Supporting Information

Parkinson's Disease Associated H50Q Mutation Accelerates α-Synuclein Aggregation *in Vitro*.

Dhiman Ghosh, Mrityunjoy Mondal, Ganesh M. Mohite, Pradeep K. Singh, Priyatosh Ranjan, A. Anoop, Saikat Ghosh, Narendra Nath Jha, Ashutosh Kumar and Samir K. Maji*

Department of Biosciences and Bioengineering, IIT Bombay, Powai, Mumbai India 400076

***Materials and Methods*:**

***Chemicals and reagents*:** All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) except silver staining kit (purchased from Invitrogen, USA) and of highest purity grade. Water was double distilled and deionized using a Milli-Q system (Millipore Corp., Bedford, MA).

***Cloning of α-synuclein (α-Syn) mutants*:** H50Q and H50A mutants were created based on pRK172 plasmid encoding wild type human α Synuclein (α-Syn) by changing corresponding nucleotides using site directed mutagenesis. The mutants were selected using DpnI digestion. The primers used for generating in PCR recation were 5'-GAGGGAGTGGTGCAAGGTGTGGCAACAGTG-3' and 5'-CACTGTTGCCACACCTTGCACCACTCCCTC-3' for H50Q and 5'-CAAGGAGGGAGTGGTGGCTGGTGTGGCAACAGTG-3' and 5'-CACTGTTGCCACACCAGCCACCACTCCCTCCTTG-3' for H50A. Mentioned oligonucleotides were used to prime DNA synthesis on the denatured plasmid template contained the desired mutation and had the same starting and ending positions on opposite strands of the plasmid template. The entire lengths of both strands of the plasmid template were amplified during the extension step of PCR. After 30 rounds of thermal cycling the linear form of mutated plasmid containing staggered nicks on opposite strands was obtained. 1 μg of PCR products were treated with restriction enzyme DpnI (NEB, England) at 37 °C for 5 hours. DpnI specifically cleaves fully methylated Gm6ATC sequence. DpnI thus digests the bacterially generated plasmid DNA used as template for amplification but not the DNA synthesized during the course of PCR *in vitro*. The DpnI digested PCR product was thus enriched in desired mutant plasmids. 20 μl of this DpnI digested PCR products were used to transform competant *E.coli* (X10 Gold) cells and plated on Luria agar plates containing ampicilin (1μg/ml) which were then incubated at 37 °C for 14 hours. The trasformed *E.coli* cells repair nicks and the linearized plasmid get self ligated to give intact circular plasmid containing our desired mutation. The plasmids were isolated from the transformants by miniprep kit (Qiagen). Mutation was confirmed by DNA sequencing.

***Protein expression and purification*:** wt α-Syn and mutants of H50Q, H50A, A53T, A30P and E46K were expressed in *E. coli* BL21 (DE3) strain according to the established protocol

described by Volles *et al.* with little modification (1). For ^{15}N labeled α-Syn expression, minimal media containing ^{15}N-labeled ammonium chloride was used as a nitrogen source. Briefly, the IPTG induced bacterial cells were pelleted down by centrifugation (4000g, 45 minutes). The pellet was resuspended in buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 150 mM NaCl). Protease inhibitor cocktail (Roche), was added to avoid any proteolytic cleavage. It was then sonicated using probe sonicator (Sonics & Materials INC, USA) at 50% amplitude and 45 pulse/min for 10 min followed by heating in boiling water for 20 minutes at 95°C. Supernatant was collected after centrifugation (14000g, 30 minutes). 10% Streptomycin sulfate (136 µl/ml of supernatant) and glacial acetic acid (228 µl/ml of supernatant) were added to the supernatant and kept for 30 minutes at 4°C, followed by centrifugation for 30 minutes. The resulting supernatant was precipitated by equal volume of saturated ammonium sulfate, prepared at 4°C. Precipitated protein was washed with ammonium sulphate solution (saturated ammonium sulfate and water, 1:1 v/v at 4°C). The washed pellet was resuspended in 100 mM ammonium acetate, stirred for 10 minutes. The α-synuclein proteins were precipitated by adding an equal volume of absolute ethanol. Ethanol precipitation was repeated twice more. Proteins were again resuspended in 100 mM ammonium acetate, lyophilized and stored at -20°C for further use.

***Preparation of low molecular weight (LMW)*:** Solid lyophilized protein was dissolved in 20 mM Gly-NaOH buffer at a concentration of 10 mg/ml. Since synucleins were not fully soluble at pH 7.4, few drops of 2 mM NaOH were added and finally pH was adjusted to 7.4 by adding few drops of HCl. Final pH of the resulting solution was confirmed by micro pH meter (Mettler-Toledo, Switzerland, Model S20 Seven easy). The protein solution was dialyzed using 10 KDa MWCO mini-dialysis units (Millipore) against same buffer at 4°C overnight. Then LMW form of the proteins were isolated using centricon YM-100 filter (100 kDa MWCO, Millipore) as described previously (2). LMW α-Syn may contain monomeric α-Syn in equilibrium with low-order, unstructured oligomers < 100 kDa molecular weight. For NMR study we used 30kDa cutt-off filter (YM-30) where LMW α-Syn may contain mostly monomeric and/or dimeric protein. The supernatant was collected and used for the study. The concentrations of synucleins in the filtrates were determined by absorbance at 280 nm, considering the molar absorptivity (ε) is 5960 for α-Syn and its all mutants.

***Amyloid fibril formation*:** After preparation of LMW, final protein concentration was adjusted to 300 µM. Then the assembly reaction was started with LMW protein in 1.5 ml

eppendorf tube in 20 mM Gly-NaOH buffer pH 7.4, 0.01% sodium azide. The eppendorf tubes containing protein solutions were placed into an EchoTherm model RT11 rotating mixture (Torrey Pines Scientific, USA) with a speed corresponding 50 r.p.m. inside a 37°C incubator. The fibril formation was monitored using CD and ThT binding at regular intervals. The fibril formations were conformed by morphology characterization using AFM and EM at the end of assembly reaction. Three independent experiments were performed for each sample.

***Circular dichroism spectroscopy (CD)*:** 5 µl of incubated protein solution was diluted to 200 µl in 20 mM Gly-NaOH, pH 7.4 with 0.01% sodium azide. The final concentration of protein was 7.5 µM. The solution was placed into a 0.1 cm path-length quartz cell (Hellma, Forest Hills, NY). Spectra were acquired using JASCO-810 instrument. Spectra were recorded over the wavelength range of 198-260 nm. Three independent experiments were performed with each sample. Raw data were processed by smoothing and subtraction of buffer spectra, according to the manufacturer's instructions. All measurements were done at 25°C. Three independent sets of measurements were performed.

***ThT fluorescence assay*:** 1 mM ThT was prepared in Tris-HCl buffer, pH 8.0, 0.01% sodium azide. 2 µl of 1 mM ThT solution was added to the 7.5 µM protein solution in 150 µl Gly-NaOH buffer, pH 7.4, 0.01% sodium azide. ThT fluorescence assay was done using Horiba-Jobin Yvon (Fluomax4) with excitation at 450 nm and emission in the range of 460-500 nm. The slit width for both excitation and emission were kept at 5 nm. ThT fluorescence obtained at 480 nm was plotted for all proteins against incubation time and the data were fitted in a sigmoidal curve. The lag time (t_{lag}) was calculated according to the published protocol (3) using equation $y = y_0 + (y_{max} - y_0)/ (1+ e^{-(k(t-t_{1/2})}$), where y is the ThT fluorescence at a particular time point, y_{max} is the maximum ThT fluorescence and y_0 is the ThT fluorescence at t_0 and t_{lag} was defined as $t_{lag} = t_{½} - 2/k$. The fluorescence data described in this study were obtained from 3 different set of studies.

***Atomic force microscopy (AFM)*:** Time dependent monitoring of the synuclein aggregation was performed using AFM. At regular intervals of aggregation, a small aliquot of incubated samples were diluted to a final concentration of 10 µM and spotted on freshly cleaved mica sheet and incubated at room temperature for 2-3 minutes. A subsequent washing with distilled water was performed to remove the unbound and excess protein/aggregate. The washed sample was dried for ~40 minutes in vacuum desiccator at room temperature. The

imaging was performed in tapping mode with silicon nitride cantilever using VeecoNanoscope IV multimode AFM. The scan rate was kept 1.5 Hz and around 6-8 different areas were scanned randomly. The experiment was performed in duplicate and at least 6-8 random areas were imaged.

***Electron microscopy (EM)*:** 40 μM of wild-type α-Syn and its all mutant fibrils were spotted on carbon-coated Formvar grid (Electron Microscopy Sciences, Fort Washington, PA) and incubated for 5 min at room temperature. The samples were washed twice with autoclaved distilled water, and the remaining water was wiped gently with filter paper. The samples were then stained with 10 μl of 1% (w/v) aqueous uranyl formate solution for 5 min, followed by air-drying. The images were taken at 43,000 X magnification at 120 kV using a transmission electron microscope (TECNAI12 D312 FEI, Netherlands). For protofibril samples (isolated from SEC) was used as it is with out any concentration normalization. Imaging was done at a magnification of 60,000 X. The experiment was done in duplicate and repeated twice.

***Size exclusion chromatography (SEC)*:** Purified synucleins were dissolved in 10 mg/ml concentrations in PBS, pH 7.4, with 0.01% sodium azide. The protein solutions were then centrifuged for 30 min at 14000g using a bench top microcentrifuge (HITACHI, himac CT15RE, JAPAN). The resulting solutions were clear and free of any larger aggregates. 500 μl of supernatant were loaded on a S200-Superdex gel filtration column attached with AKTA purifier (GE Healthcare) and eluted isocratically at 4°C in the same buffer with a flow rate of 0.25 ml/min. 200 μl fractions were collected.

***FTIR study*:** For FTIR analysis, 5 μl of the fibrillar solutions of synucleins (WT and H50Q) were spotted on thin translucent KBr pellets, kept below the IR lamp and dried immediately. For background spectrum, 5 μl of the buffer was used. The FTIR spectra were measured using average of 32 scans at the resolution of 4 cm^{-1} in the range of 1800-1500 cm^{-1} by using BrukerVertex-80 instrument equipped with DTGS detector. For secondary structure analysis, the FTIR spectra corresponding to amide-I region (1700-1600 cm^{-1}) were subjected to Fourier Self Deconvolution (FSD) followed by Lorenzian curve fitting procedure in opus-65 software. FTIR studies were performed thrice using three independent samples.

***MTT assay*:** MTT assay is routinely used to test cellular toxicity of α-Syn aggregates/ fibrils (4, 5, 6). For toxicity study of WT and mutants α-Syn fibrils, SH-SY5Y cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Himedia, India) supplemented with 10%

FBS (Invitrogen, USA), 100 units/ml penicillin and 100 μg/mL streptomycin in a 5% CO_2 humidified environment at 37°C. Cells were seeded in 96-well plates in 100 μL medium at a cell density of ~10,000 per well. After 24 hr of incubation, the old media was replaced with fresh media containing 30 μM of each α-Syn fibrils (WT, A30P, E46K, H50Q and A53T). Same volume of buffer was used as control. To calculate the effect of these amyloid fibrils on cell viability, cells were incubated with test samples for 24 hr in a 5% CO_2 humidified environment at 37°C. After incubation, 10 μl of a 5 mg/ml MTT (prepared in PBS) was added to each well and the incubation was continued for 4 hr. After 4 hr, 100 μl of a solution containing 50% dimethylformamide and 20% SDS (pH 4.8) was added to each well and incubated for overnight at 37°C. After this, absorption values at 560 nm were determined with spectraMax M2 microplate reader (Molecular Devices, USA). The assay was done in triplicate.

ROS measurement: Cytoslolic reactive oxygen species (ROS) assessment was done as described by Cremades et al. (7) with some modifications. In brief, SH-SY5Y cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS (Invitrogen, USA), 100 units/ml penicillin and 100 μg/mL streptomycin in a 5% CO_2 humidified environment at 37°C. For cytosolic reactive oxygen species (ROS) measurement, SH-SY5Y cells were seeded in 24-well plates at a density of ~10,000 cells per well and cultured for 24 hr. After 24 hr, the old media was replaced with fresh media containing 30 μM of each α-Syn fibrils (WT, A30P, E46K, H50Q and A53T). Same volume of buffer was used as control. Cells were incubated for 24 hr in a 5% CO_2 humidified environment at 37°C. After 24 hr, media was gently removed and incubated with 9 μM of 2-hydroethidium (Sigma) in 1 ml of PBS for 10 min in dark at RT. After 10 min, the cells were immediately imaged under confocal microscope to visualize the fluorescence of oxidized hydroethidium (hydroxyethidium) with an excitation at 543 nm and emission above 565 nm. Three independent experiments were performed for ROS measurement.

***Cross linking of proteins*:** PICUP experiment of α-Syn and its mutants was carried out according to the protocol established by Bitan *et al.* (8) . The PICUP was carried out with 100 kDa LMW of α-Syn and its mutants. 18 μl, 20 μM of protein solution was taken in a clear, thin walled 0.2 mL PCR tube. 1 μl of 3 mM $[Ru(bpy)_3]Cl_2$ and 1 μl of 60 mM APS were added to the protein solution. The reaction mixture was irradiated to light for 1 s, controlled by camera shutter. After irradiation, the reaction mixture was quenched immediately by

addition of 5 µl of 5x SDS-PAGE loading dye containing 5% β-mercaptoethanol. The solution was then boiled in water bath at 95ºC for 5 min. For viewing the cross-linked products, SDS-PAGE and silver staining were performed. 4 µl of reaction volume was added to each well along with standard protein ladder. Silver staining was performed using silver staining kit from Invitrogen (SilverXpress). To ensure the reproducibility of data, cross linking experiment was repeated thrice.

***Nuclear Magnetic Resonance study*:** All NMR spectra were recorded at 10 °C on a Bruker Avance 800 MHz spectrometer equipped with cryogenically cooled triple-resonance probe having Z-axis gradient and Deuterium decoupling facility. All spectra were processed with Topspin 2.1 version and analysed with Sparky 3.113. Calibration of proton chemical shifts was done using DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) as an internal reference whereas nitrogen chemical shift was calibrated indirectly as per BMRB protocols. Two-dimensional ^{1}H-^{15}N correlation heteronuclear single quantum coherent (HSQC) experiments were recorded on ^{15}N-labeled protein samples (30 kDa LMW) in 20 mM sodium phosphate buffer, pH 6.0 with a (90:10) H_2O/D_2O ratio. Various sets of ^{1}H-^{15}N HSQC correlation spectra were recorded for 400 µM α-Syn and its H50Q mutant. 2D HSQC spectra for wild type and mutant proteins were recorded for 256 data points in the indirect dimension with 8 scans. Chemical shift perturbations were calculated using $(5\Delta\delta^{1}H^{N)})^{2} + (\Delta\delta^{15}N^{2})^{1/2}$ to identify the significant perturbations due to single point mutation. NMR experiment was performed twice with two independent sets of proteins (isolated independently).

***Statistical analysis*:** The statistical significance was determined by one-way ANOVA followed by Newman-Keuls Multiple Comparison post hoc test; *P<0.05; **P<0.01; NS P>0.05.

Results

α-Syn fibrils are toxic and increase the production of reactive oxygen species (ROS) in SH-SY5Y cells

The extra cellular toxicity of 30 μM fibril of wt, A30P, E46K, H50A, H50Q and A53T using MTT assay was done using SH-SY5Y cells. MTT assay is not only used routinely for determining the cytotoxicity of extracellular amyloids such as Aβ (associated with Alzheimer's diseases) (5), but also for intracellular amyloids including α-synuclein (4, 5, 6). It is recently established that extracellular fibrillar α-synuclein can get internalized into the cells (7, 9, 10). Therefore extracellular amyloid toxicity assay using MTT assay could also be sufficiently appropriate for studying fibrillar α-synuclein toxicity.

The MTT assay revealed that all α-Syn fibrils under this study are toxic to SH-SY5Y cells (Fig S2A of the supporting information). However, we did not observed any significant difference in the toxicity level of these fibrils (WT, A30P, E46K, H50Q and A53T) under study. After treating with the fibrils for 24 hr, SH-SY5Y cells showed ~60% viability compared to control. This indicates that in presence of fibrils the cellular viability is reduced. This result is consistent with many recently published reports (4, 5, 6). Further, we assessed the elevation of intracellular ROS level on exposure of SH-SY5Y cells to α-Syn fibrils. For this, the cytosolic ROS production was evaluated. The 2-hydroethidium is a dye which is routinely used to assess the intracellular reactive oxygen species. It is readily permeable to cells and gets oxidized (2-hydroxyethidim) in presence of cytosolic super oxide anions. The oxidized product binds to DNA and becomes highly fluorescent. This elevated fluorescence is a measure of ROS level in cytosol (7, 11). The fluorescence microscopy images revealed that significantly high amount of ROS was produced (as indicated by highly fluorescent cells) in SH-SY5Y cells after treating with fibrils (Fig S2B of the supporting information). Whereas control cells (treated only with buffer in similar conditions) did not produce substantial amount of fluorescence. Our extracellular toxicity assays using MTT and ROS measurements collectively suggest that the α-Syn fibrils increase the level of intracellular ROS which may leads to cell death.

References:

1. Volles, M. J., and Lansbury, P. T., Jr. (2007) Relationships between the Sequence of α-Synuclein and its Membrane Affinity, Fibrillization Propensity, and Yeast Toxicity. *J. Mol. Biol. 366*, 1510-1522.

2. Singh, P. K., Kotia, V., Ghosh, D., Mohite, G. M., Kumar, A., and Maji, S. K. (2013) Curcumin Modulates alpha-Synuclein Aggregation and Toxicity. *ACS Chem. Neurosci. 4*, 393-407.

3. Willander, H., Presto, J., Askarieh, G., Biverstal, H., Frohm, B., Knight, S. D., Johansson, J., and Linse, S. (2012) BRICHOS domains efficiently delay fibrillation of amyloid beta-peptide. *J. Biol. Chem. 287*, 31608-31617.

4. Yonetani, M., Nonaka, T., Masuda, M., Inukai, Y., Oikawa, T., Hisanaga, S., and Hasegawa M. (2009) Conversion of wild-type α-synuclein into mutant-type fibrils and its propagation in the presence of A30P mutant. *J. Biol. Chem. 284*, 7940- 7950.

5. Bieschke, J., Russ, J., Friedrich, R.P., Ehrnhoefer, D.E., Wobst, H., Neugebauer, K., and Wanker, E.E. (2010) EGCG remodels mature α-synuclein and amyloid-beta fibrils and reduces cellular toxicity. *Proc. Natl. Acad.Sci. U.S.A. 107*, 7710−7715.

6. Lesage, S., Anheim, M., Letournel, F., Bousset, L., Honoré, A., Rozas, N., Pieri, L., Madiona, K., Dürr, A., Melki, R., Verny, C., and Brice, A. (2013) G51D α-synuclein mutation causes a novel parkinsonian-pyramidal syndrome. *Ann. Neurol.73*, 459-471.

7. Cremades, N., Cohen, S. I., Deas, E., Abramov, A. Y., Chen, A.Y., Orte, A., Sandal, M., Clarke, R. W., Dunne, P., Aprile, F. A., Bertoncini, C. W., Wood, N. W., Knowles, T. P., Dobson, C. M., and Klenerman, D. (2012) Direct observation of the interconversion of normal and toxic forms of α-synuclein. *Cell 149*, 1048−1059.

8. Bitan, G., Lomakin, A., and Teplow, D. B. (2001) Amyloid beta-protein oligomerization: prenucleation interactions revealed by photo-induced cross-linking of unmodified proteins. *J. Biol. Chem. 276*, 35176-35184.

9. Luk, K.C., Song, C., O'Brien, P., Stieber, A., Branch, J.R., Brunden, K.R., Trojanowski, J.Q., and Lee, V.M. (2009) Exogenous α-synuclein fibrils seed the formation of Lewy body-like intracellular inclusions in cultured cells. *Proc. Natl. Acad. Sci. U.S.A. 106*, 2051-2056.

10. Volpicelli-Daley, L.A., Luk, K.C., Patel, T.P., Tanik, S.A., Riddle, D.M., Stieber, A., Meaney, D.F., Trojanowski. J.Q., and Lee, V.M. (2011) Exogenous α-synuclein fibrils induce Lewy body pathology leading to synaptic dysfunction and neuron death. *Neuron 72,* 57-71.

11. Zhao, H., Joseph, J., Fales, H. M., Sokoloski, E. A., Levine, R. L.,Vasquez-Vivar, J., and Kalyanaraman, B. (2005) Detection and characterization of the product of hydroethidine and intracellular superoxide by HPLC and limitations of fluorescence. *Proc. Natl. Acad. Sci. U.S.A. 102*, 5727−5732.

***Figure legends*:**

Figure S1. Fibrilization of E46K and A53T familial mutants of α-Syn. (A) Secondary structural transion of E46K and A53T mutnats during fibrilization. The CD data revealed that both the mutants converted to β-sheet conformation, however E46K showed faster conversion. (B) Morphology analysis of α-Syn aggregates after β-sheet conversion. The EM micrographs revealed that both the mutants formed fibrillar aggregates. The scale bars are 500 nm.

Figure S2. Cell toxicity assays of α-Syn fibrils. **(**A) MTT assay of different α-Syn fibrils (WT, A30, E46K H50Q and A53T). The data revealed that all the fibrils are toxic to SH-SY5Y cells. However, there is no significant difference was observed among the fibrils. (B) Intracellular ROS assessment in the SH-SY5Y cells after treating the cells with different α-Syn fibrils (WT, A30, E46K H50Q and A53T). The fluorescence intensity of oxidized hydroethidium (hydroxyethidium) was assessed under confocal microscope. Relatively higher fluorescence in the treated samples indicates the generation of ROS. Scale bars are 50 μm.

Figure S3. Morphology assessment of cross-linked products of α-Syn. The morphology of *α-Syn* (WT, H50Q and H50A) was examined before and after crosslinking using EM. – PICUP and +PICUP indicates non-cross-linked and cross-linked samples, respectively. Scale bars are 500 nm.

Fig S1.

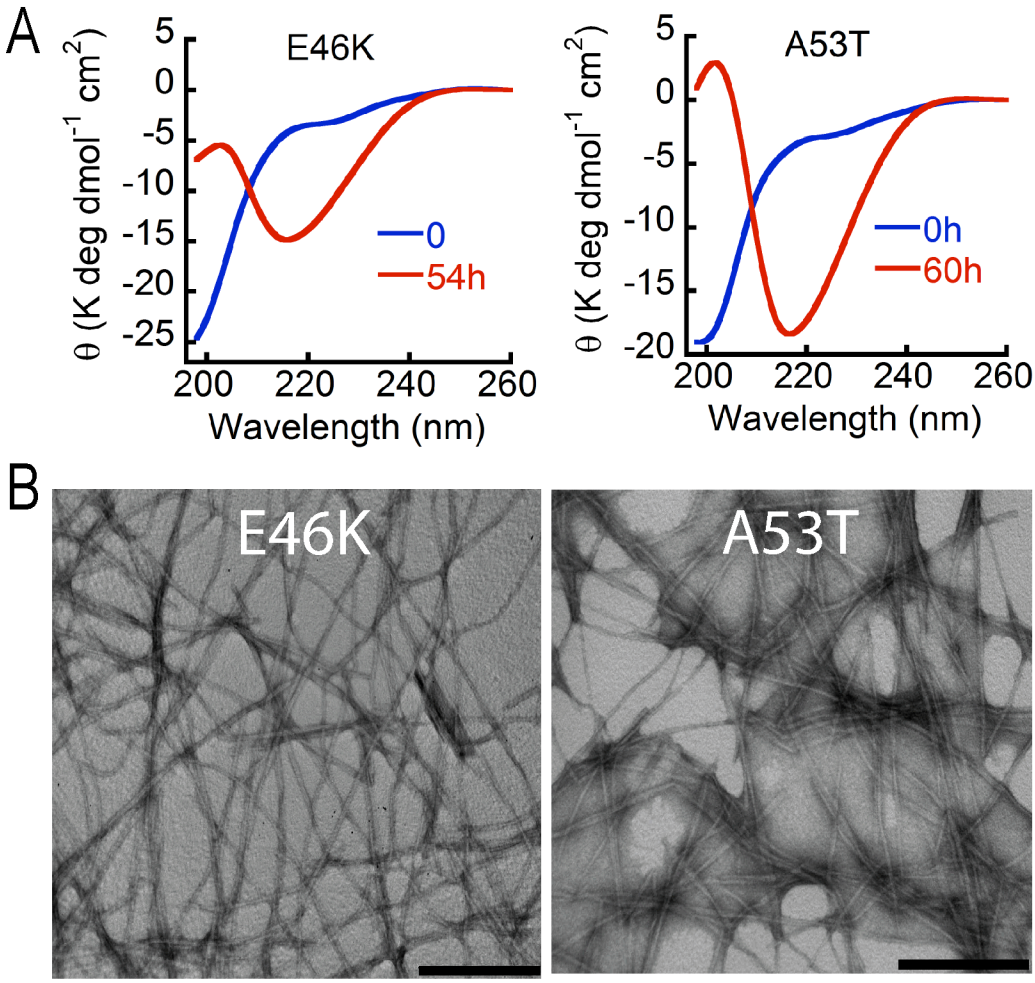
A
E46K
θ (K deg dmol-1 cm2)
5
0
-5
-10
-15
-20
-25
200
220
240
260
Wavelength (nm)
0
54h
A53T
θ (K deg dmol-1 cm2)
5
0
-5
-10
-15
-20
200
220
240
260
Wavelength (nm)
0h
60h
B
E46K
A53T

Fig S2.

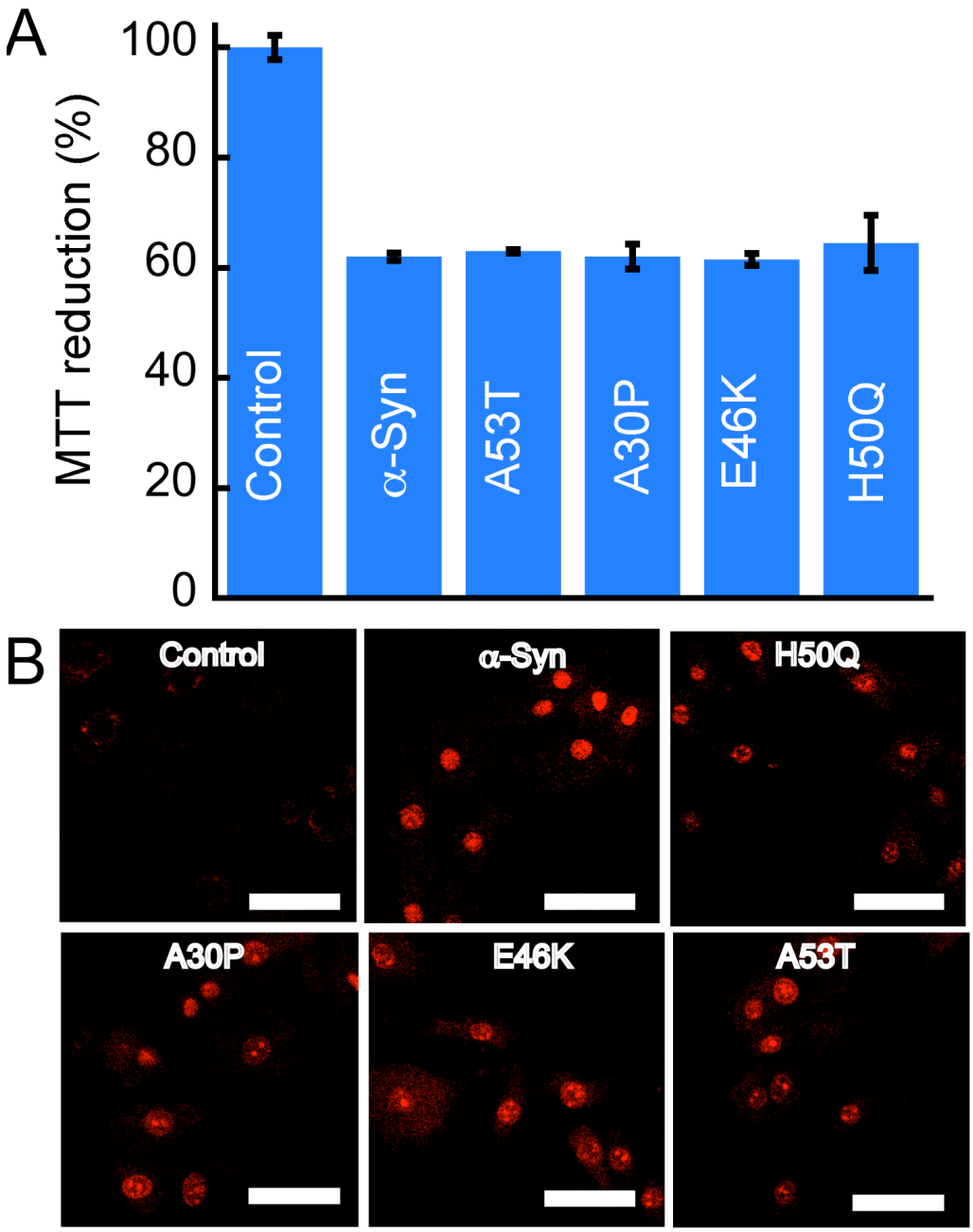
A
MTT reduction (%)
100
80
60
40
20
0
Control
α-Syn
A53T
A30P
E46K
H50Q
B
Control
α-Syn
H50Q
A30P
E46K
A53T

Fig S3.