Supplementary information

Cryo-EM structure of full-length α-synuclein amyloid fibril with Parkinson's disease familial A53T mutation

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Materials and Methods

Preparation of Ac-A53T and Ac-WT α-syn

Overexpression and purification of Ac-WT and Ac-A53T α-syn were conducted by a previously published procedure[1]. The genes of human wild-type and A53T mutant α-syn (1–140) were inserted into pET22 vector, respectively, and transformed into *E. coli* BL21(DE3) with the gene of Fission yeast N-acetyltransferase complex B co-transformed to obtain the N-terminally acetylated α-syn[2]. Protein expression was induced once the OD_{600} of bacteria reached ~0.6 by adding 1 mM isopropyl-1-thio-D-galactopyranoside (IPTG) at 37 °C for 4h. The harvested bacteria were lysed by sonication in lysis buffer (50mM Tris-HCl, pH 8.0, 1mM phenylmethylsulphonyl fluoride, 1mM EDTA). The cell debris was removed by centrifugation at 16,000×g for 30 min and the supernatant was further purified by heating at 100 °C for 10 min. After centrifugation, nucleic acid was removed by adding Streptomycin (20 mg/mL) into the supernatant. Next, the protein was purified by adjusting the PH of the supernatant to 3.5 to precipitate other unwanted components followed by another round of centrifugation. Then the supernatant was harvested and dialyzed in the dialysis buffer (50mM Tris-HCl, pH 8.0) overnight. After dialysis, the sample was purified by Q column (GE Healthcare, 17-5156-01) and further purified by Superdex 75 (GE Healthcare, 28-9893-33) to obtain the protein with high purity. Acetylation of the proteins was confirmed by an on-line EASY-nL-LC 1000 coupled with an

Orbitrap Q-Exactive HF mass spectrometer.

Fibril formation

Amyloid fibrils were prepared by incubating recombinant Ac-A53T α-syn (300 μM) at 37 °C with constant agitation (1,000 rpm) in ThermoMixer (Eppendorf) for 5 days in D-PBS buffer (BBI Life Sciences) and recombinant Ac-WT α-syn (300 μM) at 37 °C with constant agitation (900 rpm) in ThermoMixer (Eppendorf) for 3 days in fibril growth buffer (50 mM Tris, pH 7.5, 150 mM KCl). The α-syn preformed fibril seeds (PFFs) were obtained by sonicating the fibrils at 20% power for 22 times (1 s/time, 1 s interval) on ice. To obtain homogenous fibril sample for cryo-EM study, 100 μM α-syn monomer was incubated in the presence of α-syn PFFs (1%, v/v) at 37 °C with constant agitation (1,000 rpm, 5 days for Ac-A53T; 900 rpm, 3 days for Ac-WT). As for primary neuron related experiments, the fibrils were further washed by PBS buffer for three times and sonicated into α-syn PFFs (20% power, 22 times with 1 s/time, 1 s interval).

Negative-staining transmission electron microscopy

Fibril solution (5 μl aliquot) was added on the glow discharged 200 meshed carbon coated copper grids (Beijing Zhongjingkeyi Technology Co., Ltd.) and incubated for 45 s, then sequentially washed by 5 μl double-distilled water and 5 μl 3% w/v uranyl acetate. The grid was stained by 5 μl 3% w/v uranyl acetate for 45 s. Filter paper was applied to remove excess solution. The images were collected by Tecnai T12 microscope (FEI Company).

Rat primary neuron experiment

Primary cortical neurons were prepared from the cortex of embryonic day (E) 16–E18 of Pregnant Sprague-Dawley (SD) rats purchased from Shanghai SIPPR BK Laboratory Animals Ltd, China. The experiments were followed by a previously published procedure[1]. In brief, 150,000 cells from dissociated cortical neurons were cultured onto coverslips in each hole of 24-well plate coated with poly-D-lysine. Primary cortical neurons were cultured in Neurobasal media with B-27 supplement, 0.5 mM L-glutamine and 1% penicillin-streptomycin at 37 °C, 5% CO_2. Neurons were then treated with Ac-WT, Ac-A53T α-syn PFFs or PBS at 8 days in vitro (DIV) with the final concentrations of 250 nM of α-syn monomer equivalent. The neuron samples were collected for immunocytochemistry at 14/18/22 days post-treatment. All rat experiments were performed according to the protocols approved by Animal Care Committee of the Interdisciplinary Research Center on Biology and Chemistry (IRCBC), Chinese Academy of Sciences (CAS). The experiment was repeated for more than three times.

Immunofluorescence imaging

Cultured neurons on day 14,18 and 22 post-treatment were fixed with fixation buffer (4% paraformaldehyde and 4% sucrose in PBS) for 10 minutes and permeabilized with buffer containing 0.15% Triton X-100 for 15

minutes after three times PBS washing. The fixed coverslips were blocked with 5% bovine serum albumin in PBS for 30 minutes at room temperature. Neurons were stained with anti-p129-α-syn (Abcam, ab51253, 1:1,000 dilution) and anti-MAP2 (Abcam, ab5392, 1:1,000 dilution) antibodies, respectively. Alexa Fluor 488- and Alexa Fluor 568-conjugated secondary antibodies (Life Technologies, A11039 and A11036, 1:1,000 dilution) were incubated for 1 h at room temperature after washing with PBS for three times. Finally, coverslips were mounted onto glass slides with ProLong Gold antifade reagent containing DAPI. SP8 confocal microscope was used for collecting images. The statistical analysis of fluorescent gray value of pS129 α-syn signal was quantified by using ImageJ software.

Lactate dehydrogenase (LDH) assay

Primary neurons were treated with Ac-WT, Ac-A53T α-syn PFFs or PBS at 8 DIV with α-syn concentrations of 250, 500, 1,000 nM (monomer equivalent), and harvested for LDH measurement at 23 days post-treatment. The LDH cytotoxicity assay was performed following the manufacturer's instructions. The absorbance was recorded at 490 nm and 680 nm (background) with BioTek™ Synergy™ 2 Multi-Mode Microplate Readers (Thermo Scientific). The experiment was repeated for more than three times, independently. Statistical significance was determined by one-way ANOVA using GraphPad Prism.

Cryo-EM sample preparation and data collection

The Ac-A53T α-syn fibril sample (4 μl aliquot) was adsorbed to glow discharged 300 meshed cryo-EM copper Quantifoil grids (R1.2/1.3) twice. Filter papers were pretreated in FEI Vitrobot Mark IV with 95% humidity at 16 °C. Grids were plunge frozen in liquid ethane cooled by liquid nitrogen after being blotted for 6.5 s. The grids with fibril samples were imaged on a FEI Titan Krios transmission electron microscope at 300 kV equipped with a GIF Quantum energy filter (slit width 20 eV). The cryo-EM images were captured and recorded using a Gatan K2 Summit camera at a magnification of 105,000× and in super-resolution mode with physical pixel size 0.678 Å. Defocus values were set in a range of -2.1 to -1.3 μm. Micrographs are collected in dose fractionation mode (32 frames for each micrograph) by the Serial EM software[3]. A total dose of ~ 35 electrons per square angstrom ($e^-/Å^2$) was used for each micrograph and total exposure time was set as 8 s (0.25 s per frame).

Image processing

MotionCorr2 was performed to correct beam induced sample motion with dose weighting implement[4]. 32 frames of each micrograph were aligned and summed, resulting in a physical pixel size of 1.356 Å. CTF estimation was performed on movie stacks using CTFFIND4.1.8 to estimate the defocus values of dose-weighted micrographs. All filaments were manually selected using the manual picking in RELION3.0[5] and 16,736 filaments were picked from 1,338 micrographs manually. Particles were extracted into 1024- and

288-pixel boxes in RELION using the 90% overlap scheme. 291,154 segments were obtained after extraction in 288 pixel boxes and then classified by 2D (two-dimensional) classification.

Helical reconstruction

2D classification was performed with all extracted 291,154 segments. 4.8 Å was set as an initial value of helical rise of amyloid fibrils in particle extraction. Symmetry of pseudo-2_1 axis in two protofilaments was observed in 2D classification results because of a staggered arrangement between the subunits on the two sides of the filament. Thus, the rise was set as 2.4 Å in subsequent 3D classification.1024 and 288-pixel box size class averages of fibrils were used to determine crossover distance and further calculate an initial twist angle. 2D classification was performed with several iterations to exclude heterogeneous particles as well as to select segments from homogeneous classes. 3D initial model was obtained with selected homogeneous segments and applied to 3D classification as reference map in a low-pass filter. Class3D jobs with K=3 were typically used in 3D classification to generate a starting reconstructions model. Additional 3D classifications using the preliminary reconstructions helped to obtain particles in a homogeneous state. Several iterations of 3D classification with right parameters provided appropriate reference for 3D refinement and final reconstruction.

The final map of Ac-A53T α-syn fibrils was convergence with the rise of 2.42Å and the twist angle of 179.55°. Post-processing with a soft-edge mask and an estimated map sharpening B-factor of -141.697 $Å^2$ provided a map with a resolution of 3.49 Å based on the gold-standard FSC = 0.143 criteria.

Atomic model building and refinement

COOT was used to build and modify atomic model into the refined density map[6]. The model of Ac-A53T α-syn fibril was built based on the model of H50Q α-syn fibril (PDB ID: 6PES). Finally, a 3-layer model was generated for structure refinement. Structure models were refined by using the real-space refinement program in PHENIX[7].

Buried surface area

The buried surface area was calculated using areaimol from the CCP4 package[8] with a probe radius of 1.4 Å. The difference between the solvent accessible surface areas of one α-syn chain alone and within the 5-layer fibril structure represents half of the reported buried area.

Supplementary References

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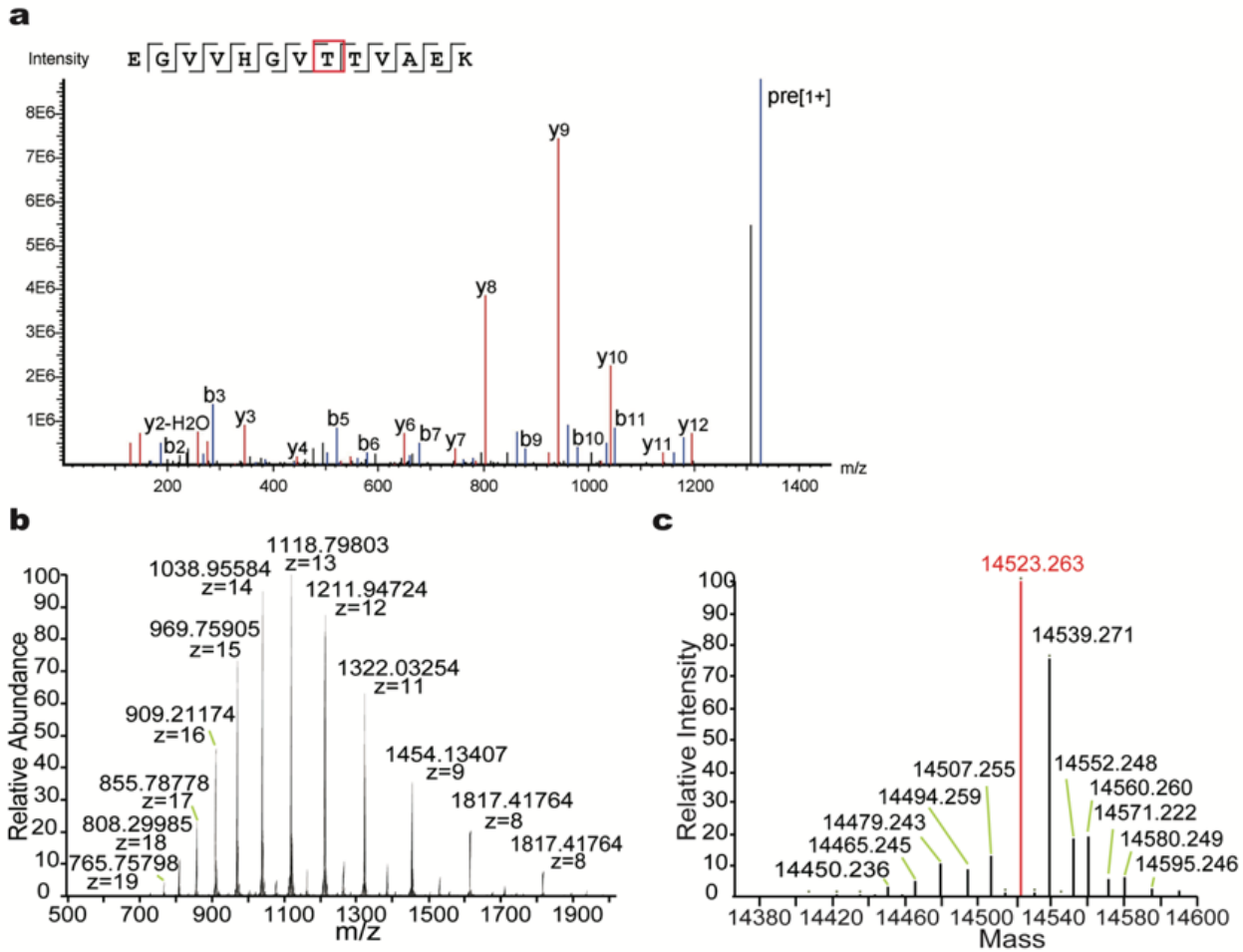

Figure S1. Mass Spectrometry analysis of the recombinant Ac-A53T α-syn. (**a**) LC-MS/MS analysis of the peptide ^{46}EGVVHGVTTVAEK^{60} from trypsin digestion of Ac-A53T α-syn confirms that Ala at position 53 was indeed replaced to Thr. (**b**) Combined ion series of the Ac-A53T α-syn. (**c**) The deconvoluted MS spectrum of Ac-A53T α-syn. The result shows that the A53T α-syn protein is acetylated as indicated by the red line.

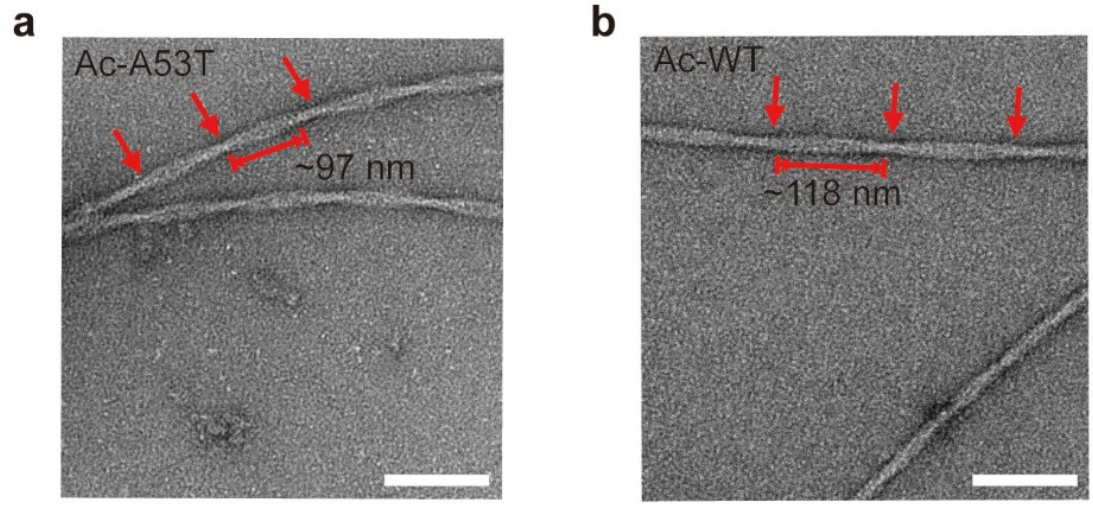

Figure S2. Different morphologies of Ac-A53T and Ac-WT α-syn fibrils. Negative-staining TEM images of the Ac-A53T α-syn fibrils (**a**) and the Ac-WT fibrils (**b**) are shown. Fibril pitch length is measured. Scale bar = 100 nm.

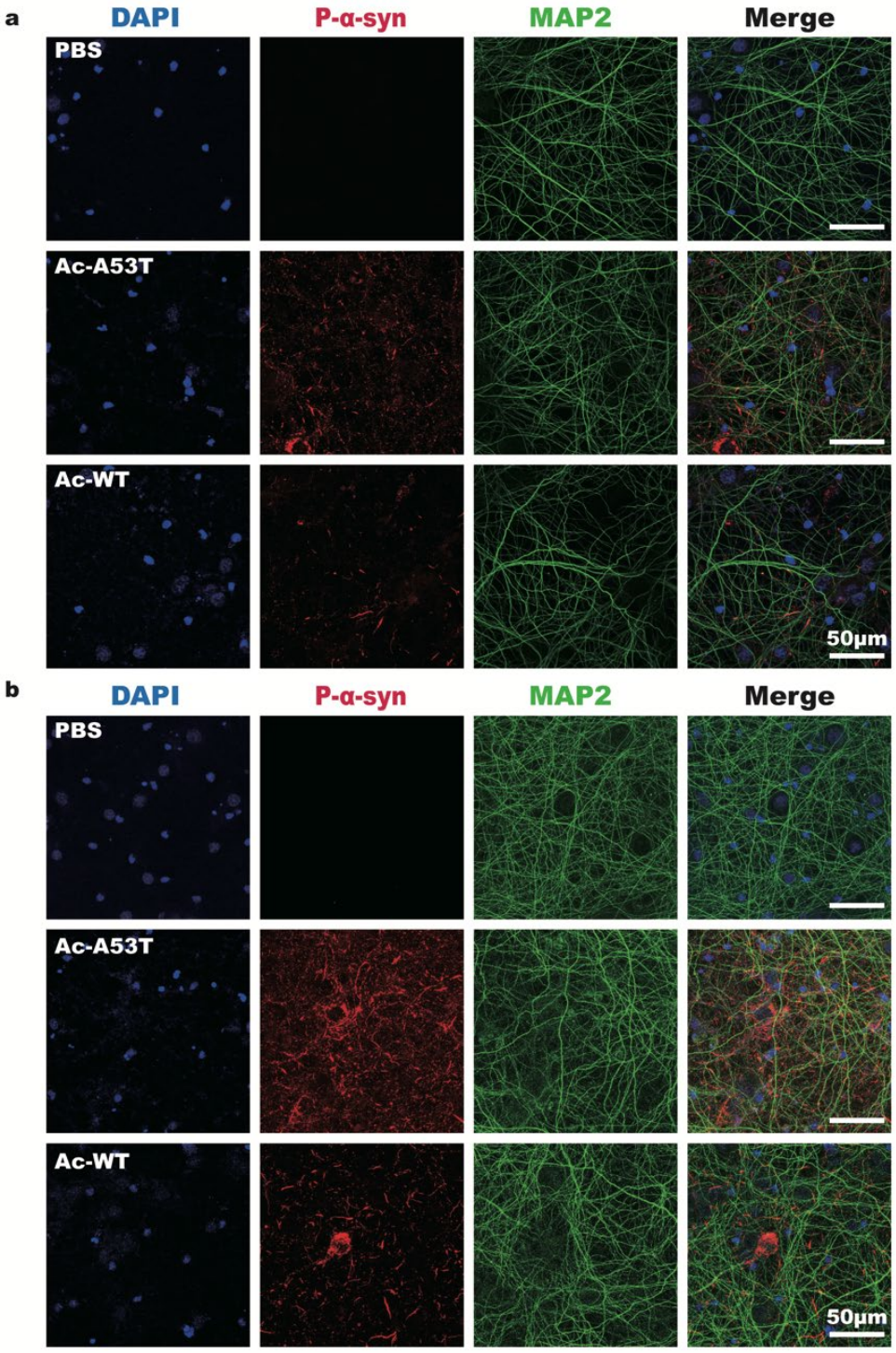

Figure S3. Induction of the endogenous α-syn aggregation in rat primary cortical neurons treated with the Ac-WT and Ac-A53T α-syn PFFs, respectively. Primary neurons at DIV8 were treated with 250 nM Ac-A53T or Ac-WT α-syn PFFs for 14 days (**a**) and 18 days (**b**). Fixed neurons were imaged by confocal microscopy. DAPI was stained in blue, MAP2 was in green and pS129 α-syn was in red.

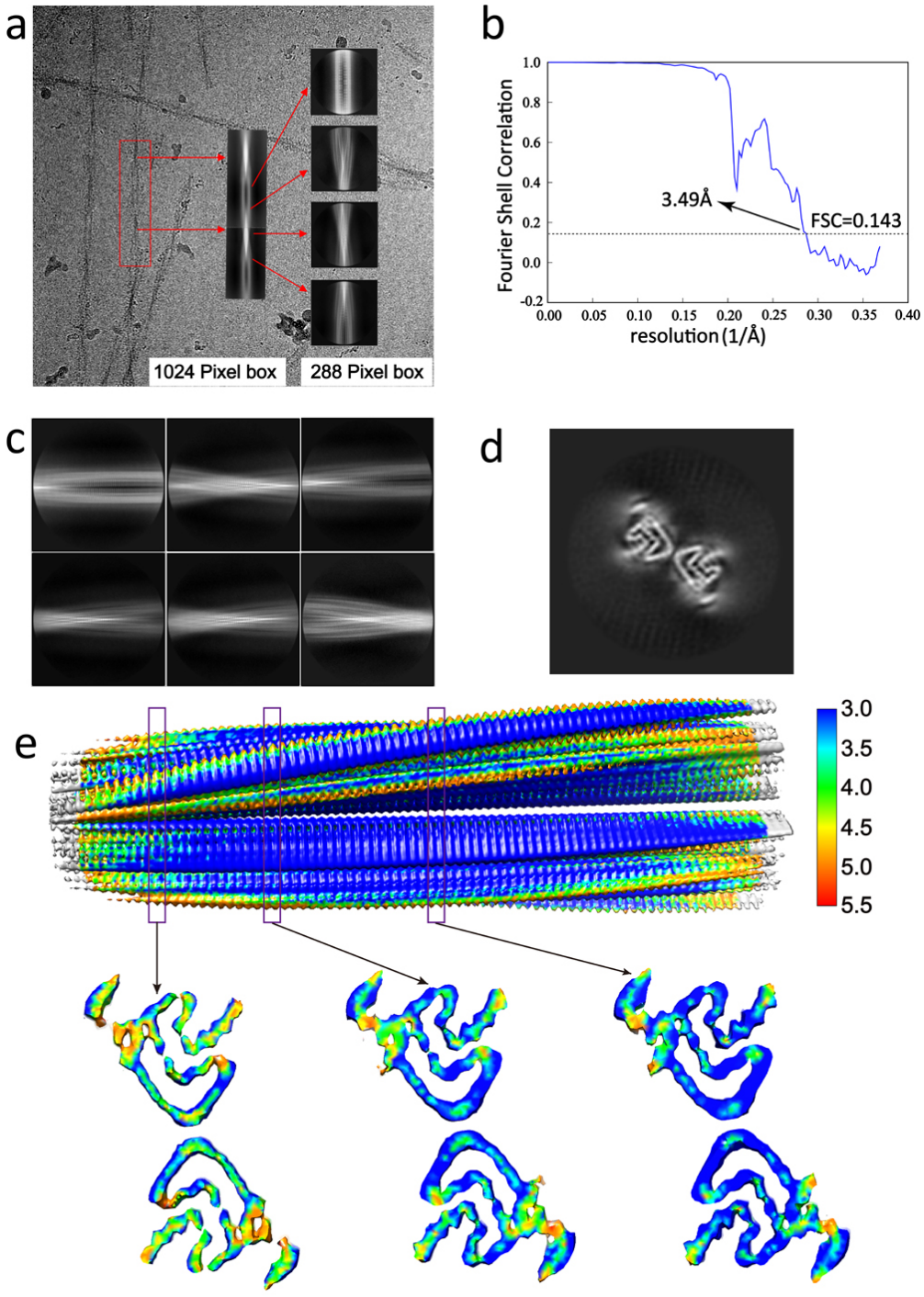

Figure S4. Cryo-EM structure determination of Ac-A53T α-syn fibril. (**a**) 2D classification of the Ac-A53T α-syn fibril was mapped on a single fibril from a cryo-EM micrograph of the Ac-A53T fibril. This morphology occupies ~77% of the total fibrils. The rest were heterogeneous and not able to be classified. (**b**) Gold-standard Fourier shell correlation curve of the Ac-A53T α-syn fibril. The overall resolution is 3.49 Å using the FSC cut-off of 0.143. (**c**) Representative 2D class averages of Ac-A53T fibril. (**d**) Representative 3D class averages of Ac-A53T fibril. (**e**) Density maps are colored according to local resolution estimation by Resmap. Structure model building was based on the central region of the density map, which exhibits the highest resolution.

Table S1. Statistics of cryo-EM data collection and structure refinement.

Name	**Ac-A53T α-syn fibril**
PDB ID	6LRQ
EMDB ID	EMD-0958
Data Collection	
Magnification	105,000
Pixel size (Å)	0.678
Defocus Range (μm)	-1.3 to -2.1
Voltage (kV)	300
Camera	K2 summit
Microscope	Titan Krios
Exposure time (s/frame)	0.25
Number of frames	32
Total dose (e^-/Å)	47
Reconstruction	
Micrographs	1,338
Manually picked fibrils	16,736
Box size (pixel)	288
Inter-box distance (Å)	39.1
Segments extracted	291,154
Segments after Class2D	178,759
Segments after Class3D	22,958
Resolution (Å)	3.487
Map sharpening B-factor ($Å^2$)	-141.697
Helical rise (Å)	2.420
Helical twist (°)	179.551
Atomic model	
Initial model used (PDB ID)	6PES
Non-hydrogen atoms	2,628
Protein residues	378
Ligands	0
r.m.s.d. Bond lengths	0.009
r.m.s.d. Bond angles	0.850
All-atom clash score	20.53

Rotamer outliers	0.00%
Ramachandran Outliers	0.00%
Ramachandran Allowed	14.75%
Ramachandran Favored	85.25%