

1 **Supplementary information for**

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

4

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21

22 **Supplementary Figure Legends**

23 **Figure S1. Structural features of the six human tau isoforms.**

24 This schematic representation illustrates the exon composition of the six tau isoforms
25 (0N3R, 1N3R, 2N3R, 0N4R, 1N4R, 2N4R) generated from the *hMAPT* gene. The
26 isoforms are defined by the presence or absence of the N-terminal insertions (N1, N2)
27 and the microtubule-binding repeat domains (R1–R4).

28

29 **Figure S2. Peripheral administration of AAV-PHP.eB encoding P301L/S320F tau**
30 **induces AT180-positive phosphorylated tau aggregates.**

31 **a.** Immunostaining of brain hemispheres with AT180 antibody in mice expressing WT tau
32 or P301L/S320F via AAV-PHP.eB, and from non-injected control mice.

33 **b.** High-magnification images of the MPtA (boxed by solid line) in **a**. Scale bars are 100
34 μm .

35 **c.** Quantification of AT180 immunostaining in the MPtA. Statistical significance was
36 determined by Welch's two-tailed *t*-test. Mean \pm SEM, $n = 3$. $*p < 0.05$.

37 **d.** High-magnification images of the hippocampal CA2 region (boxed by dashed line) in
38 **a**. Scale bars are 100 μm .

39 **e.** Quantification of AT180 immunoreactivity in the CA2 region. Statistical significance
40 was determined by Welch's two-tailed *t*-test. Mean \pm SEM, $n = 3$. $*p < 0.05$.

41

42 **Figure S3. Peripheral administration of AAV-PHP.eB encoding P301L/S320F tau**
43 **induces pS396-positive phosphorylated tau aggregates.**

44 **a.** Immunostaining of brain hemispheres with pS396 antibody in mice expressing WT tau
45 or P301L/S320F via AAV-PHP.eB and from non-injected control mice.

46 **b.** High-magnification images of the MPtA (boxed by solid line) in **a**. Scale bars are 100
47 μm .

48 **c.** High-magnification images of the hippocampal CA2 region (boxed by dashed line) in
49 **a**. Scale bars are 100 μm .

50

51 **Figure S4. Peripheral administration of AAV-PHP.eB encoding P301L/S320F tau**
52 **induces PHF-1-positive phosphorylated tau aggregates.**

53 **a.** Immunostaining of brain hemispheres with PHF-1 antibody in mice expressing WT tau
54 or P301L/S320F via AAV-PHP.eB, and from non-injected control mice.

55 **b.** High-magnification images of the MPtA (boxed by solid line) in **a**. Scale bars are 100
56 μm .

57 **c.** High-magnification images of the hippocampal CA2 region (boxed by dashed line) in
58 **a**. Scale bars are 100 μm .

59

60 **Figure S5. Peripheral administration of AAV-PHP.eB encoding 301L/S320F induces**
61 **sarkosyl-insoluble tau in wild-type mice.**

62 **a.** Experimental timeline. Two-month-old wild-type C57BL/6J mice received an

63 intravenous injection of a recombinant AAV-PHP.eB into the retro-orbital sinus. Brains
64 were collected 2 months after injection for biochemical analysis.

65 **b.** AAV-PHP.eB–injected mice expressing either WT tau or P301L/S320F human tau, as
66 well as non-injected control mice (non-injected), were processed using the sarkosyl
67 extraction procedure described in **Fig. 3a**. These fractions were analyzed by western
68 blotting. Total tau was detected using the anti-tau antibody Tau13, and GAPDH was used
69 as a loading control.

70

71 **Figure S6. FRET biosensor assay of P301L/S320F tau induced to mouse brains.**

72 **a.** Representative FACS histograms of BS cells incubated with mouse brain homogenates.
73 FRET-positive signals were displayed as yellow events within the blue triangular gating
74 region. FRET-positive cells were detected using flow cytometry with Brilliant Violet 510
75 / Brilliant Violet 421.

76 **b-d,** Representative flow cytometry plots of BS cells without homogenate (**b**), BS cells
77 incubated with brain homogenate from *App*^{NL-G-F}/*hMAPT* mice expressing WT tau (**c**),
78 and BS cells incubated with brain homogenate from *App*^{NL-G-F}/*hMAPT* mice expressing
79 P301L/S320F human tau (**d**).

80 **e,** Quantification of the proportion of FRET-positive cells (mean ± SEM; n = 3 per group).
81 Statistical significance was evaluated using a two-tailed Welch's *t*-test. For all
82 comparisons: $p < 0.05$ (*), $p < 0.01$ (**), *ns* = not significant.

83

84 **Figure S7. Summary of the image processing of P301L/S320F human tau filaments**

85 Top: Cryo-EM pipeline for P301L/S320F human tau filaments.

86 Bottom left: Local resolution map, colored from blue (high resolution) to red (lower
87 resolution).

88 Bottom right: FSC curves, demonstrating a resolution of 2.24 Å. FSC curves reflect
89 unmasked (green), masked (blue), and corrected (black) estimates of map resolution.

90

91 **Figure S8. Full uncropped images of Figure 1c.**

92 The white numbers indicate the molecular weights (kDa).

93

94 **Figure S9. Full uncropped images of Figure 3b.**

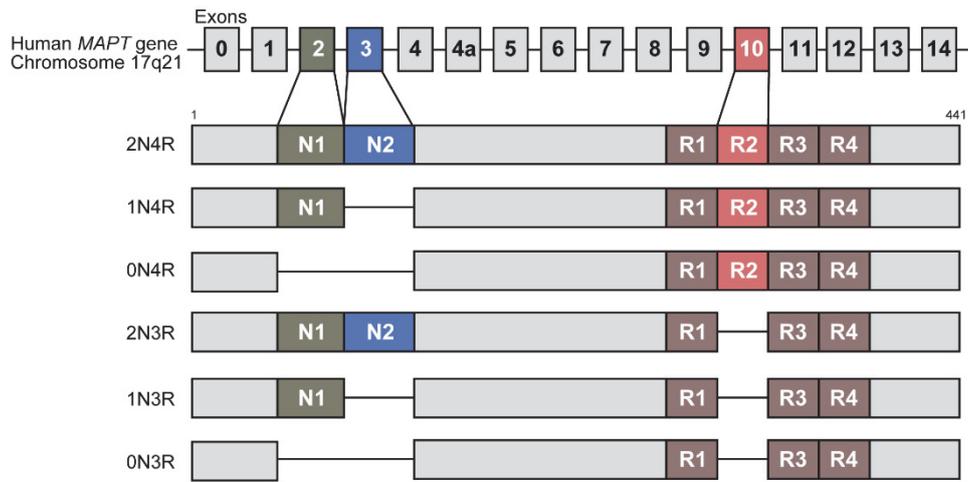
95 The white numbers indicate the molecular weights (kDa).

96

97 **Figure S10. Full uncropped images of Figure S5.**

98 The white numbers indicate the molecular weights (kDa).

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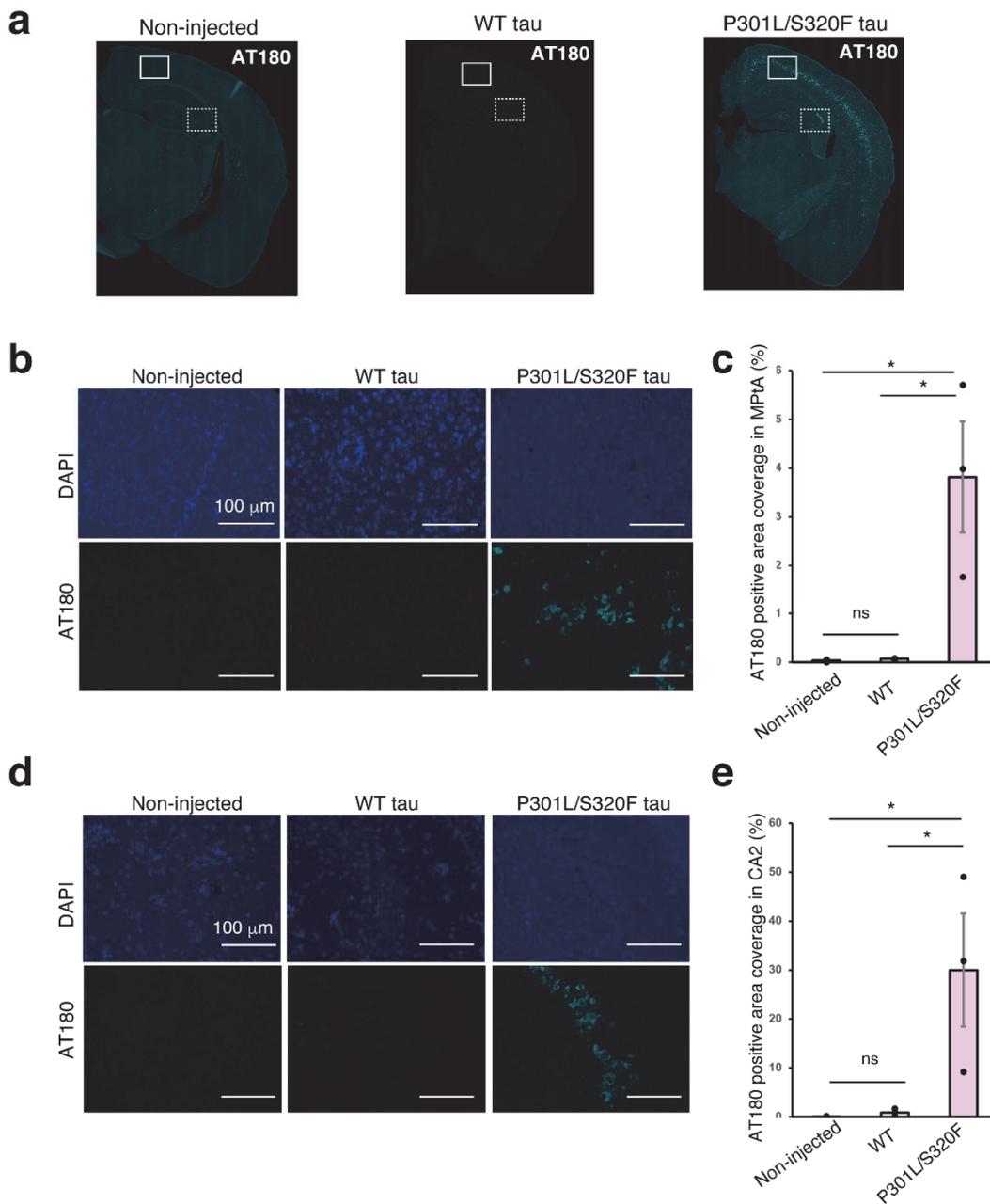


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101

102 **Fig. S1**

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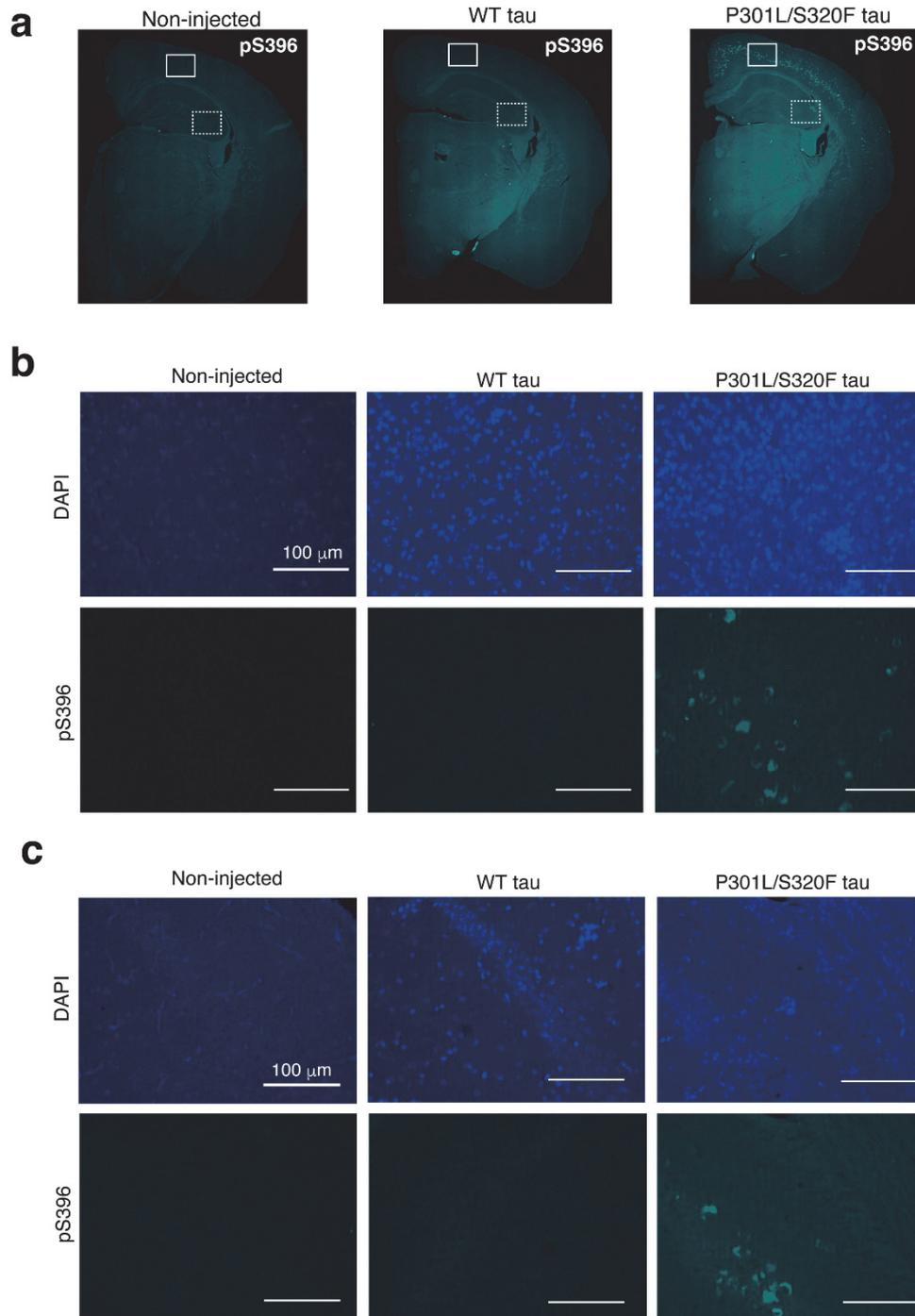


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106 **Fig. S2**

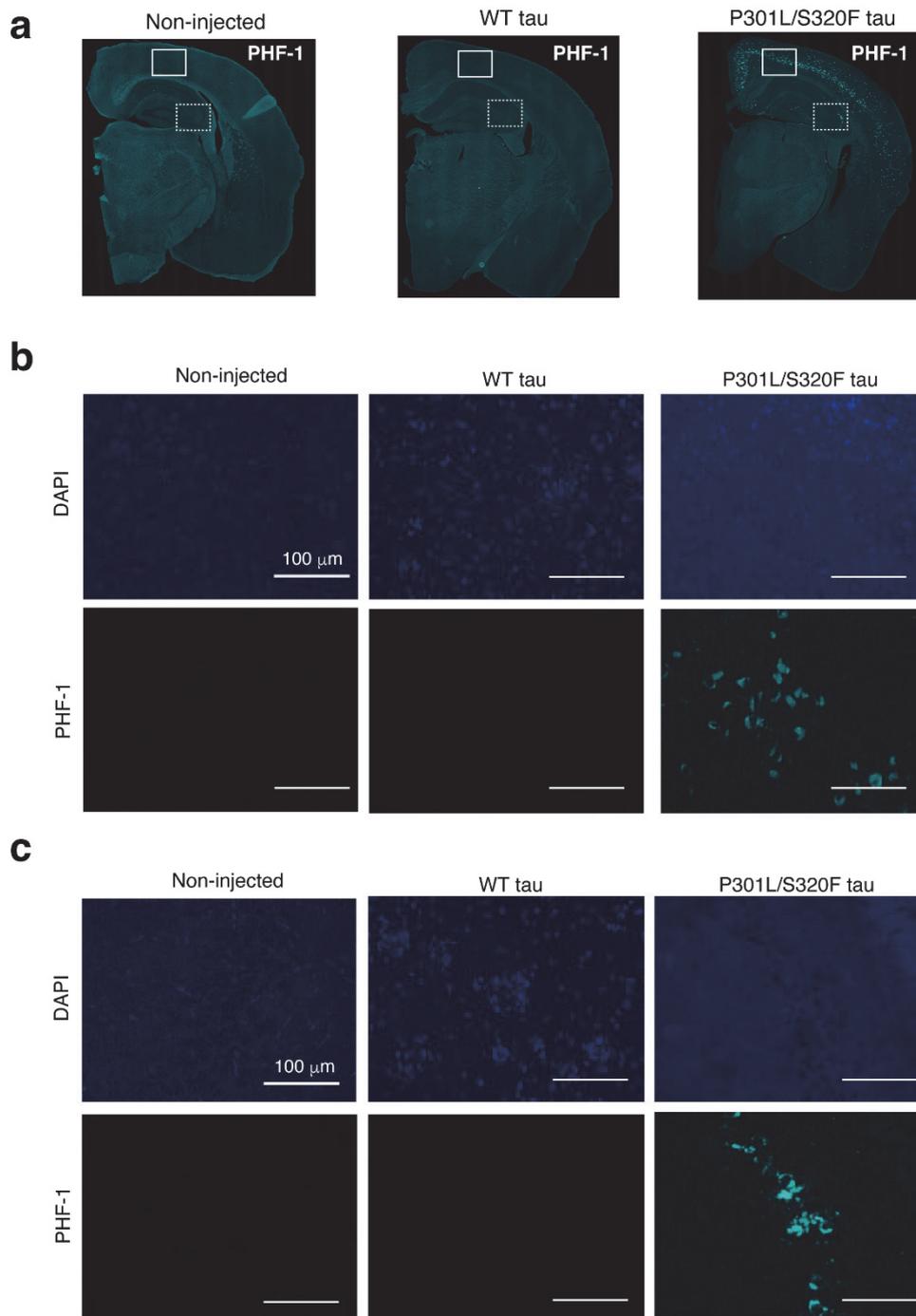
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109 **Fig. S3**

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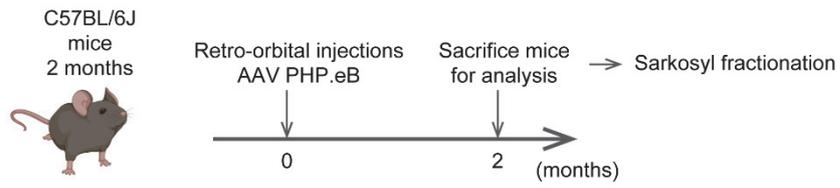


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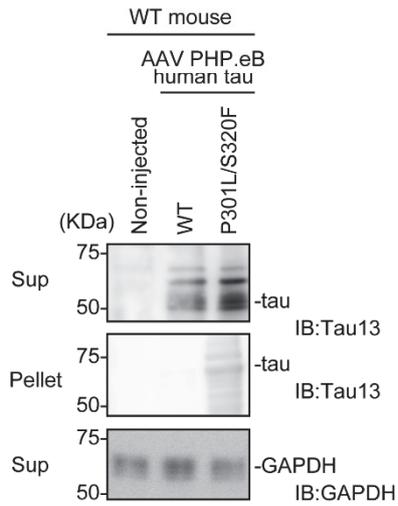
112 **Fig. S4**

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a



b

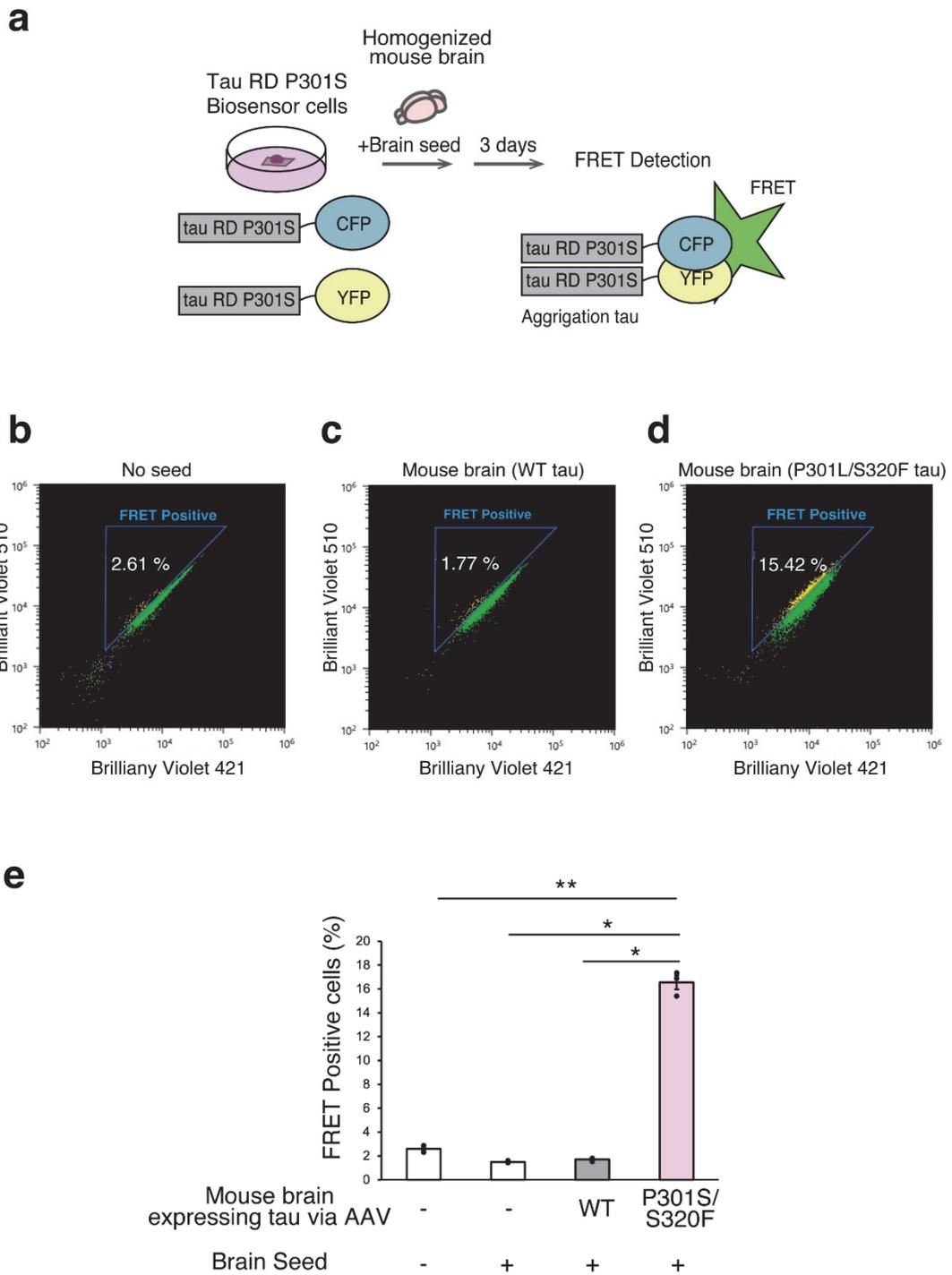


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116 **Fig. S5**

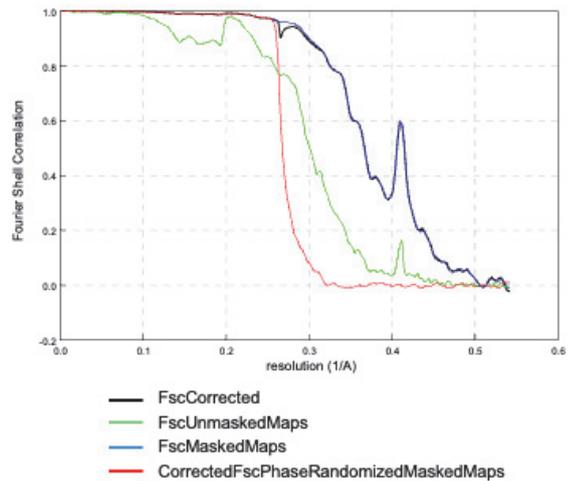
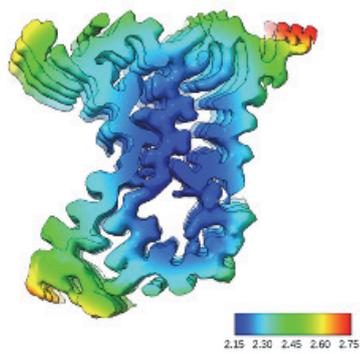
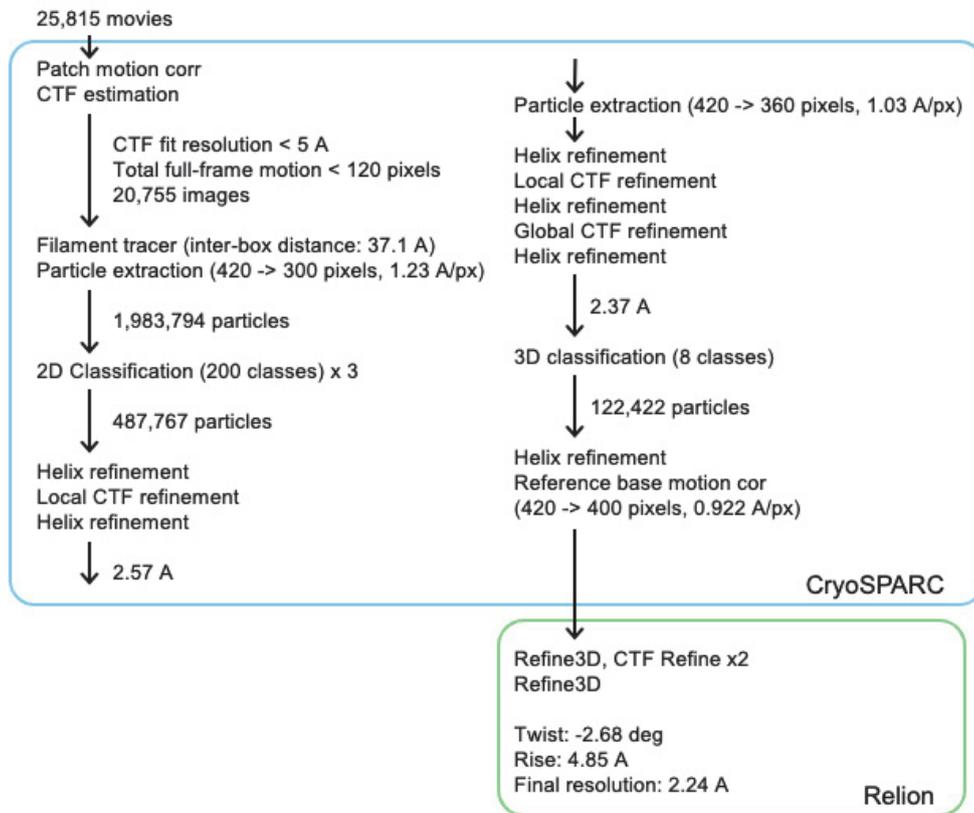
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119 **Fig. S6**

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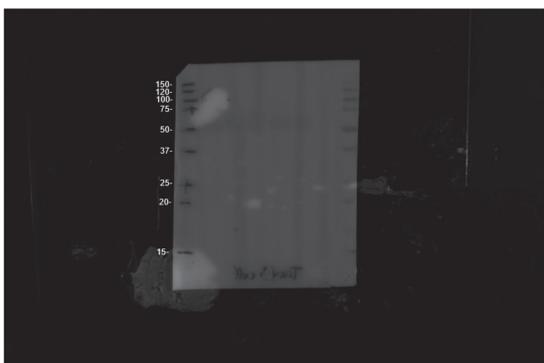


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122 **Fig. S7**

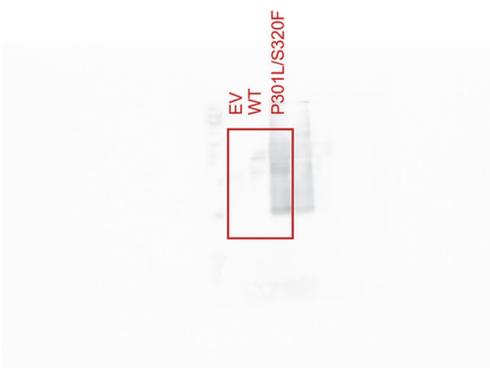
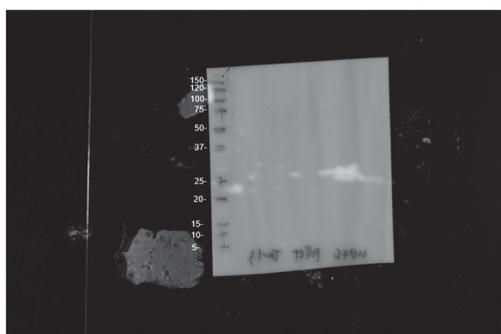
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Fig. 1c: Sup, tau13



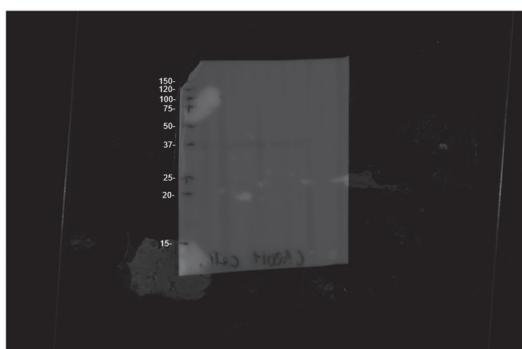
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Fig. 1c: Pellet, tau13



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Fig. 1c: Pellet, GAPDH

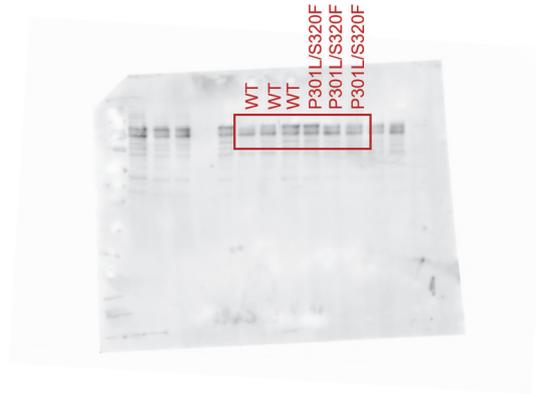


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127 **Fig. S8**

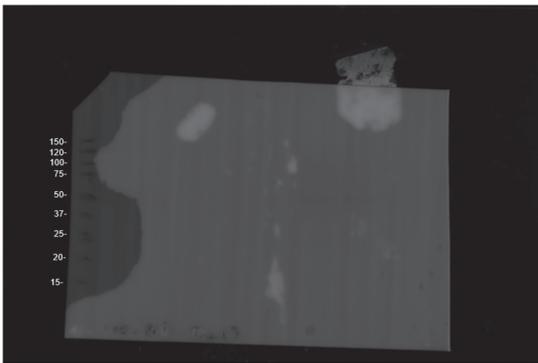
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Fig. 3b: Sup, tau13



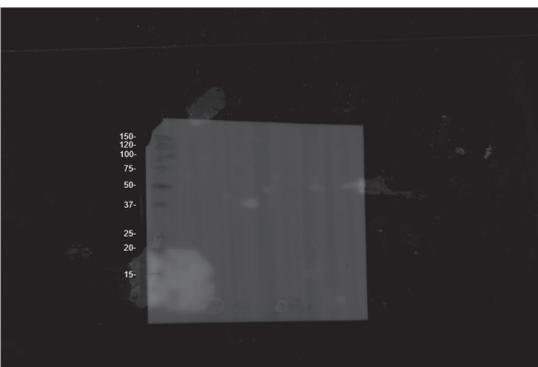
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Fig. 3b: Pellet, tau13



130

Fig. 3b: Sup, RD4



131

132 Fig. S9 cont'd

133

Fig. 3b: Pellet, RD4



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Fig. 3b: Sup, RD3



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Fig. 3b: Pellet, RD3

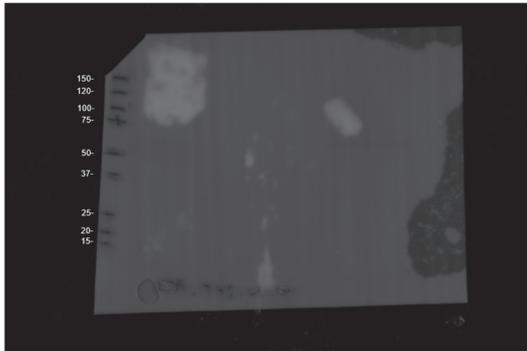


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137 **Fig. S9 cont'd**

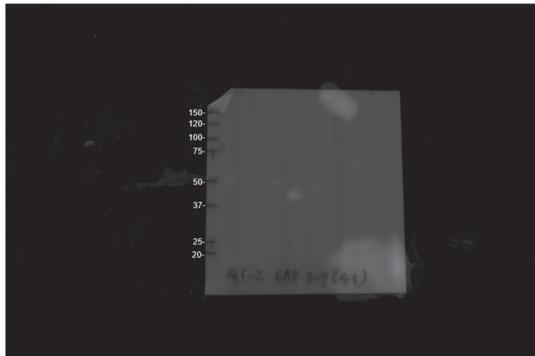
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Fig. 3b: Pellet, AT8



139

Fig. 3b: Sup, GAPDH



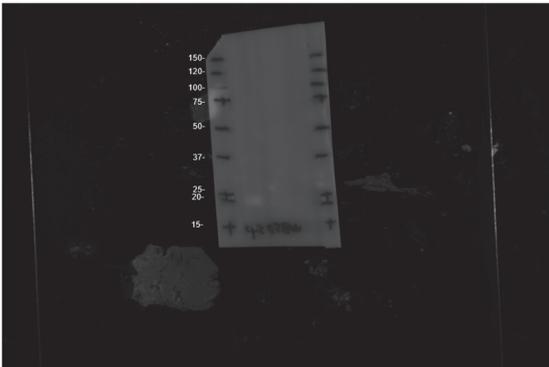
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142 **Fig. S9**

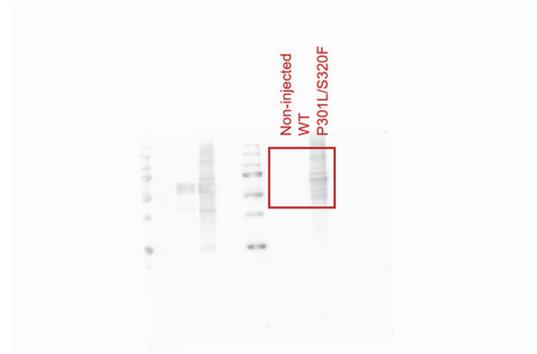
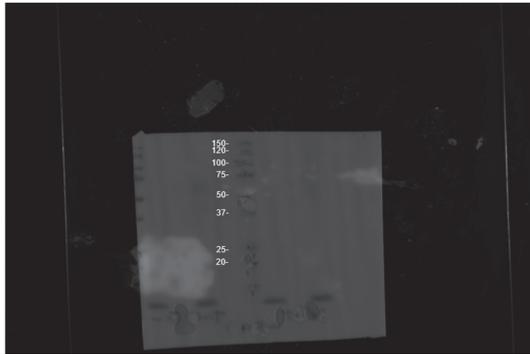
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Fig. S5: Sup, Tau13



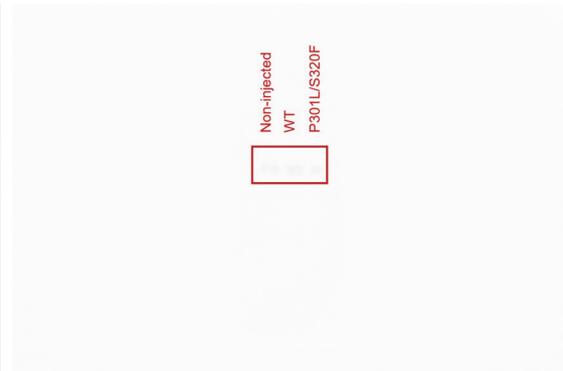
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Fig. S5: Pellet, Tau13



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Fig. S5: Sup, GAPDH



146

147 **Fig. S10**

148

149 **Table**

150 **Table S1. 1st Antibodies**

1 st antibody	Product # / REF	maker	dilution
tauC4	ABN2178	Merck Millipore	1/50 (Immuno-EM)
Tau13	Cat# 835201	BioLegend	1/1000 (WB)
AT8	# MN1020	Invitrogen	1/1000 (WB), 1/50 (Immuno-EM) 1/100 (Immuno-staining)
pS396	AB9658	Merck Millipore	1/200 (Immuno-EM), 1/2000 (Immuno-staining)
GAPDH	sc-32233	Santa Cruz Biotechnology	1/3000 (WB)
AT180	BR007	Innogenetics	1/100 (Immuno-staining)
RD3	05-803	Merck Millipore	1/1000 (WB)
RD4	05-804	Merck Millipore	1/1000 (WB)
PHF-1	A gift from Dr. P. Davies.	Provided	1/2000 (Immuno-staining)

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152

153 **Table S2. 2nd Antibodies**

2 nd antibody	Product # / REF	maker	dilution
Mouse-HRP	51503500 3	Jackson	1/1000 (WB)
Rabbit-HRP	564-71261	Fujifilm	1/1000 (WB)
EM .GAM5	GC5/100	BBI Solutions	1/100 (Immuno-EM)
EM .GAR10	GC5/100	BBI Solutions	1/100 (Immuno-EM)
Mouse IgG	BA-2000	Vector	1/1000 (Immuno-staining)
Cy5	TS- 000025	Akoya Biosciences	1/50 (Immuno-staining)
DAPI	H-1800	Vector Laboratories, Inc.	

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