406 fold, nor the Alzheimer PHF fold. Importantly, it also differs from the patient-derived
407 P301L fold, suggesting that the combination of P301L and S320F mutations stabilizes a
408 novel filament conformation not observed in any previously described human tauopathy.

409

410 **Discussion**

411 P301L/S320F double-mutant has already been established as a rapidly
412 developing tauopathy model characterized by aggregation of and accumulation of
413 phosphorylated tau in neuronal cells, NFT pathology, cognitive impairment, synaptic
414 protein abnormalities, and deficits in contextual memory [27]. In contrast, conventional
415 tau transgenic mice typically require more than 6 months to develop detectable tau tangles.
416 Therefore, AAV-mediated expression of P301L/S320F double-mutant tau can be regarded
417 as a much more robust model of tau pathology. We further demonstrate that peripheral
418 administration of the recombinant AAV-PHP.eB encoding P301L/S320F mutant tau
419 rapidly induced the neuronal intracellular aggregation and filament formation in mouse

420 brains without the injection of exogenous seeds to mouse brains. Owing to this
421 combination of mutations, P301L/S320F double-mutant tau provides an excellent and not
422 highly invasive tauopathy model in which tau pathology is markedly accelerated
423 following AAV delivery.

424 Our cryo-EM analysis revealed that the extracted filaments consisted
425 predominantly of single helical filaments, and the final reconstructed map reached a
426 resolution of 2.24 Å. The ordered core was composed of tau residues 279–329, in which
427 five β-sheets connected by loop and turn structures formed a compact three-folded
428 architecture. This fold accounted for 55.9% of all tau filaments with clearly resolved β-
429 sheets observed by cryo-EM. All filaments in which a defined helical pitch could be
430 confidently determined exhibited the structure (Fig. 5). The remaining 44.1 % of
431 filaments displayed a longer helical pitch or non-helical and disordered β-sheets, making
432 structural determination difficult. The fold observed for P301L/S320F mutant tau in this
433 study is distinct from the known tau folds reported in AD and PiD, nor from the structure
434 derived from P301L patient brains (Fig. 6). This indicates that the combination of P301L
435 and S320F generates a newly stabilized and unique three-dimensional conformation.
436 Such structural insights provide important clues for understanding the diversity of tau
437 filaments and the molecular basis of filament formation in tauopathies.

438 The S320F mutation is clinically associated with pathology resembling sporadic
439 PiD, in which both straight and twisted tau filaments have been reported. However,
440 whereas PiD inclusions are composed exclusively of 3R tau, the insoluble tau fraction
441 from S320F carriers contains both 3R and 4R tau isoforms [25]. Although S320F carriers
442 exhibit PiD-like straight filament formation and develop frontotemporal dementia, the

443 coexistence of 3R and 4R tau suggests that the underlying filament architecture in these
444 cases may differ from that of typical PiD, which is defined by the exclusive presence of
445 3R tau. Electron microscopy has further shown that the major tau species (~80%) form
446 straight filaments similar to those in AD [25,67]. *In vitro*, S320F tau has been shown to
447 increase the rate of tau nucleation, shorten the lag phase of aggregation, and promote the
448 formation of short protofibrils and oligomers [68,69], which may explain the ability of
449 this mutant to aggregate in the absence of preformed seeds [70]. Taking these previous
450 reports together with our structural data, S320F appears to form a stable loop through π–
451 π stacking between F320 aromatic rings along the Z-axis and hydrophobic interactions
452 with the surrounding residues V309, P312, L315, and V318. Because V309, P312, L315,
453 and V318 are shared by both 3R and 4R tau, tau carrying the S320F mutation is likely to
454 recruit both free 3R and 4R tau into the growing filaments. Thus, the loop structure
455 centered on S320F may accelerate amyloid assembly and core formation, providing a
456 mechanistic explanation for the presence of both 3R and 4R isoforms in the insoluble tau
457 fractions from S320F carriers. Furthermore, comparison of the S320F-containing loop
458 with the structure of tau filaments from PiD patients lacking this mutation reveals
459 similarities in the length and packing of the major hairpin-like arm around residues 306–
460 341 and in the packing of residues near position 320. Taken together, these observations
461 suggest that the S320F mutation itself, rather than tau isoform composition alone, may
462 contribute to the emergence of a PiD-like disease phenotype through its unique structural
463 effects, even in filaments containing both 3R and 4R tau isoforms.

464 However, among filaments with a clearly defined pitch, we did not observe a
465 robust fold that incorporated WT 3R or 4R tau. Such species likely belong to the

466 population of filaments with excessively long pitch and partially ordered β-sheets in
467 which structural determination was not feasible. This implies that, although S320F
468 promotes filament formation through loop stabilization, it may not by itself be sufficient
469 to generate highly ordered β-sheets within a short time frame in the model mouse. This
470 interpretation is consistent with previous observations that AAV-mediated expression of
471 S320F alone in neonatal ventricles does not produce substantial tau aggregation within
472 three months [27].

473 In the present study, the combination of P301L and S320F mutations generated
474 not only the S320F-dependent loop but also an additional hydrophobic interaction
475 between L301 and I328. This hydrophobic contact appears to rigidify the base of the
476 S320F-dependent loop, thereby defining a specific loop spanning 28 amino acids from
477 L301 to I328. Formation of this L301–I328 loop exposes the hydrophobic residues V306
478 and I308 on the outer surface of the loop, allowing L282, L284, V287, and Q288 to pack
479 against them and stabilize the three-folded architecture. Furthermore, interactions
480 between N286 and S289 are likely to support and lock in this folding pattern. Collectively,
481 these interactions contribute to the robust, compact three-folded structure observed for
482 P301L/S320F double-mutant tau.

483 One of the key advantages of our model is the rapid induction of tau pathology.
484 Whereas conventional models such as PS19 mice require more than 6 months for
485 detectable tau aggregation, our system recapitulated tau accumulation and filament
486 formation within 1–2 months. This should not be interpreted as a more faithful
487 reproduction of human pathology, but rather as a powerful experimental system that
488 enables rapid induction and analysis of tau aggregation. Because tau filament formation

489 in our model was induced within at most 2 months in mouse brains, it is plausible that we
490 captured a particularly stable and compact fold. WT 4R tau does not contain L301, and
491 3R tau lacks this region entirely; therefore, filaments that incorporate WT tau isoforms
492 are expected to adopt structures distinct from the fold identified here, and their maturation
493 may require longer periods. Long-term observation will thus be necessary to elucidate
494 how WT tau is incorporated and how these filaments structurally evolve.

495 This study has several limitations: although peripheral administration of a
496 recombinant AAV-PHP.eB achieved widespread gene delivery throughout the brain,
497 transduction efficiency varied among regions, and the distribution of tau aggregates
498 largely mirrored the pattern of AAV infection. In addition, because the number of
499 transduced cells was limited, we did not observe large-scale neurodegeneration such as
500 overt brain atrophy or extensive neuronal loss. Future studies combining region-specific
501 intracranial injections with strategies to further enhance transduction efficiency may
502 allow stronger pathology and more widespread neurodegeneration to be modeled.

503 Taken together, our findings demonstrate that P301L/S320F mutant tau promotes
504 aggregation and filament formation in the absence of exogenous seeds, and that the
505 resulting fold is clearly distinct from previously reported tau structures. These results
506 deepen our mechanistic understanding of tau aggregation and provide a structural
507 framework for exploring the basis of tauopathies and for identifying potential therapeutic
508 targets.

509

510 **Conclusions**

511 In this study, we established a non-invasive and rapid *in vivo* model of tau
512 pathology by administering a recombinant AAV-PHP.eB from the periphery. Expression
513 of the P301L/S320F double-mutant human tau in adult mouse brain induced robust tau
514 aggregation without the need for exogenous seeds, accompanied by phosphorylation
515 patterns commonly observed in AD, including AT8, AT180, pSer396, and PHF-1. The
516 concomitant detection of early (AT8 and AT180) and late (pSer396 and PHF-1)
517 phosphorylation epitopes indicates that tau phosphorylation progresses in a stepwise
518 manner, leading to the accumulation of filamentous tau species that have reached an
519 advanced pathological maturation stage. Our cryo-EM analysis of sarkosyl-insoluble tau
520 filaments demonstrated that the combined P301L and S320F mutations cooperatively
521 remodel the tau core to generate previously unreported filament fold distinct from known
522 patient-derived structures. Together, these findings reveal the strong intrinsic aggregation
523 propensity and unique fibrillization mechanism of P301L/S320F tau *in vivo*. The model
524 presented here faithfully reproduces tau pathology on a background of humanized tau and
525 Aβ deposition, providing a powerful platform for dissecting disease mechanisms and
526 advancing therapeutic strategies for tauopathies.

527 **Abbreviations**

528 AAV: adeno-associated virus

529 AD: Alzheimer disease

530 APP: amyloid precursor protein

531 BS cells: biosensor cells

532 CA2: Cornu Ammonis 2

533 cryo-EM: cryo-electron microscopy

534 DAPI: 4',6-diamidino-2-phenylindole

535 EM: electron microscopy

536 FRET: fluorescence resonance energy transfer

537 FTD: frontotemporal dementia

538 FTDP-17: frontotemporal dementia with Parkinsonism linked to chromosome 17

539 HEK293A: human embryonic kidney 293A

540 hMAPT: human microtubule-associated protein tau

541 MPtA: medial parietal association area

542 PBS: phosphate-buffered saline

543 RD: repeat domain

544 Sup: sarkosyl-soluble fraction

545 Pellet: sarkosyl-insoluble fraction

546 vg: viral genomes

547 WPRE: woodchuck hepatitis virus post-transcriptional regulatory element

548 WT: wild-type

549

550 **Figure Legends**

551 **Figure 1. P301L/S320F double-mutant tau forms sarkosyl-insoluble tau filaments in cultured**
552 **cells.**

553 **a**. Schematic representation of human 1N4R tau, showing the amino acid sequences of
554 the microtubule-binding repeats (R1–R4), and the mutation sites P301L and S320F.

555 **b**. Experimental design for assessing tau aggregation in HEK293A cells. Cells stably
556 expressing 1N3R and 1N4R WT tau isoforms were transfected with plasmids encoding
557 either 1N4R WT tau or 1N4R P301L/S320F double-mutant tau, followed by sarkosyl
558 extraction to separate the soluble (Sup) and insoluble (Pellet) fractions.

559 **c**. Western blot analysis of each fraction. Tau in the sarkosyl-insoluble fraction was
560 prominently detected in the P301L/S320F group compared with the WT and empty-vector
561 (EV) groups. GAPDH served as a loading control.

562 **d**. Quantification of sarkosyl-insoluble tau normalized to soluble tau (mean ± SEM; n =
563 3 per group). Statistical significance was evaluated using a two-tailed Welch's t-test. For
564 all comparisons: $p < 0.05$ (*), ns = not significant.

565 **e**. Resuspended sarkosyl-insoluble pellets obtained as in b were negatively stained with
566 2% phosphotungstic acid (pH 7.0) and observed under a transmission electron microscope.
567 Scale bar is 100 nm.

568

569 **Figure 2. P301L/S320F tau induces phosphorylated tau aggregates in the brains of *App*$^{NL-G-}$**
570 **^{F}/*hMAPT* mice.**

571 **a.** Experimental design. *App*$^{NL-G-F}$/*hMAPT* double knock-in mice aged 9–11 months were
572 intravenously injected via the retro-orbital sinus with the recombinant AAV-PHP.eB
573 (2×10^{11}vg/mouse). Brains were collected 2 months after injection for histological analysis.

574 **b**. Immunostaining of brain hemispheres with AT8 antibody in mice expressing WT tau
575 or P301L/S320F via AAV-PHP.eB, and from non-injected control mice.

576 **c**. High-magnification images of the MPtA (boxed by a solid line) in b. Scale bars are 100
577 mm.

578 **d.** Quantification of AT8-positive area in the MPtA. Statistical significance was
579 determined by Welch's two-tailed *t*-test. Mean ± SEM, n = 3. *$p < 0.05$, ns = not
580 significant.

581 **e**. High-magnification images of the hippocampal CA2 region in b. Scale bars are 100
582 mm.

583 **f**. Quantification of AT8-positive area in the CA2 region. Statistical significance was
584 determined by Welch's two-tailed *t*-test. Mean ± SEM, n = 3. *$p < 0.05$, ns = not
585 significant.

586

587 **Figure 3. Evaluation of tau aggregation propensity and seeding activity in mouse brains**
588 **expressing P301L/S320F tau**

589 **a**. Experimental workflow. Following systemic administration of AAV-PHP.eB encoding

590 either WT or P301L/S320F tau, mouse brain hemispheres were fractionated into soluble
591 (Sup) and insoluble (Pellet) fractions by the sarkosyl extraction method.

592 **b**. Western blot analysis of each fraction.

593 **c**. Ratio of sarkosyl-insoluble to soluble tau (mean ± SEM; n = 3 per group), showing a
594 significant increase in the P301L/S320F group ($p < 0.05$). Statistical significance was
595 determined by Welch's two-tailed *t*-test.

596 **d**. Sarkosyl-insoluble pellets were negatively stained with 2% phosphotungstic acid (pH
597 7.0).

598 **e-g**. Filaments positive for TauC4 (e), AT8 (f), and pS396 (g) antibodies were observed
599 in the P301L/S320F group. Secondary antibodies used were 10 nm gold-conjugated anti-
600 rabbit IgG for TauC4 and pS396 and 5 nm gold-conjugated anti-mouse IgG for AT8.

601

602 **Figure 4. Cryo-EM structure of P301L/S320F tau filaments extracted from mouse brains.**

603 **a.** Representative cross-sections through the cryo-EM reconstruction of P301L/S320F tau
604 filaments, shown perpendicular to the helical axis with a projected thickness of
605 approximately one rung. The map resolution is 2.24 Å. Scale bar is 5 nm.

606 **b**. Three-dimensional reconstruction of the P301L/S320F tau filaments obtained by
607 helical reconstruction. Scale bar is 5 nm.

608 **c**. Amino acid sequence of human 1N4R tau (residues 255–367) corresponding to the
609 reconstructed region. The five β-strand regions (β1–β5) are indicated by green arrows.
610 Mutation sites P301L and S320F are shown in red.

611 **d**. Rendered view of the secondary structure elements in two successive rungs.

612

613 **Figure 5. Local structural effects of P301L and S320F mutations on tau filaments.**

614 **a**. Sharpened cryo-EM density map of the P301L/S320F tau filament with the atomic
615 model fitted. Major side chains are shown, and mutated residues L301 and F320 are
616 highlighted in red.

617 **b**. Schematic representation of the P301L/S320F tau filament fold. β-strands are indicated
618 by thick lines with arrowheads. Orange semicircles indicate additional densities observed
619 near the filament crossover region.

620 **c**. Structure surrounding the P301L mutation site. The L301 residue (red) is positioned
621 within the β-strand core and forms hydrophobic interactions with nearby residues,
622 including V300 and I297.

623 **d**. Structure surrounding the S320F mutation site. The F320 residue (red) is embedded
624 within the loop region and forms hydrophobic interactions with V309, P312, L315, and
625 V318, which likely contribute to the stabilization of the filament.

626

627 **Figure 6. Comparison with previously reported patient-derived tau filament structures.**

628 **a**. Structural comparison between P301L/S320F mouse tau filaments (magenta) and
629 P301L patient tau filaments (cyan, PDB: 9GG0), with an RMSD of 4.23 Å over 40
630 residues.

631 **b**. Structural comparison between P301L/S320F mouse tau filaments (magenta) and PiD

632 patient tau filaments (cyan, PDB: 6GX5), with an RMSD of 3.87 Å over 42 residues.

633 **c**. Structural comparison between P301L/S320F mouse tau filaments (magenta) and AD

634 patient tau filaments (cyan, PDB: 6HRE), with an RMSD of 2.09 Å over 16 residues.

635

636 **Declarations**

637 **Ethics approval and consent to participate**

638 All animal experiments were conducted in accordance with the guidelines of the
639 Institutional Animal Care Committee of the Graduate School of Pharmaceutical Sciences
640 at the University of Tokyo (protocol no. P3-20).

641

642 **Consent for publication**

643 Not applicable.

644

645 **Availability of data and materials**

646 Cryo-EM maps have been deposited in the Electron Microscopy Data Bank
647 (EMDB) with the accession numbers EMD-68389 (P301L/S320F tau filament).
648 Corresponding refined atomic models have been deposited in the Protein Data Bank
649 (PDB) under accession numbers PDB: 22JY. The datasets used and analyzed during the
650 current study are available from the corresponding author on reasonable request.

651

652 **Competing interests**

653 The authors declare that they have no competing interests.

654

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Authors' contributions

M.Ka performed cell and biochemical experiments, extracted filaments from mouse brains, analyzed the data, prepared figures, and wrote the original draft of the manuscript. T.K. supervised the biochemical experiments and provided technical guidance. H.Y. conducted cryo-EM imaging, performed structural reconstruction, and assisted in figure preparation. L.T. contributed to the optimization of experimental conditions for cell-based and mouse experiments. K.Y. performed immunohistochemical staining of mouse brain sections. A.S. carried out quantitative analyses of Western blot and immunohistochemical staining data. X.L. extracted tau filaments from cultured cells. S.T. constructed the P301L/S320F tau plasmid and established the AAV-PHP.eB systemic delivery protocol in mice. T.C.S. and T.S. established and provided $App^{NL\text{-}G\text{-}F}$/*hMAPT*

677 double knock-in mice. M.Ki supervised the structural analysis and provided critical input
678 on the manuscript. T.T. conceived and supervised the overall project, secured funding,
679 and served as the corresponding author.

680

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689

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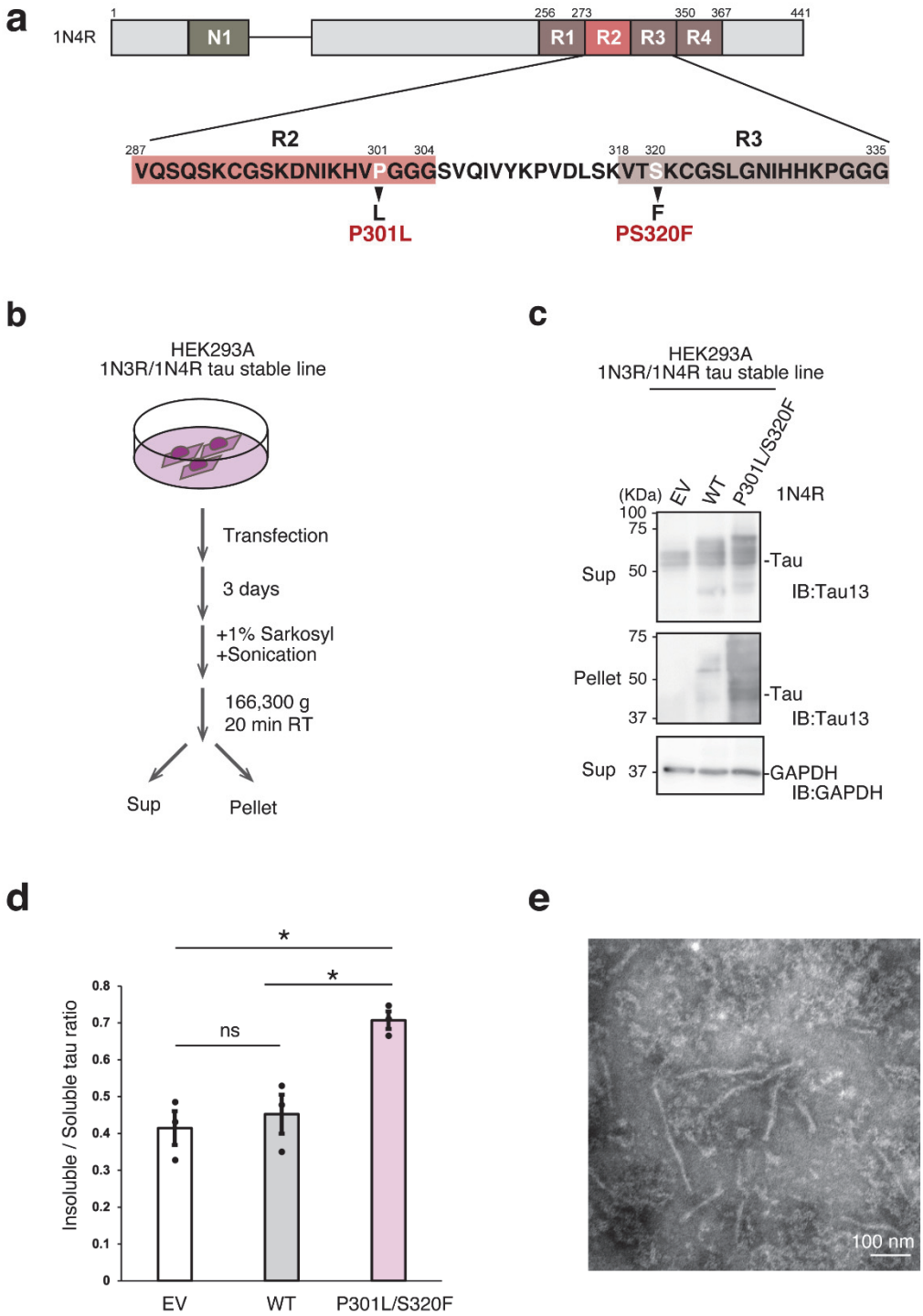
935 **Figures**



936

937 **Fig. 1**

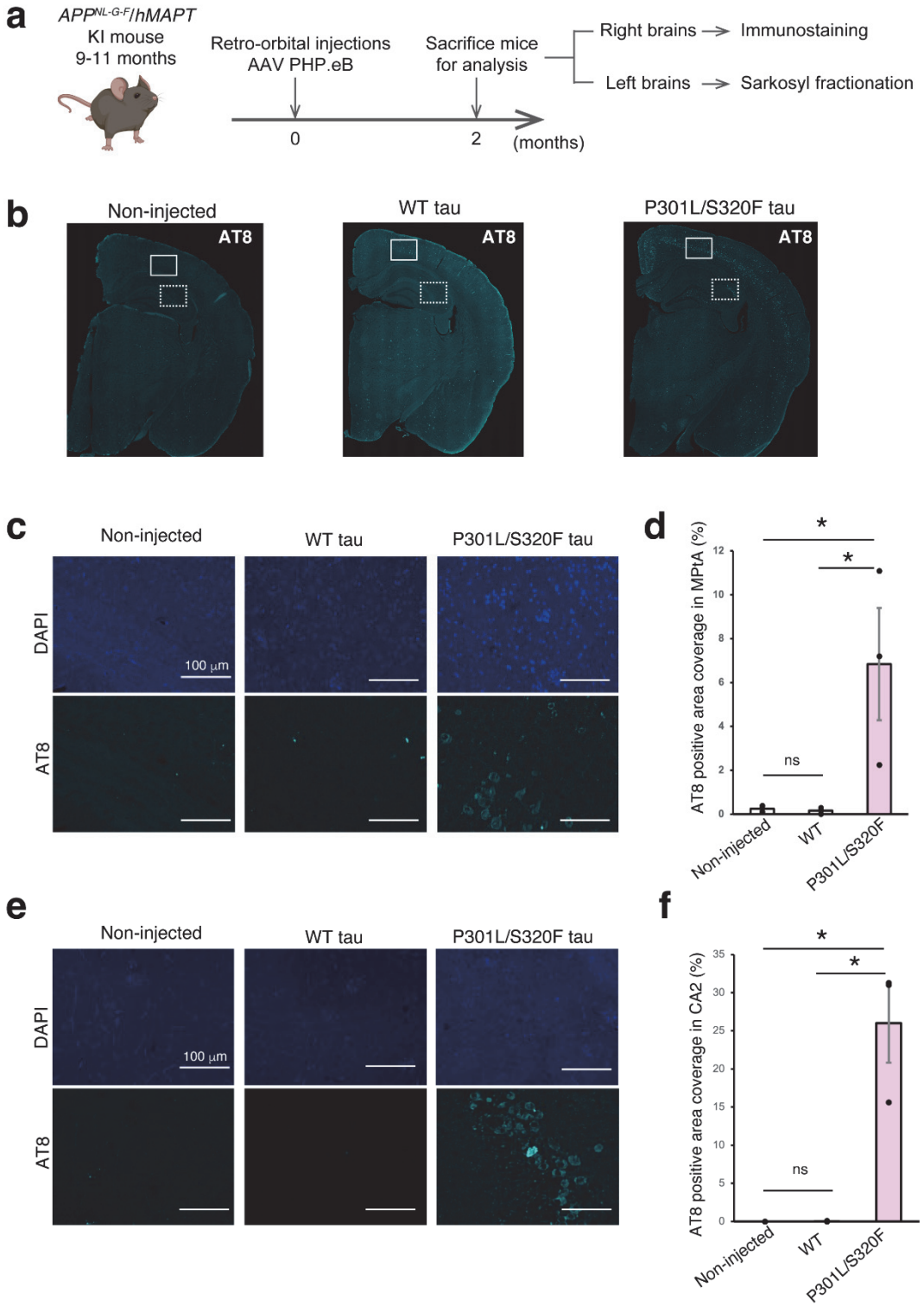

a
APPNL-G-F/hMAPT
KI mouse
9-11 months
Retro-orbital injections
AAV PHP.eB
Sacrifice mice
for analysis
Right brains → Immunostaining
Left brains → Sarkosyl fractionation
0
2
(months)
b
Non-injected
WT tau
P301L/S320F tau
AT8
c
Non-injected
WT tau
P301L/S320F tau
DAPI
AT8
100 μm
d
AT8 positive area coverage in MPtA (%)
ns
Non-injected
WT
P301L/S320F
e
Non-injected
WT tau
P301L/S320F tau
DAPI
AT8
100 μm
f
AT8 positive area coverage in CA2 (%)
ns
Non-injected
WT
P301L/S320F

Fig. 2

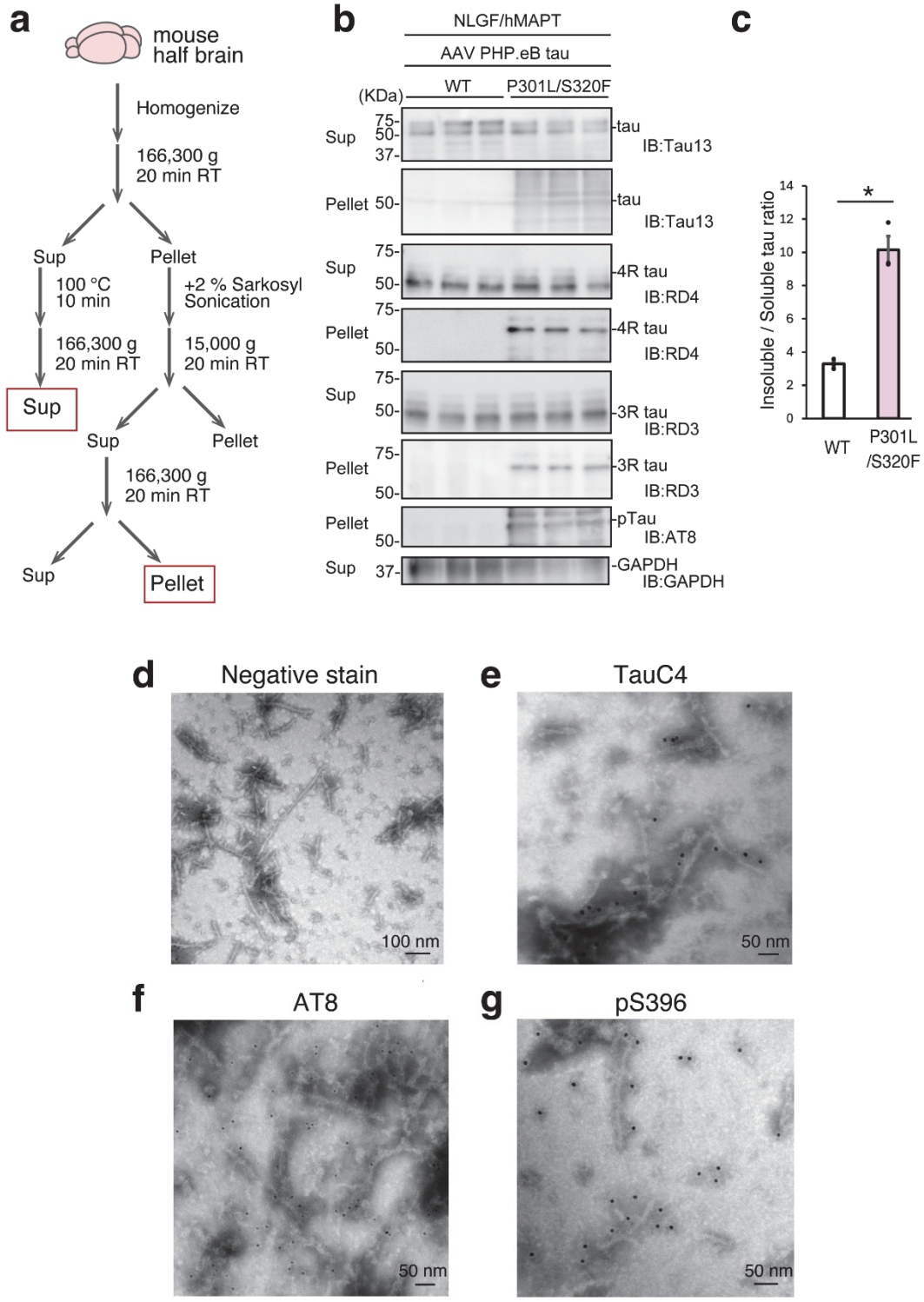

a
mouse
half brain
Homogenize
166,300 g
20 min RT
Sup
Pellet
100 °C
10 min
+2 % Sarkosyl
Sonication
166,300 g
20 min RT
15,000 g
20 min RT
Sup
Sup
Pellet
166,300 g
20 min RT
Sup
Pellet
b
NLGF/hMAPT
AAV PHP.eB tau
WT
P301L/S320F
(KDa)
Sup
Pellet
tau
IB:Tau13
4R tau
IB:RD4
3R tau
IB:RD3
pTau
IB:AT8
GAPDH
IB:GAPDH
c
Insoluble / Soluble tau ratio
WT
P301L
/S320F
d
Negative stain
100 nm
e
TauC4
50 nm
f
AT8
50 nm
g
pS396
50 nm

Fig. 3

Fig. 4

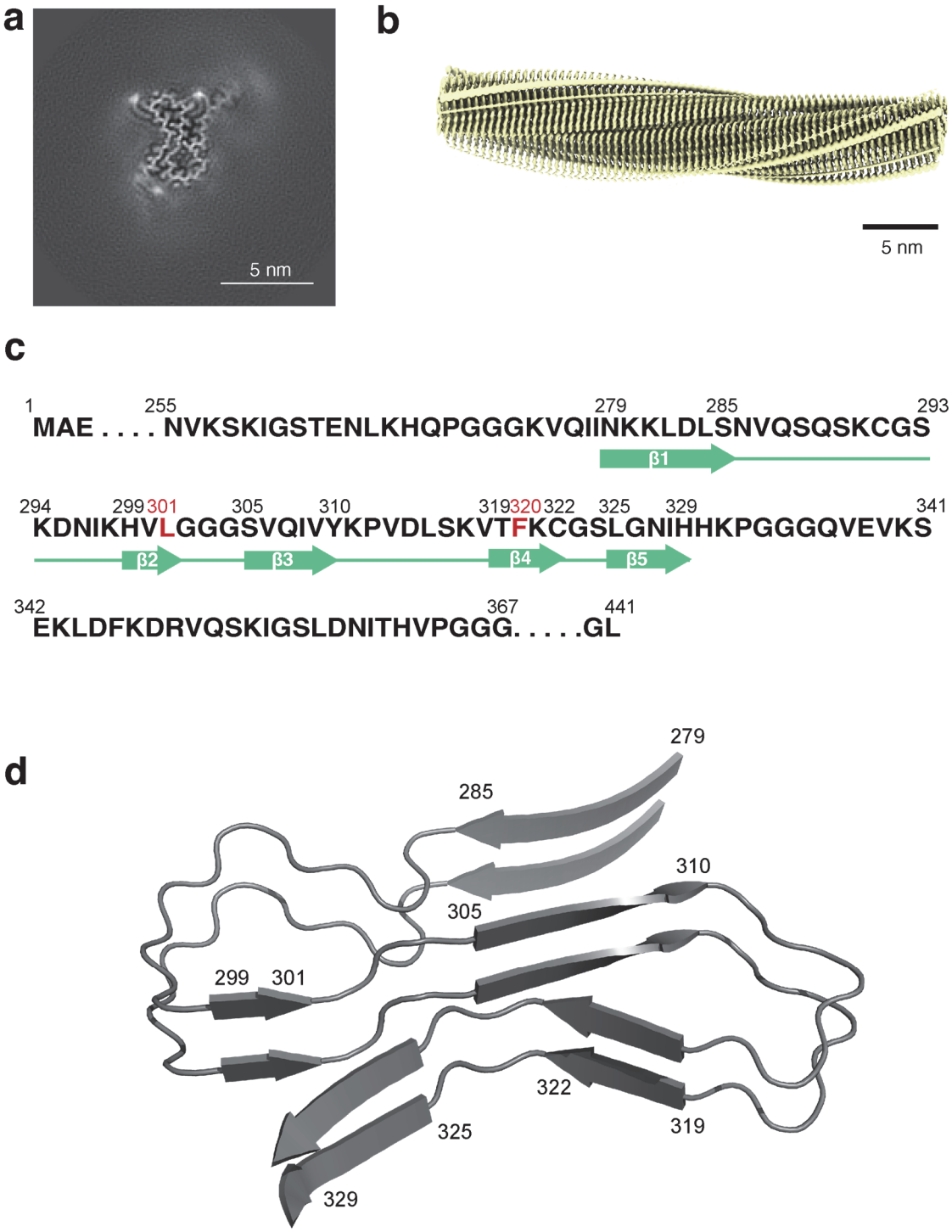

a
5 nm
b
5 nm
c
1 255 279 285 293
MAE . . . . NVKSKIGSTENLKHQPGGGKVQIINKKLDLSNVQSQSKCGS
β1
294 299 301 305 310 319 320 322 325 329 341
KDNIKHVLGGGSVQIVYKPVDLSKVTFKCGSLGNIHHKPGGGQVEVKS
β2 β3 β4 β5
342 367 441
EKLDFKDRVQSKIGSLDNITHVPGGG. . . . .GL
d
279
285
310
305
299 301
322
325
319
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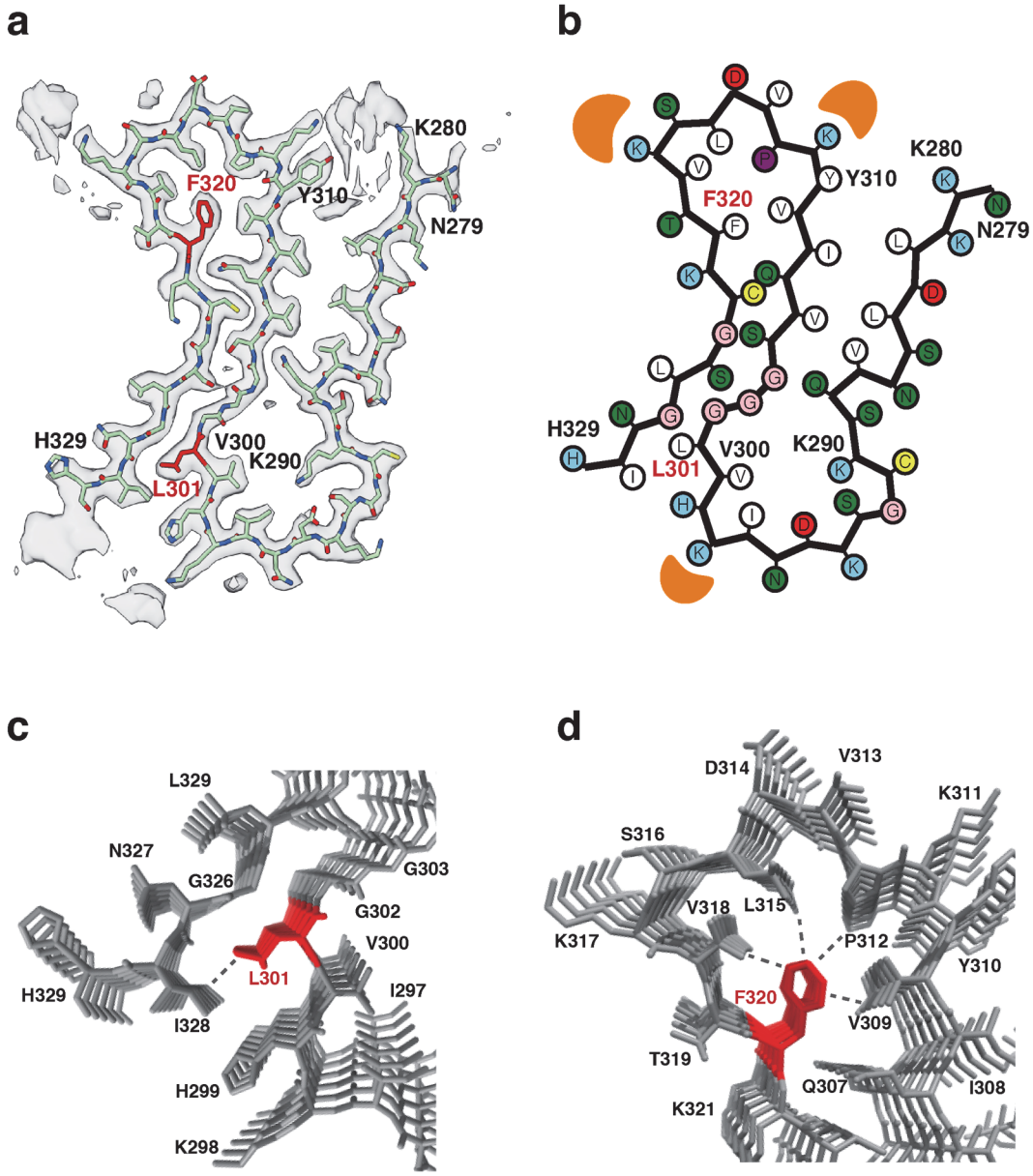
Fig. 4

945

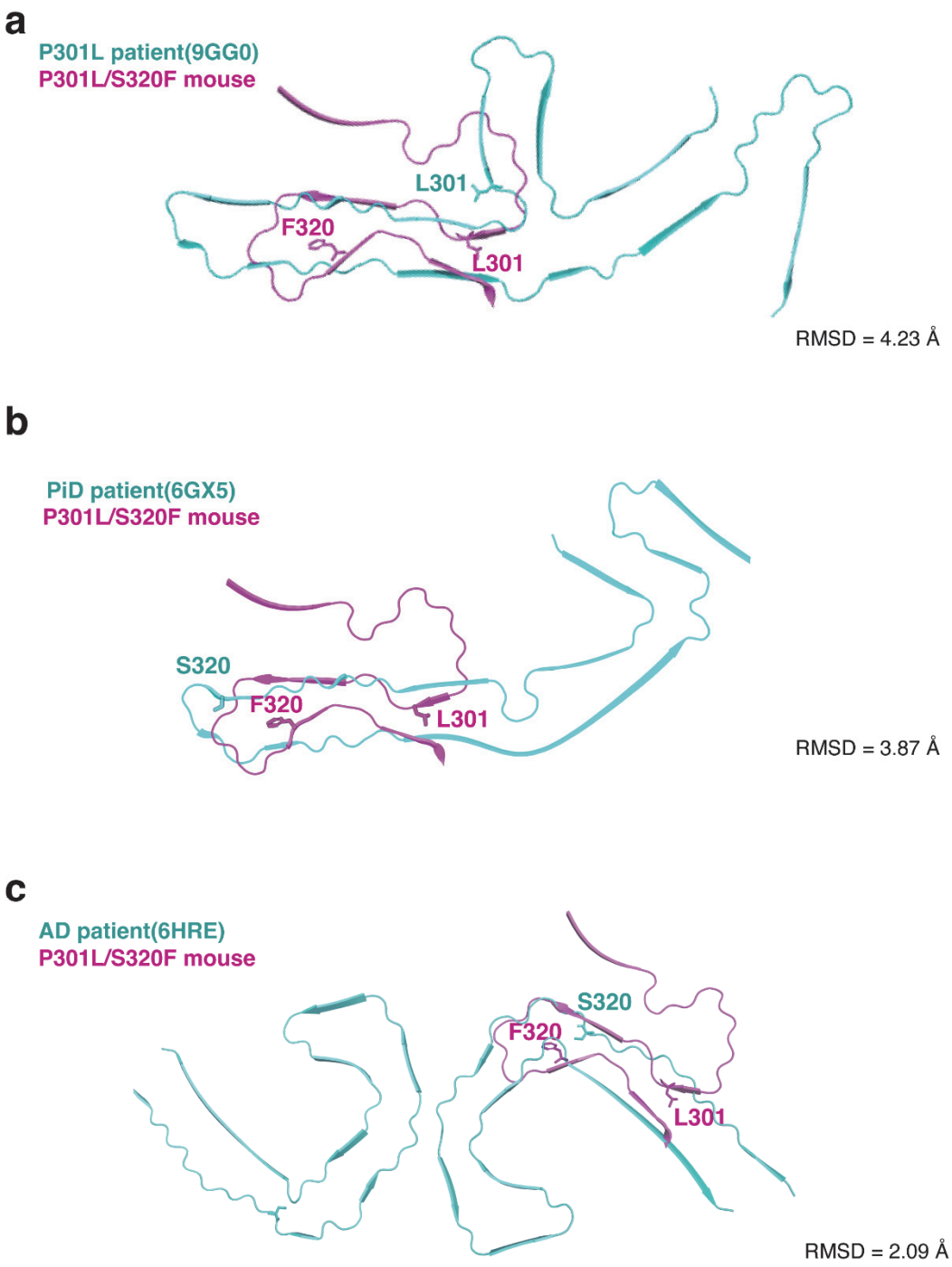
Fig. 5



946

947 **Fig. 5**

948


a
P301L patient(9GG0)
P301L/S320F mouse
L301
F320
L301
RMSD = 4.23 Å
b
PiD patient(6GX5)
P301L/S320F mouse
S320
F320
L301
RMSD = 3.87 Å
c
AD patient(6HRE)
P301L/S320F mouse
S320
F320
L301
RMSD = 2.09 Å

949

950 **Fig. 6**