



Cryo-EM structure of amyloid fibril formed by α -synuclein hereditary A53E mutation reveals a distinct protofilament interface

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Synucleinopathies like Parkinson's disease (PD), dementia with Lewy bodies (DLB), and multiple systems atrophy (MSA), have the same pathologic feature of misfolded α -synuclein protein (α -syn) accumulation in the brain. PD patients who carry α -syn hereditary mutations tend to have earlier onset and more severe clinical symptoms than sporadic PD patients. Therefore, revealing the effect of hereditary mutations to the α -syn fibril structure can help us understand these synucleinopathies' structural basis. Here, we present a 3.38 Å cryo-electron microscopy structure of α -synuclein fibrils containing the hereditary A53E mutation. The A53E fibril is symmetrically composed of two protofilaments, similar to other fibril structures of WT and mutant α -synuclein. The new structure is distinct from all other synuclein fibrils, not only at the interface between proto-filaments, but also between residues packed within the same proto-filament. A53E has the smallest interface with the least buried surface area among all α -syn fibrils, consisting of only two contacting residues. Within the same protofilament, A53E reveals distinct residue rearrangement and structural variation at a cavity near its fibril core. Moreover, the A53E fibrils exhibit slower fibril formation and lower stability compared to WT and other mutants like A53T and H50Q, while also demonstrate strong cellular seeding in α -synuclein biosensor cells and primary neurons. In summary, our study aims to highlight structural differences – both within and between the protofilaments of A53E fibrils – and interpret fibril formation and cellular seeding of α -synuclein pathology in disease, which could further our understanding of the structure-activity relationship of α -synuclein mutants.

Synucleinopathies, like Parkinson's disease (PD), dementia with Lewy bodies (DLB), and multiple systems atrophy (MSA), are all linked to the accumulation, deposition and dysfunction of misfolded α -synuclein protein (α -syn) (1, 2). There is

compelling evidence that the hallmark feature of Parkinson's disease is the aggregation of alpha-synuclein in Lewy bodies (LBs) and Lewy neurites (LNs). In the patients diagnosed with MSA, the same fibrillar form of α -syn from Glial cytoplasmic inclusions (GCIs) is found in the oligodendrocytes of white matter tracts (3, 4). Moreover, α -syn's genetic variability in the SNCA gene (including single point mutation and genomic duplication or triplication of SNCA) is robustly linked to familial parkinsonism and plays a crucial molecular role in the early onset of PD (5–9). So far, multiple single point mutations in α -syn have been found to cause familial Parkinson's disease, including A18T, A29S, A30P, E46K, H50Q, G51D, A53E, A53V, A53T, and E83Q, leading to an earlier onset and more severe clinical symptoms and pathology (9–15).

A53E is a novel α -synuclein hereditary mutation that was first discovered from a biopsy of a Finnish patient with atypical Parkinson's disease (PD) (14). Previous studies have shown that the A53E mutation delays α -syn fibril formation and enhances toxicity in cell cultures under stressful conditions (16). *In vitro* studies indicate that the A53E mutation imparts a lower membrane binding affinity than the wild-type (WT) protein (17). Moreover, the patient with this novel SNCA A53E mutation showed highly abundant α -syn pathology throughout the brain and spinal cord with both MSA and PD features.

Various PD mutations have been linked to different forms of disease with distinct pathologies. The A30P and H50Q mutations are linked to patients with classic PD, E46K is linked to DLB, while A53E, A53T, and G51D bring about MSA and severe PD symptoms (16, 18–20). This noticeable variation in severity and disease development between different α -syn mutations highlights the importance of key residues that influence the fibril fold and overall negative symptomatic effects caused by fibrils. By characterizing and determining the near-atomic resolution structure of fibrils with mutant α -syn, we might help elucidate the molecular mechanisms in the formation and spread of this synucleinopathy.

Previously, we showed that WT full-length α -syn fibrils could exist in two distinct polymorphs, termed: rod and twister (21). The rod's protofilaments are formed by the ordered

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residues 38 to 97 of α -syn, where residues 50 to 57 from the preNAC region form the interface between protofilaments. On the other hand, the twister polymorph protofilaments are formed by ordered residues 43 to 83 and residues 66 to 78 from its NACore form the interface. For both WT polymorphs, rod and twister, residue A53 is located at the hydrophobic interface of the protofilament. It's logical to speculate that the A53E mutation might alter key contacts at the protofilament interface, potentially leading to a different fibril structure and downstream pathology. In this study, we look to highlight these differences in a structural context and have determined the cryo-EM structure of α -syn fibrils containing this A53E hereditary mutation. By comparing WT fibril structures to those of other mutant fibril structures, we might unveil conserved or labile structural similarities and differences between α -syn mutants; identify key interactions between residues within fibrils and their influence on the fibrillar energetic fold; and explain how single mutations at key locations within α -syn changes its core morphology and influences the symptomatic severity between wild-type and heritable Parkinson's diseases.

Results

Cryo-EM structure and architecture of A53E fibril

In order to further study the influence of this hereditary mutation on the α -syn amyloid fibril formation, we expressed and purified recombinant full-length α -syn containing the A53E hereditary mutation and grew fibrils using our previously established protocol (21). Cryo-EM imaging and subsequent 2D classification revealed that the fibril sample is morphologically homogeneous with one major species (Figs. 1A and S1). By helical reconstruction, we obtained a

cryo-EM structure of A53E α -syn fibrils at a resolution of 3.38 Å (Table S1). The A53E fibril polymorph has a pitch of ~ 880 Å with a width of ~ 120 Å. The fibril contains two intertwined protofilaments related by an approximate 2_1 screw axis of symmetry, with a helical rise 2.42 Å and a helical twist of 179.5° between α -syn subunits (Figs. 1, B and C and S2, A and B).

The quality of our cryo-EM density map was sufficient for us to unambiguously build an atomic model for the A53E fibril (Fig. 2A and Table S1) and shows that the A53E fibrils are wound together by two protofilaments. Within each subunit of the protofilament, residues Leu38 to Gln99 form a Greek-key-like fibril core, which is similar to that of a WT α -syn fibril structure with two turns. Each α -syn subunit comprises seven in-register parallel β -strands [*i.e.*, residues 48–50 (β_1), 52 to 56 (β_2), 61 to 64 (β_3), 69 to 72 (β_4), 75 to 77 (β_5), 81 to 83 (β_6), 94 to 96 (β_7)]. The A53E fibril core features a loose fold containing 7 short β -strands; on the other hand, WT α -syn fibril core contains a long β -strand (β_1) and 4 short β -strands. This A53E fibril core forms a flat, two-dimensional layer, stacked every 4.8 Å along the fibril axis to form a mature β -sheet fibril that extends up to hundreds of nanometers (Fig. 2, C–E). Such packing schema of the stabilized A53E fibril leads to a hydrophobic core, surrounded by hydrophilic residues (Fig. 2B).

In the A53E fibril structure, we also observed two unidentified densities flanking the two protofilaments, which we term 'islands.' Amino acid side chains located on these islands, possibly from residues 106 to 109 or 111 to 114, could form a hydrophobic interaction with residues T⁶⁴-N⁶⁵-V⁶⁶ on the A53E β -arch to stabilize A53E fibrils (Fig. S3, A–C). Similar islands are also observed in the structure of α -synuclein hereditary disease mutant H50Q (22). The presence and absence of different islands among different fibril polymorphs also

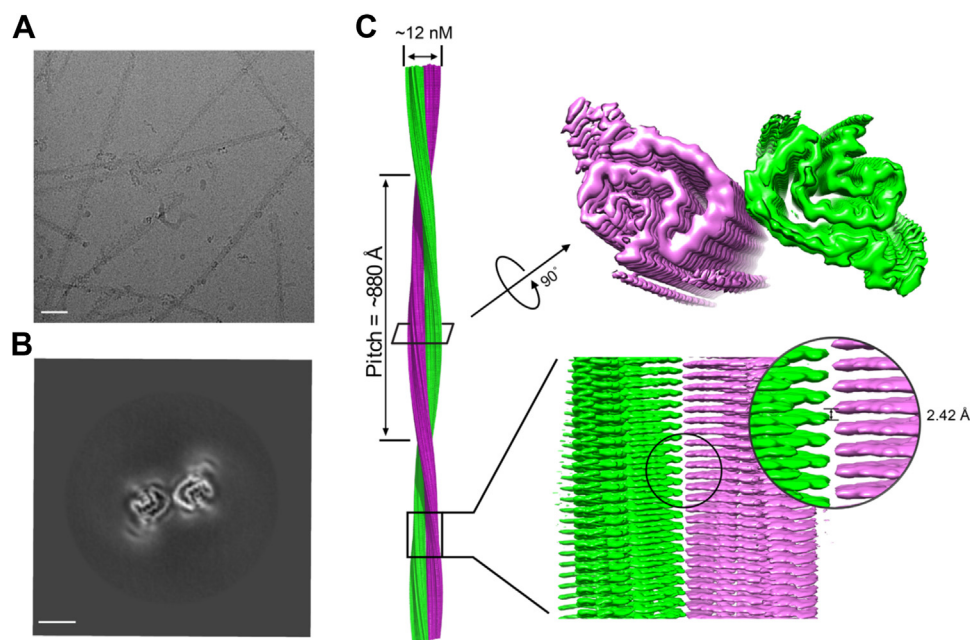


Figure 1. Cryo-EM structure of A53E α -syn fibril. A, A raw cryo-EM micrograph of amyloid fibrils from full-length human A53E α -syn. Scale bar, 500 Å. B, cross-sectional view of the 3D reconstruction of these fibrils showing two protofibrils forming a dimer. Scale bar, 500 Å. C, Cryo-EM 3D density map of the A53E α -syn fibril. Fibril width, length of half pitch (180° helical turn), and helical rise is indicated. The two protofibrils are colored in green and purple.

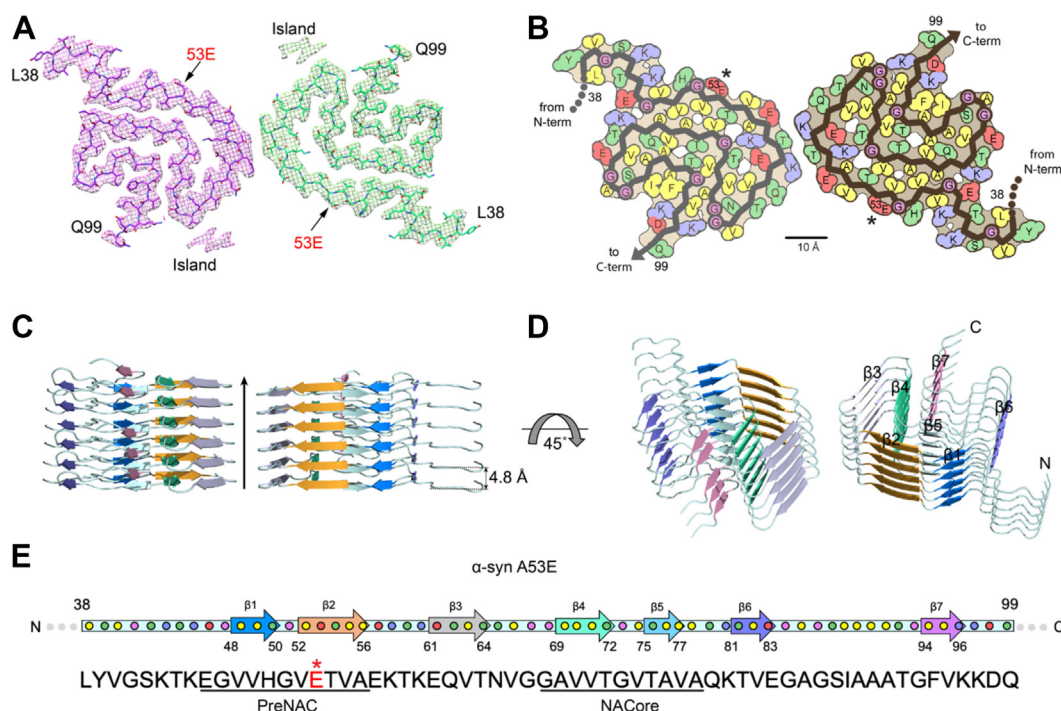


Figure 2. Overall structure of the α -syn A53E fibril. *A*, Top view of the A53E fibril. One layer of the structure is shown, which consists of two α -syn molecules covering residues 38 to 99. The two molecules are colored differently. *B*, schematic representation of fibril structure with amino acid side chains colored as follows: hydrophobic (yellow), negatively charged (red), positively charged (blue), and polar uncharged (green). *C*, side views of the multi-layer structure of A53E fibril, the distance between neighboring layer in one protofilament is indicated. *D*, ribbon representation of the structure of an A53E fibril core in *b*). β -strands on the top layer are shown. *E*, primary and secondary structure of α -syn A53E fibril. Arrows in the secondary structure indicate that conservative residues adopting β -strand conformation, and each colored dot represents a residue. PreNAC comprises residues 46 to 56; NACore comprises residues 68 to 78. The A53E mutation lies in the preNAC region, asterisk indicates the location of residue 53 (red).

reflect the structural diversity of amyloid protein outside of the conserved fibril kernel and may influence the significant differences seen in α -syn pathology.

Comparison of A53E and wild type α -Syn protofilament folds

Compared to the other α -synuclein fibril polymorphs, the structure of the A53E has a common conserved β -arch-like protofilament kernel, but a different interface between the two protofilaments (Fig. 3D). In the WT rod structure, the residue Ala53 is involved in a steric-zipper interface between the two protofilaments formed by preNAC region residues 50 to 57 ($^{50}\text{HGVA}/\text{ETVAE}^{57}$), which is assumed to be disrupted by A53E mutation. In the WT twister polymorph, NACore residues 66 to 78 ($^{66}\text{VGGA VVTGVTAVA}^{78}$) form a homotopical steric-zipper interface which bundles the two protofilaments together. However, the A53E fibril contains a protofilament interface formed by only two residues ($^{59}\text{TK}^{60}$) not previously seen in the WT α -syn structures. Residues $^{59}\text{TK}^{60}$ are located at a sharp turn in both rod and twister WT protofilament polymorphs, making its interface between protofilaments extremely small and without obvious interaction (Fig. 3, A–C). Further analysis revealed the buried surface area to be $10.3 \text{ \AA}^2$ with a shape complementarity of 0.06, which is in stark contrast to the buried surface area of $65\sim 92 \text{ \AA}^2$ and shape complementarity of ~ 0.7 in the WT α -syn polymorph(s) as well as the buried surface area of $34\sim 50 \text{ \AA}^2$ and shape complementarity of $0.17\sim 0.57$ in other synuclein mutants (H50Q, A53T) fibrils (Fig. S6). The A53E fibril interface is very

small compared to the more extensive preNAC and NACore interfaces seen in wild-type structures. Even compared with the protofilament interface of A53T fibrils, which contains the same two residues interfaced, A53E still has the smallest fibril protofilament interface observed to date.

The second key difference between A53E and WT α -syn structures is their pattern of electrostatic interactions. In the WT rod fibril structure, residues E46 and K80 form a salt bridge, which is crucial for the stabilization of the Greek-key-like structure conformation (Fig. 4, B and E). In the WT twister fibril, the E57–K58 residues also form an essential surface-exposed salt bridge to stabilize fibril conformation (Fig. 4, C and F). In contrast, the A53E mutant rearranges the electrostatic interactions in the fibril and imparts obvious changes in its electrostatic interactions. We note the formation of one new salt bridge not seen in WT fibrils formed by K96 and D98 residues to stabilize the conformation of the C-terminal region (Fig. 4, A and D).

Cavity difference in α -syn fibril structures

The formation of a cavity within the protofilament is a common feature in all α -syn fibril structures determined to date. In our previously obtained WT α -syn fibril structure, the cavity is surrounded by residues T54, A56, T59, E61, T72, G73, and T75 at the center of the β -arch. However, in the mutant A53E fibril, only residues T54, A56, K58, G73, and V74 are involved in the formation of the cavity, making it smaller than that of the WT (Fig. 2, A and B). Moreover, this is probably

Cryo-EM structure of α -synuclein mutant A53E fibrils

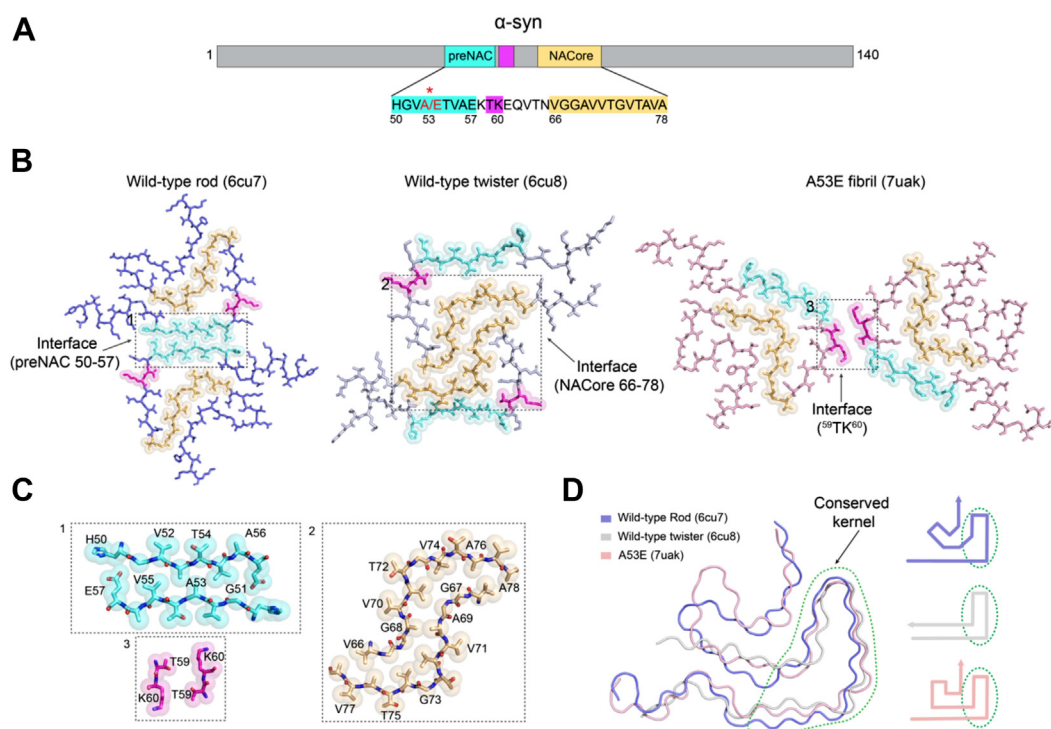


Figure 3. Comparison of the WT and A53E α -syn polymorphs. A, primary structure schematic highlighting residues of the conserved kernel (50–78) that form protofilament interfaces in α -syn polymorphs. The preNac region is shown in cyan, residues ⁵⁹TK⁶⁰ are shown in purple, and NACore region is shown in orange. B, protofilament interface of the WT rod, twister, and A53E fibrils. Residues involved in the interfaces are highlighted with spheres. The fibril interface of WT rod fibril is colored in cyan, that of the WT twister fibril is in magenta, and that of the A53E fibril is in orange. The interface and electrostatic interactions are zoomed in C, zoomed-in views of the fibril interface interactions. Interface residues are labeled. D, compare structures of every single α -syn subunit from the A53E, WT rod, and WT twister fibrils. A53E fibril is in light pink; WT rod fibril is in slate. E46K fibril is in gray. The conserved kernel region with a similar structure shared by three different fibrils is marked with a green dashed circle.

caused by a conformational change with residues K58 and T59's side chain orientation. Compared with the wild-type structure and the MSA brain extracted structure, the side chain of K58 faces inwards towards the cavity, while T59's flips away from the cavity into the solvent. This inversion is speculated to promote the stability of the hereditary mutant fibril and creates a more compact fibril core. Similarly, we see this flipped orientation in other mutants like H50Q (Fig. S4, A–F) (22, 23).

In the cavity of the A53E α -syn structure, we observe an unidentified density. A larger but similarly shaped density was also found in our previous H50Q fibril structure (22). However, these densities in the A53E and H50Q α -syn structures don't align with each other, and thus are likely distinct in identity. Although the resolution of the cryo-EM density map is insufficient to determine whether this density is part of the protein structure or bound solvent molecules, density diversification at the cavity near the fibril core reflects the conformational variation amongst multiple α -syn fibril structures, which may play a role in fibril formation and α -synuclein seeding.

Comparison of the stability and pathological characteristics of WT and A53E α -syn fibrils

To assess the role that the mutation A53E plays in synuclein fibril formation and stability, we monitor the fibril formation of WT and A53E synuclein *via* thioflavin-T (ThT)

fluorescence. Our ThT experiments indicate that, both A53E and WT aggregation proceeded with a typical sigmoidal curve. A53E reached maximum intensity in $\sim$ 52 h, while it only took $\sim$ 38 h for WT α -syn. This experiment, in agreement with previous studies (24), confirms that the fibril formation of A53E is slower than WT α -synuclein (Figs. 5A and S8). We next asked if the structural differences that we observe in the A53E mutant affects its stability, compared to wild-type fibrils. The fibril stability is assessed using a sodium dodecyl sulfate (SDS) denaturation assay. Both WT and A53E fibrils were incubated with various concentrations of SDS at 37 °C, and later measured using ThT fluorescence and SDS-PAGE analysis. Both results demonstrated that the A53E fibril is slightly more susceptible to chemical denaturation than WT, A53T and H50Q fibrils (Figs. 5B and S9), which is distinct from other α -synuclein mutants showing stronger fibril stability. Furthermore, structural analysis of A53E fibrils when compared to other mutant and WT fibrils highlights its smaller, and energetically weak interface between the two protofilaments (Fig. S6), even as solvation energy calculations indicates equivalent stability in their overall fibril structures (Fig. S7 and Table S2). We speculate that the poor stability of A53E fibrils in the presence of SDS denaturation may stem from A53E's weak interface between protofilaments, consisting of only 2 interacting residues.

We hypothesized that the lower stability of the A53E fibril might alter the cellular seeding of α -synuclein in a pathologic

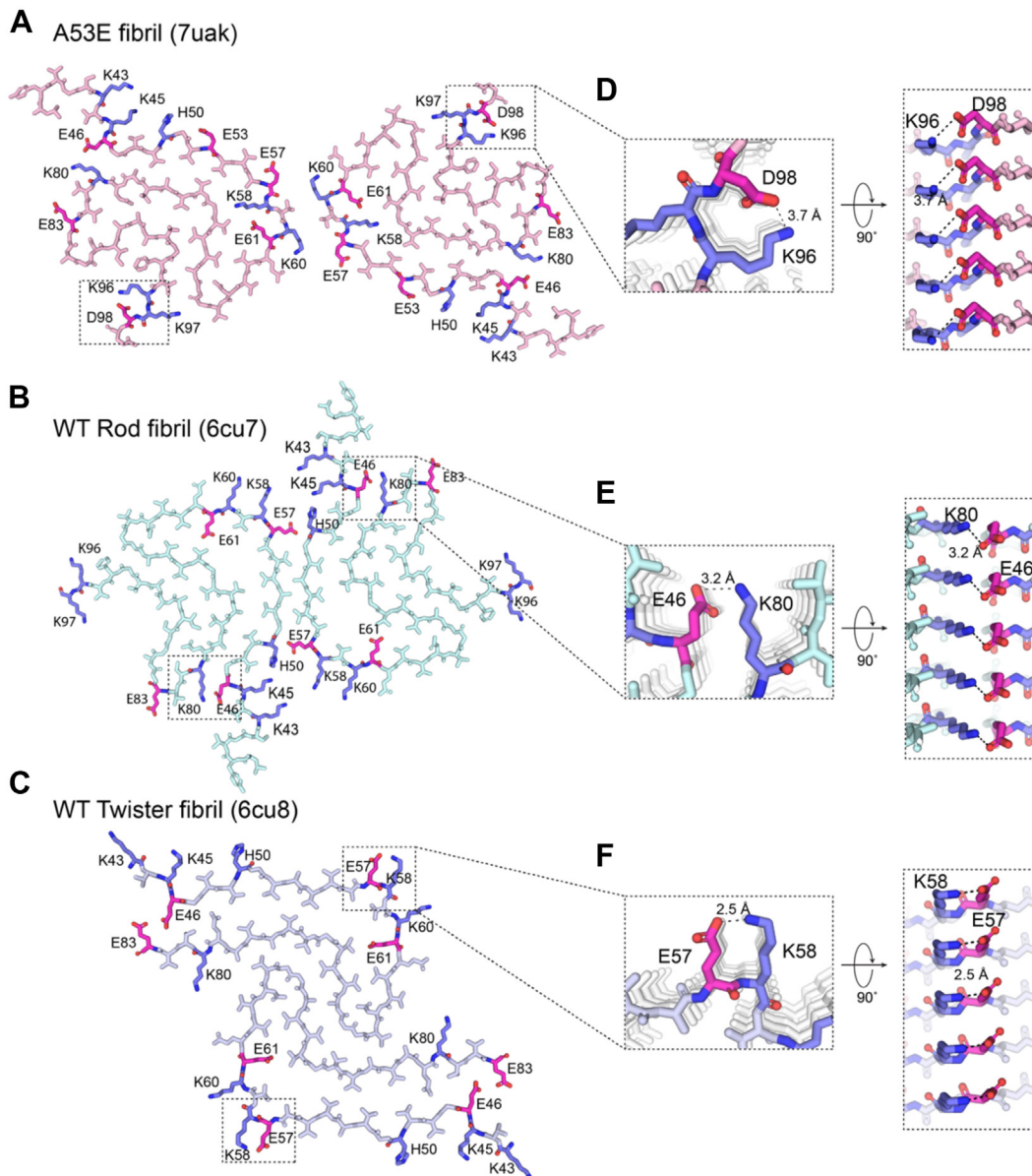


Figure 4. Electrostatic interactions in wild type and A53E α -syn fibril polymorphs. A–C, one layer of A53E, WT rod and twister fibrils are shown by sticks [colored by *light pink* (A), *cyan* (B) and *pale blue* (C), respectively], charged and ionizable residues are colored as follows: K and H are colored in slate, E and D in *magenta*. D–F, a magnified top view of the boxed region of (A–C), respectively, showing the electrostatic interactions in A53E and wild type fibrils, where two pairs of amino acids (Lys 96 and Glu 98 in A53E; Glu 46 and Lys 80 in Rod; Glu 57 and Lys 58 in twister fibril) from opposing subunits form salt bridges. A side view (*right*) highlighting a strong salt bridge between two residues from its opposing subunit, with a distance of 3.7, 3.2, and 2.5 Å (*black*).

environment, so we designed an assay to monitor this change. To examine the cellular seeding, A53E and WT fibrils were introduced to HEK293T α -syn-A53T-YFP biosensor cells and mouse primary neurons, respectively (25–27). When transduced with A53E or WT fibrils, the biosensor cells tended to develop intracellular synuclein aggregates in a dose-dependent manner. Treatment with A53E fibrils at low concentrations (~ 20 nM) induced slightly higher level of α -synuclein aggregation than WT fibrils (Figs. 5C and S10). To monitor the kinetics of α -synuclein aggregation in these biosensor cells, we performed a time course measurement of α -synuclein aggregation with the treatment of A53E compared to WT fibrils, as

a reference (Figs. 6 and S11). We found that treatment with A53E fibrils at a high concentration of 200 nM induced intracellular aggregation slower than WT fibrils, which may stem from the slower aggregation rate of A53E fibril structures. In addition to biosensor cells, we monitored the cellular seeding of A53E and WT fibrils using mouse primary neurons which more closely mimics the native cellular environment. The results show that A53E fibrils have higher seeding capacity at a lower dose (4 ng per a 50 μ l well) than WT fibrils (Figs. 5D and S12), which is consistent with previous demonstrations of the potential pathogenic enhancement of A53E over WT α -syn fibrils (16, 28).

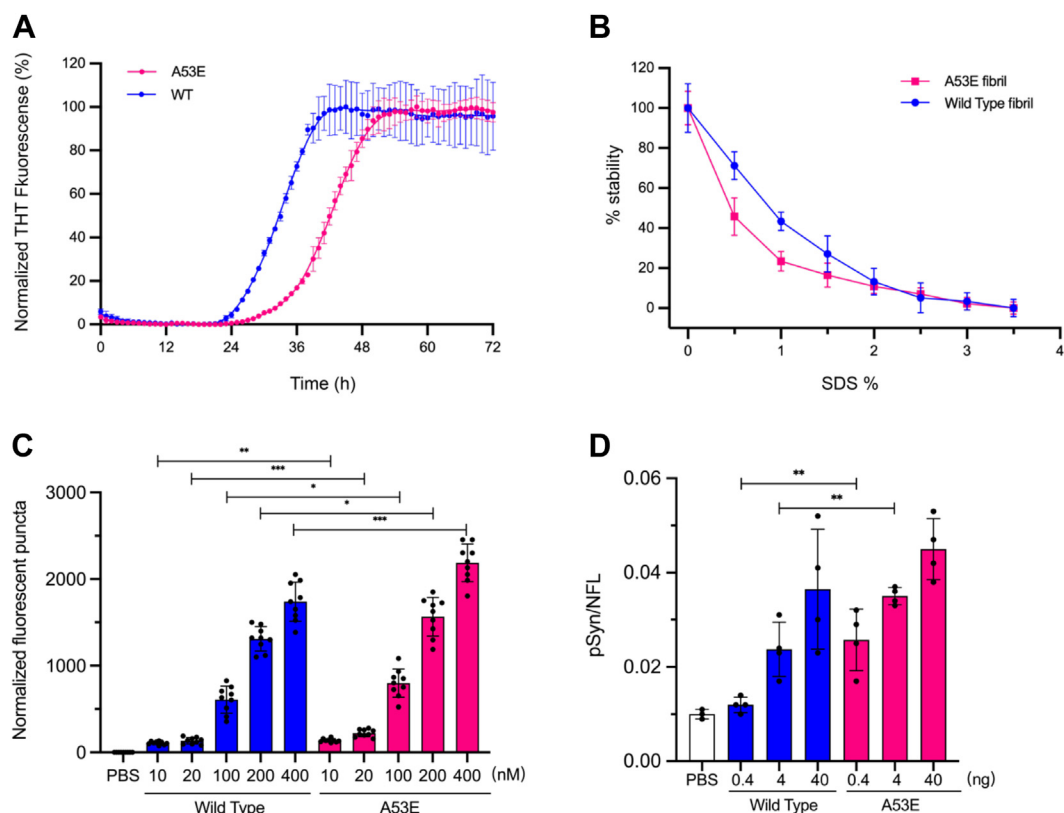


Figure 5. Comparison of the stability and pathological characteristics of WT and A53E α -syn fibrils. A, THT assay measuring kinetics of A53E and wild-type α -syn aggregation. Wild-type aggregation plateaus at 38 h whereas A53E aggregates slower and plateaus at 50 h. Data are shown as mean $\pm$ s.d., $n = 3$ independent experiments. B, stability assay of A53E and wild-type α -syn. A53E and wild-type fibrils were heated to 37 $^{\circ}$ C and incubated with varying concentrations of SDS. A53E fibrils show more instability to SDS than wild-type fibrils. Individual triplicate measurements are shown, and the plotted line represents the average of the triplicates. Data are shown as mean $\pm$ s.d., $n = 3$ independent experiments. C, HEK293T α -syn A53T-YFP biosensor cells were used to perform the cell seeding assay of wild-type α -syn and A53E fibrils. Each of sonicated fibril samples were transfected into biosensor cells using Lipofectamine 3000. After 48 h, the fluorescence microscopy images of α -syn biosensor cells were taken and the number of fluorescent puncta indicating aggregated endogenous α -syn YFP was counted. Data are presented as mean $\pm$ SD ($n = 9$, *** $p < 0.005$, one way ANOVA). D, quantification of total pS129 α -syn (stained with 81A) normalized with total NFL (stained with anti-neurofilament antibody) induced by various concentrations of wild-type and A53E α -syn fibrils in primary neurons (experiment repeated 4 times). A53E fibrils have a higher seeding capacity than wild-type α -syn fibrils. Data are presented as mean $\pm$ SD ($n = 4$, ** $p < 0.05$, one way ANOVA).

Discussion

Structural polymorphism of fibrils is a common feature of pathologic amyloids spanning proteins like α -syn, Tau, and A β . Evidence has shown that the α -synuclein fibrils found in MSA, Parkinson's disease, Parkinson's disease with dementia, and dementia with Lewy bodies (DLB) are distinct (29). The diversity of fibril structures has also been shown to cause significant differences in biological activity and clinical pathology (29–35). We plan to explore additional structures of mutant synuclein fibrils to elucidate the structure-activity relationship of synuclein fibrils in toxicity and pathology. In support of this, multiple near-atomic fibril structures have suggested that hereditary mutations may disrupt the stability normally seen with WT fibril fold conformation, as is the case with H50Q, E46K, A53T, G51D, etc. - which show distinct morphologies from WT fibrils (23, 30, 36–38). With our newly determined structure of A53E, we can further contribute to the characterization of each mutant fibril structure and infer what properties influence its pathology and clinical significance.

The cryo-EM structure of α -syn fibril with hereditary mutation A53E presented here reveals a conserved kernel

β -arch-like fold as in WT α -syn protofilaments. However, A53E α -syn forms a different and less stable protofilament interface compared to both WT rod and twister fibril interfaces, which only utilizes two residues ⁵⁹TK⁶⁰ to ensemble protofilaments (21). Moreover, the introduction of a negatively charged residue - glutamate - in the A53E structure seems to create a kinetics barrier that results in a significant decrease in the aggregation rate of α -syn fibril formation. The A53E mutation is commonly seen in mixed PD and MSA pathology in patients, as well as another α -syn mutant, G51D (28, 39, 40), both of which introduce negative charge at a residue in this same spatial region. This alteration might influence the kinetic aggregation or stability of the fibril in such a way that predisposes the patient to these particular mixed PD and MSA pathologies.

In the A53E mutant α -syn fibril structure, there is a strong effect of internal electrostatic interaction that maintains the stability of the protofilament. For example, E46 and K80 form a key salt bridge to stabilize the fibril core. But in A53E protofilament, those two residue's side chains are too far away to form a strong salt bridge. Instead, a new salt bridge formed

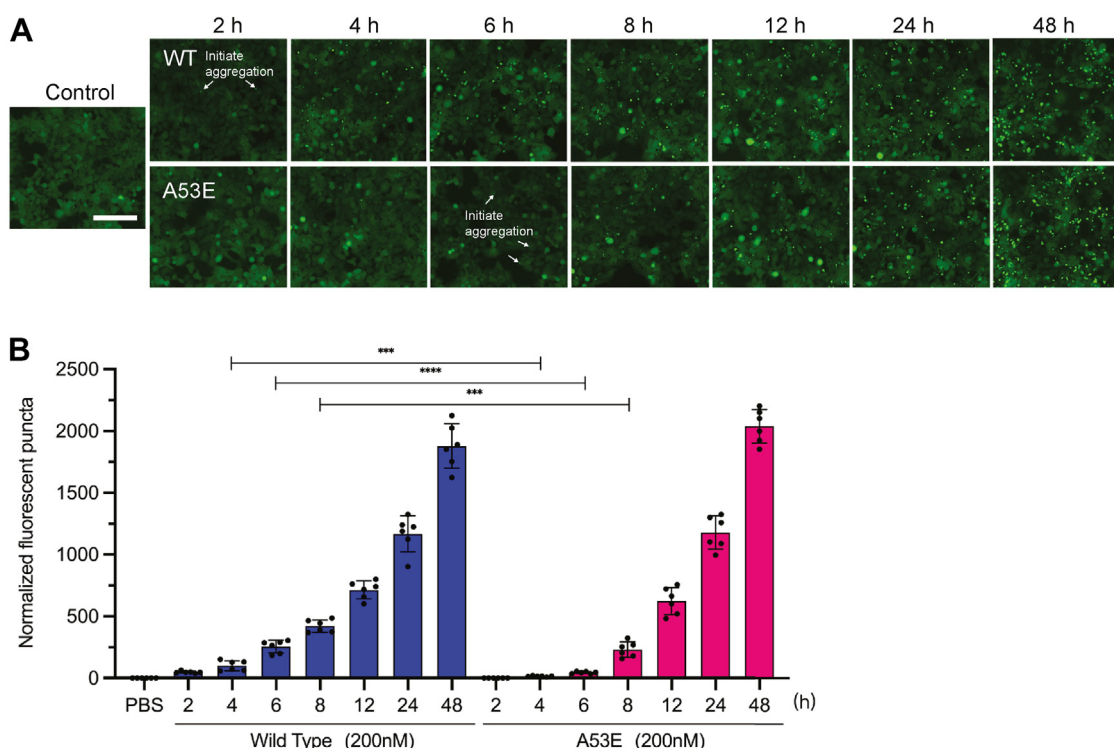


Figure 6. Time-dependent cell seeding assay of wild-type α -syn and A53E fibril via HEK293T α -syn A53T-YFP biosensor cells. A, representative fluorescence microscopy images of α -syn biosensor cells at different time points post-seeding (0–48 h). Each of the mildly sonicated 200 nM wild-type and A53E α -syn fibril sample was added to the cell culture medium, Scale bar: 100 μ m. B, aggregation of endogenous α -syn YFP was quantified using the number of fluorescent puncta in fluorescent images. Data are presented as mean $\pm$ SD ($n = 6$, *** $p < 0.005$, one-way ANOVA).

between K96 and D98 stabilizes the C-terminal region of A53E fibril. In contrast to these regions of strong interaction and stability, the A53E mutant also shares sites of relatively high flexibility. In every mutant fibril structure, there are positions where amino acid side chains flip opposite what is seen in the wild-type structures. The three sites, where this occurs in A53E and other mutants (H50Q, A53T, E46K and G51D), involve the residues K58/T59, V74/T75, and T81/V82 (Fig. S5, A and B). The pair, K58 & T59, is flipped from the outward & inward orientation to inward & outward orientation, which is similar to the H50Q and A53T protofilaments (22, 23). We speculate that this inversion may cause the cavity seen in mutant fibrils to become smaller, and thus accommodate fewer co-factors in this cavity or adopt a more compact conformation, making the fibril structure more compact. The pair of T81 & V82 also undergoes a similar inversion, where the hydrophobic side chain of V82 is flipped to the inward side, which increases the hydrophobicity of the fibril core. While these flips don't entirely change the overall fold between each mutant, it does provide insight into the sequence positions where most backbone strain occurs. In all three cases, the flipped sites occur at a turn in the layer and are places where we'd expect the most torsional strain when conforming a peptide to a flat layered structure. We guess that the minute change in a single amino acid causes a downstream shift in the overall structure, allowing the most strained amino acid conformations to relax into another spatial orientation compared to WT. By focusing on regions of variable instability between mutants with single point mutations, we can identify regions of

flexible sites which may be of interest for those looking to design therapeutics to interact and destabilize α -syn fibrils.

In the first *ex vivo* α -syn fibril structure, which was extracted from the brains of patients with MSA by Schweighauser et al, the brain-derived Type I and Type II filaments have a cavity, that contains non-proteinaceous density (29). Type I filaments have a larger cavity containing more additional densities than the cavity of Type II filaments. The authors also note that the density within the cavity could contain an unknown molecule that binds to the filaments. These finding indicates that a possible event of co-factor binding located near the cavity may contribute to maintaining fibril formation and/or stability (22, 29). The side-chain inversions which we have noted may also play a role in creating or closing the cofactor cavity by shifting neighboring residues to accommodate or exclude an accessory, or even competitive, cofactor. The binding of a cofactor molecule might nucleate the fold and pull α -syn residues around itself to transition into its filament conformation, a mechanism that begins with cofactor binding and forces the amino acids to pack around it, rather than an insertion of the cofactor into the fibril after layering. The presence of a cavity in fibril structures may affect and contribute to the overall stability of the fibril and thus influence the symptoms, kinetics, spread of fibril seeds within patients – and may explain why A53E has a weaker stability.

In summary, we have determined the first near-atomic resolution structure of the hereditary mutation A53E α -syn fibril. Compared with the wild-type α -syn fibril, A53E α -syn fibril exhibits a novel polymorphism with a different interface

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and electrostatic interactions. This structure enriches the fibril polymorphism within the α -syn family of mutants and provides a structural basis for understanding the influence of hereditary mutations on fibril formation and probing their pathogenic mechanisms.

Experimental procedures

α -syn expression and purification

Full-length α -syn wild-type, A53E, A53T and H50Q mutant proteins were expressed and purified according to a published protocol (21). Transformed bacteria were induced at an OD₆₀₀ of 0.6 to 0.8 with 1 mM IPTG for 6 h at 30 °C. The bacteria were then lysed with a probe sonicator for 10 min in an iced water bath. After centrifugation, the soluble fraction was heated in boiling water for 10 min and then titrated with HCl to pH 4.5 to remove the pellet. After adjusting to neutral pH, the protein was dialyzed overnight against Q Column loading buffer (20 mM Tris-HCl, pH 8.0). The next day, the protein was loaded onto a HiPrep Q 16/10 column and eluted with elution buffer (20 mM Tris-HCl, 1 M NaCl, pH 8.0). The eluent was concentrated using Amicon Ultra-15 centrifugal filters (10 NMWL; Millipore Sigma) to ~5 ml. The concentrated sample was further purified with size-exclusion chromatography through a HiPrep Sephacryl S-75 HR column in 20 mM Tris, pH 8.0. The purified protein was dialyzed against water, concentrated to 3 mg/ml, and stored at 4 °C. The concentration of the protein was determined using the Pierce BCA Protein Assay Kit (cat. No. 23225; Thermo Fisher Scientific).

Fibril preparation and optimization

Both wild-type, A53E, A53T and H50Q fibrils were grown under the same condition: 300 μ M purified monomers, 15 mM tetrabutylphosphonium bromide, shaking at 37 °C for 2 weeks.

Negative stain transmission electron microscopy (TEM)

3.5 μ l fibril sample was spotted onto a freshly glow-discharged carbon-coated electron microscopy grid for 2 min, then 5 μ l uranyl acetate (2% in aqueous solution) was applied to the grid for 1 min. The excess stain was removed by filter paper. Another 5 μ l uranyl acetate was applied to the grid and immediately removed. The samples were imaged using an FEI T12 electron microscope.

ThT kinetic assay

All the purified α -syn monomers (50 μ M) were adequately mixed with 10 μ M ThT and added into a 96-well-plate (final volume of 100 μ l). Samples were incubated at 37 °C for over 3 days with 600 rpm double orbital shaking. The ThT signal was monitored using the FLUOstar Omega Microplate Reader (BMG Labtech) at an excitation wavelength of 440 nm and an emission wavelength of 490 nm.

SDS stability assay

The SDS stability of the wild-type and A53E α -syn were evaluated using the aggregation tendency after the treatment

of SDS with different concentrations (0.5%~3.5%). The ThT signals were measured to monitor the aggregation of the wild-type and A53E α -syn after 5 min of SDS treatments at 37 °C with double orbital shaking at 600 rpm. The 10% SDS was added to reach different SDS final concentrations ranging from 0.5% to 3.5%. The ThT signals with 0% SDS treatment were used for normalization.

Measurement of α -syn fibril seeding using biosensor cells

HEK293T α -syn YFP biosensor cells were generously provided from the laboratory of M. Diamond (24). The cells were grown in DMEM (Dulbecco's modifications of eagle's medium with L-glutamine & 4.5 g/l glucose) supplemented with fetal bovine serum (FBS), 100 units/ml of penicillin G, and 0.1 mg/ml of streptomycin sulfate, in a humidified atmosphere of a 5% CO₂ at 37 °C. Trypsin-treated HEK293T cells were plated on collagen-coated flat 96-well plates at a density of 2.5×10^4 cells/well in 200 μ l culture medium and incubated at 37 °C in 5% CO₂. α -syn fibrils were diluted with Opti-MEM (GIBCO) and sonicated for 10 min in an ultrasonic water bath. The α -syn fibril samples were then mixed with Lipofectamine 3000 (Thermo Fisher Scientific) and incubated for 15 min and then added to the cells. The actual volume of Lipofectamine 3000 was calculated based on the dosing of 0.3 μ l per well. α -syn fibrils aggregation in the biosensor cells was visualized at 48 h by fluorescence microscopy and fluorescent images were processed in ImageJ to count the number of seeded cells. An Opti-MEM-treated control was used for normalization.

Primary neuron cultures and fibril transduction

Primary mouse neurons were prepared from the hippocampus of embryonic day (E) 16–E19 CD1 mouse embryos as previously described (27). Wild-type and A53E α -syn fibrils were diluted in Dulbecco's PBS (without Mg²⁺ or Ca²⁺) and sonicated with 20 pulses before being added to neuron medium. Sonicated fibrils were added into each well to reach final concentration of 0.4 ng, 4 ng, or 40 ng per well (50 μ l) at 10 days *in vitro* (DIV).

Immunocytochemistry and quantification

Neurons were collected for immunocytochemistry at 14 days post-treatment. Cells were washed with DPBS then fixed with 4% PFA (PFA; Electron Microscopy Sciences) containing 1% Triton X-100 for 15 min to remove soluble proteins. Following 1 h in 3% BSA and 3% FBS in DPBS blocking buffer at room temperature, neurons were incubated with anti-phospho- α -syn (81A) and anti-neurofilament antibody (NFL) overnight at 4 °C followed by staining with secondary antibodies for 2 h at room temperature. The plate was scanned on an ImageXpress Pico system scanner. Quantification of area occupied by Wild-type and A53E α -syn fibrils induced pathology was performed by Cell Reporter Xpress.

Cryo-EM imaging and drift correction

An aliquot of 2.5 μ l of fibril solution was applied to a Quantifoil Holey carbon grid (2/1, 300 mesh), that was glow

discharged for 40 s with a PELCO Easy Glow system. The grid was blotted and plunge-frozen in liquid ethane with a Vitrobot IV (Thermo Fisher) at 4 °C under 100% humidity. The frozen grids were stored in liquid nitrogen before use. For data collection, the cryo-EM grids were loaded into an FEI Titan Krios electron microscope equipped with Gatan Quantum imaging filter (GIF) and a post-GIF K2 Summit direct electron detector. Movies were recorded as dose-fractionated frames in super-resolution mode by SerialEM automation software package, with image shift induced-beam tilt correction (41). The slit width in the GIF system was set to 20 eV to remove inelastically scattered electrons. A total of 3334 movies were recorded for the data set, the nominal magnification is 1,300,00 $\times$, corresponding to a calibrated pixel size of 0.535 Å at the specimen scale. An exposure time of 7 s was used for each movie at a rate of 0.2 s per frame, and the dose rate was set to 1.35 e⁻/Å²/frame (a total dosage of 48 e⁻/Å²).

Frames in each movie were aligned for drift correction with the graphics processing unit (GPU)-accelerated program MotionCor2 (42). The first and last frame were discarded during drift correction. Two averaged micrographs, one with dose weighting and the other one without dose weighting, were generated for each movie after drift correction. The averaged micrographs were binned 2 $\times$ 2 to yield a pixel size of 1.07 Å. The micrographs without dose weighting were used for contrast transfer function (CTF) estimation and particle picking, while those with dose weighting were used for particle extraction and in-depth processing.

Cryo-EM data processing

The CTF estimation of each micrograph was performed by CTFFIND4 (43). By discarding the micrographs with under-focus values outside the allowed range (1.0–3.5 μ m), or those containing crystalline ice, we selected 2767 good micrographs from the dataset. Then a total of 46,626 filaments were picked using crYOLO (44, 45). Fibril particles were first extracted using a large box size (1024 pixels) and a 10% inter-box distance in RELION 3.1, then the particles were subjected to two-dimensional (2D) class averaging to determine the pitch (46). Helical parameters were deduced from the pitch with the assumption that each helix had a twisted twofold screw axis (21). The 2D classes reveal that A53E α -syn forms fibrils of a single morphology with a pitch of $\sim$ 880 Å, which gives the calculated helical twist to be 179.5° (helical rise of 2.4 Å) (Fig. S2C). Subsequently, we extracted all fibrils particles with a 512-pixel box size and 10% inter-box distance, yielding 226,604 particles. Using the calculated helical twist (179.5°) and helical rise (2.4 Å), these particles were subjected to a three-dimensional (3D) class averaging with a single class and a featureless cylinder created by EMAN2 as initial model (47). The cylinder was refined to a model in which two separated and twisted protofilaments could be seen. This model was then used to classify good and bad particles with a 3D class averaging with three classes. Particles in the best class of the previous 3D classification were re-extracted with a 300-pixel box size for further 3D classification. Two additional 3D

classifications were performed to select particles with better homogeneity, and a final subset of 31,916 helical segments were selected and subjected to 3D auto-refinement, CTF refinement, and post-processing, yielding a map at 3.38 Å resolution (Fig. S2, A and B). Details of the data processing are summarized in Table S1.

The global resolution reported above is based on the ‘gold standard’ refinement procedures and the 0.143 Fourier shell correlation (FSC) criterion. Local resolution evaluation was performed with RELION3.1.

Atomic model building

Atomic model building was accomplished in an iterative process involving Chimera, Coot, and Phenix (48–50). Briefly, the structure of A53T α -syn (PDB: 6LRQ) was fitted into the cryo-EM map as an initial model by Chimera (23). This fit revealed the mismatch segments of the main chain. After deleting the mismatched parts, the model was refined by ‘real-space refinement’ in Phenix. We then manually built the missing residues and adjusted side chains to match the cryo-EM map with Coot. This process of real space refinement and manual adjustment was repeated iteratively until the peptide backbone and sidechain conformations were optimized. Ramachandran, secondary-structure restraints, and NCS restraints were used during the refinement. Refinement statistics are summarized in Table S1. The model was also evaluated based on Ramachandran plots and MolProbity scores (51, 52).

Data and materials availability

Atomic coordinates have been deposited in the Protein Data Bank under accession number 7UAK. The cryo-EM density map has been deposited in the Electron Microscopy Data Bank under accession number EMD-26427.

Supporting information—This article contains supporting information.

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Author contributions—L. J., C. S. and K. Z. designed experiments and performed data analysis. C. S. expressed and purified the α -syn protein. C. S. grew fibrils of α -syn and performed biochemical experiments. K. Z. and Z. H. Z. performed cryo-EM data collection. C.

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S. and K. Z. prepared cryo-EM samples, processed cryo-EM data and built the atomic models. W. S. S. and T. H. performed cell experiments. M. R. S. carried out solvation energy calculations. L. J. and Z. H. Z. supervised and guided the project. C. S. wrote the manuscript with input from all authors.

Competing interests—The authors declare that no competing interests exist.

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