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Broad-Spectrum Suppression and Disassembly of α-Synuclein Variant Aggregates Mediated by a Ruthenium Metallodrug via Conserved Metal-Coordination

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MATERIALS and METHODS

Materials. NAMI-A was synthesized according to literature methods.[1] Thioflavin T (ThT) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Reactive oxygen species assay kit was purchased from Beyotime (Shanghai, China). Isopropyl beta-D-1-thiogalactopyranoside was purchased from BioFroxx (Einhausen, Germany).

Protein expression and purification. WT α-Syn and five familial variants (E46K, H50Q, T72M, G51D, A53E) were expressed and purified according to literature methods,[2] with modifications. Briefly, N-terminal $(His)_6$-GB1-tagged fusion protein was expressed in *E. coli* BL21 (DE3) cells and induced by 0.4 mM IPTG at 37°C for 4 h. Proteins were initially purified using Ni-NTA affinity chromatography (GE Healthcare). The $(His)_6$-GB1 tag was cleaved using TEV enzyme. α-Syn was further purified using a Superdex 75 pg 10/300 GL size exclusion chromatography (SEC) column (GE Healthcare) on an ÄKTA Purifier (GE Healthcare). The purified protein was dialyzed into 20 mM sodium phosphate buffer (pH 7.4). The protein concentration was determined by measuring the absorbance at 280 nm on a Quickdrop spectrometer with an extinction coefficient of 5960 M^{-1} cm^{-1}. The protein was subsequently stored at -80°C. For NMR measurements, the ^{15}N-labeled protein was obtained by culturing *E. coli* in M9 medium containing $^{15}NH_4Cl$ as the sole nitrogen source.

Preparation of α-Syn oligomers and fibrils. The α-Syn oligomer/fibril mixtures were generated by incubating 1 mM protein in 20 mM phosphate buffer (pH 7.4) at 37°C with constant shaking (900 rpm) for 12 h. The mixtures were centrifuged at 13000 g for 30 min to pellet fibrils. The supernatant containing oligomers was concentrated with ultrafiltration (30 kDa cutoff). The oligomer concentration was determined by UV absorbance at 280 nm using the extinction coefficient (5960 M^{-1} cm^{-1}). The formation of oligomers was verified by ThT fluorescence assay. The fibril morphology was analyzed by TEM.

ThT assay. Aggregation was monitored by incubation of α-Syn (70 μM) in the presence or absence of NAMI-A (350 μM) in 20 mM phosphate buffer at 37°C. After incubation for specified times, ThT fluorescence emission spectra (460–570 nm) were recorded with excitation at 450 nm on a Hitachi F-4600 fluorescence spectrometer.

Intrinsic Fluorescence Quenching. α-Syn was prepared at given concentration in a 20 mM phosphate buffer. After addition of NAMI-A, the emission spectra (285–420 nm) were recorded at 37°C with excitation at 280 nm.

Transmission Electron Microscopy (TEM). Fiber pellets were resuspended in ultrapure water and sonicated for 40 min to obtain homogenous samples. Aliquots (10 µL) were applied to carbon-coated copper grids for 5 min. Excess liquid was carefully removed with filter paper, followed by two rapid washes with pure water. Grids were negatively stained with 5 µL of 2% (w/v) phosphotungstic acid for 2 min and excess stain was removed with filter paper. The grids were air-dried for 30 min prior to imaging using a JEOL 1400 microscope (Peabody, MA, USA) at an acceleration voltage of 120 kV.

Electrospray ionization Mass Spectrometry (ESI-MS). ESI-MS measurements were conducted on an Exactive Plusmass spectrometer (Thermo Fisher Scientific). α-Syn was incubated with NAMI-A in 20 mM ammonium acetate buffer (pH 7.4) at 37°C for 2 h. The unreacted NAMI-A and salts were removed by ultrafiltration (3 kDa cutoff) with 20 mM ammonium acetate buffer (pH 7.4). The final protein samples were prepared in 20 mM ammonium acetate buffer (pH 7.4). The data were analyzed employing the XCalibur software, version 2.0 (Thermo Finnigan).

Circular Dichroism (CD) Spectroscopy. Far-UV CD spectra (190–260 nm) were recorded on a Jasco J-810 CD spectrometer equipped with 1 mm path length cuvette. A scan speed of 200 nm·min^{-1} was applied with a data pitch of 1 nm, and represent the average of 3 accumulations.

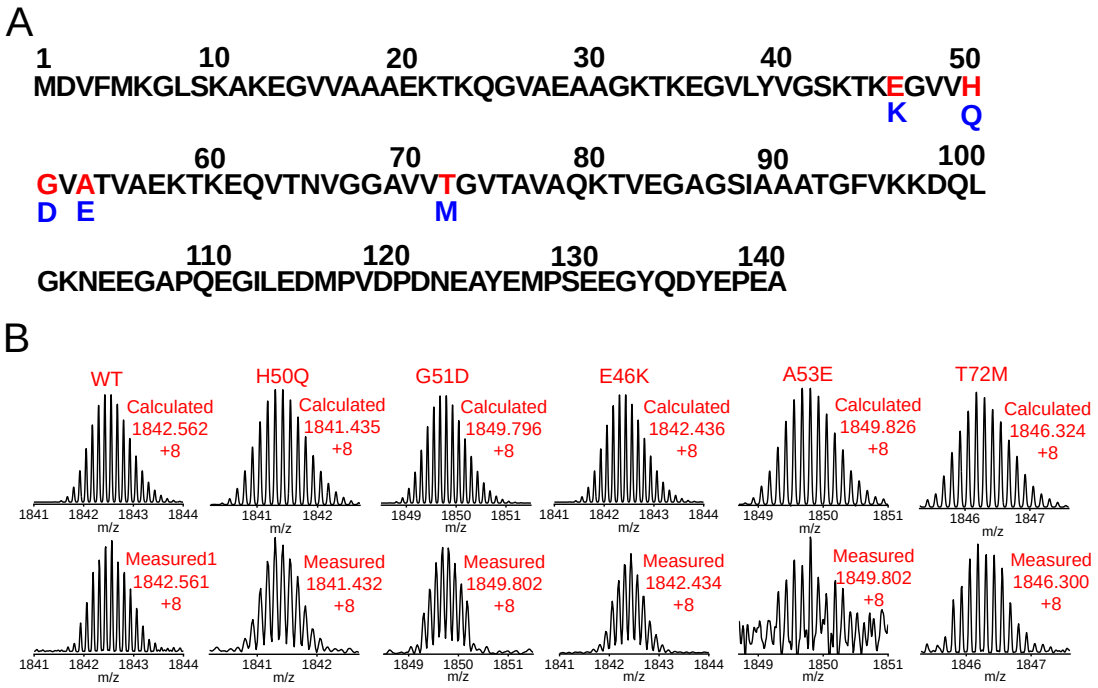
Nuclear Magnetic Resonance (NMR) Spectroscopy. NMR spectra were acquired at 25°C on a Bruker Avance 600 MHz spectrometer equipped with a TCI CryoProbe. ^{15}N-labeled α-Syn (0.2 mM, WT or variants) were prepared in 20 mM sodium phosphate buffer (pH 7.4) containing 10% D_2O. Two-dimensional ^{1}H-^{15}N heteronuclear single quantum correlation (HSQC) spectra were recorded with standard WATERGATE water suppression pulse sequence. The NMR spectra of 2048 and 200 data points were obtained in the direct and indirect dimensions, respectively, with 8 scans. The data were processed and analyzed using Sparky software.

Cell Viability Assay. The cytotoxicity of α-Syn oligomers and the protective effect of NAMI-A were assessed using the MTT assay. Briefly, SH-SY5Y cells were seeded in 96-well plates at a density of 5 × 10^3 cells per well and allowed to grow for 12 h. Cells were then treated for 24 h with either various concentrations (0.1, 1, 2, and 5 µM) of pre-formed α-Syn oligomers (WT, G51D, or H50Q), or with α-Syn oligomers (1 µM) that had been pre-incubated with or without 5 µM NAMI-A for 2 h at 37°C. Thereafter, MTT solution (1 mg/mL) was added to each well and incubated for 4 h at 37°C. The formed formazan crystals were dissolved in 100 µL of DMSO and quantified through the absorbance at 490 nm by using a Bio-Rad 680 microplate reader.

Detection of ROS in Cells. Intracellular ROS levels were detected using the fluorescent probe 2',7'-

dichlorodihydrofluorescein diacetate (DCFH-DA). SH-SY5Y cells were seeded in 12-well plates at a density of 8×10^4 cells per well and allowed to grow for 24 h. Cells were treated with α-Syn oligomers (1 μM) that had been pre-incubated with or without NAMI-A (5 μM) for 12 h. After treatment, the cells were washed with PBS and then co-incubated with a working solution containing 10 μM DCFH-DA and 2 μM DAPI in serum-free DMEM medium at 37°C for 30 min in the dark. Subsequently, the cells were washed twice with PBS to remove excess probes prior to fluorescence imaging.

Live/Dead Cell Staining Assay. SH-SY5Y cells were seeded in 12-well plates at a density of 8×10^4 cells per well and allowed to grow for 24 h. Cells were then treated with α-Syn oligomers (1 μM) that had been pre-incubated with or without NAMI-A (5 μM) for 6 hours. Following the treatment, the cells were washed three times with PBS and subsequently stained with a working solution containing 10 μM fluorescein diacetate (FDA) and 20 μM propidium iodide (PI) for 20 min at 37°C in the dark. After staining, the cells were washed again with PBS to remove excess dye. Fluorescence images were immediately captured.



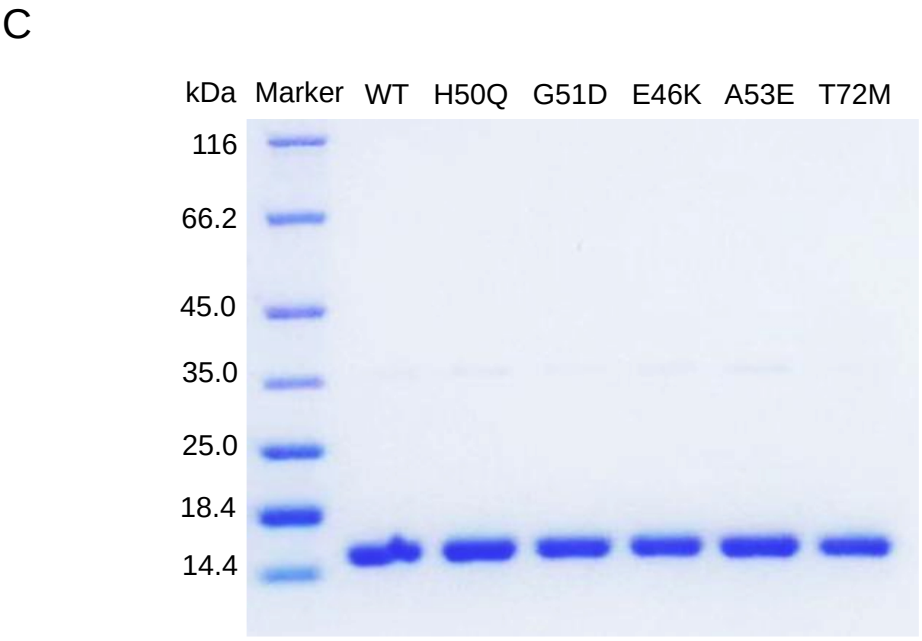


Figure S1. Protein purity and molecular weight characterization. (A) The wildtype α-Syn sequence, and the mutation sites are marked in red. (B) Selected peaks of ESI-MS show the +8 charged signals of the wildtype α-Syn and familial variants, the theoretical isotopic pattern is shown for comparison. (C) Gel electrophoresis analysis of the wildtype α-Syn and familial variants.

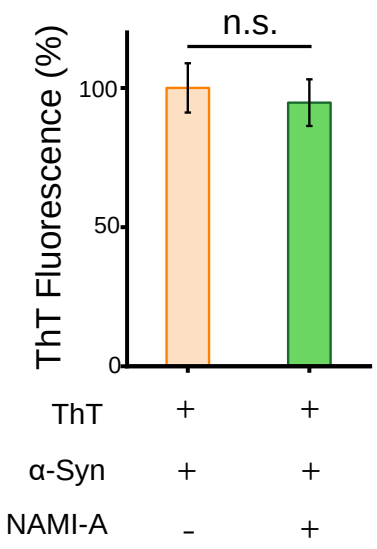


Figure S2. Effect of NAMI-A on ThT fluorescence assay. Fluorescence of 20 µM ThT was measured in the presence of pre-formed α-Syn fibrils (70 µM) with or without 350 µM NAMI-A. Data are presented as the mean ± s.d. from three independent experiments. Statistical significance was determined by Student's t-tests (n.s. = not significant).

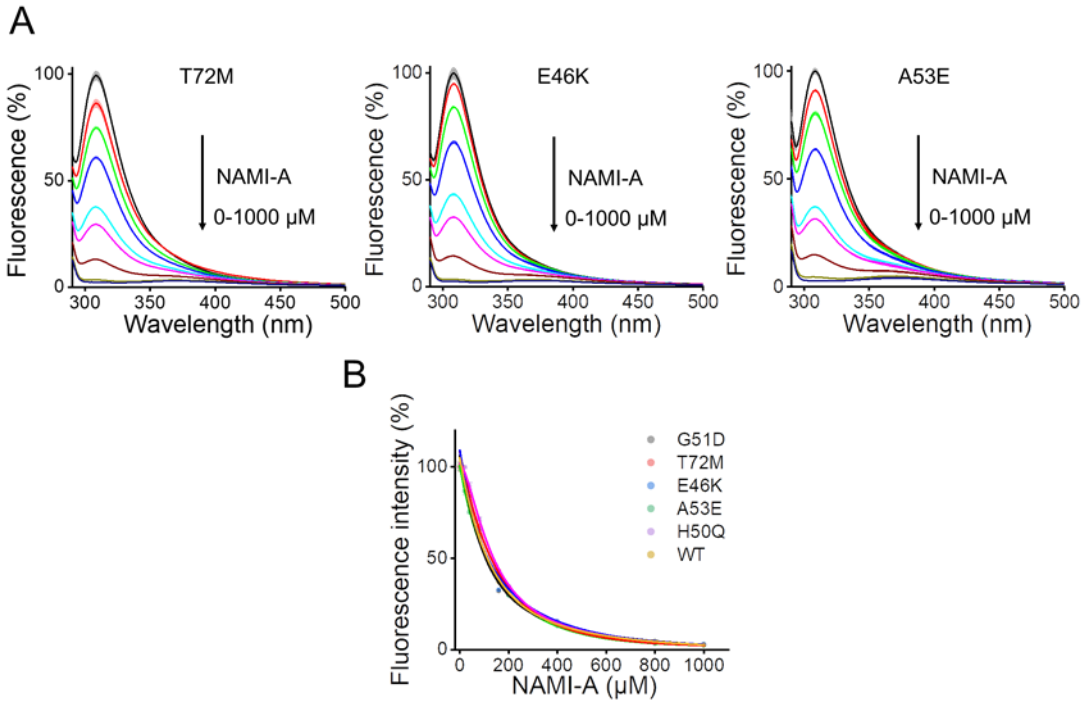


Figure S3. NAMI-A quenches α-Syn fluorescence in a concentration-dependent manner. (A) Normalized fluorescence spectra of other three familial variants (T72M, E46K, A53E) after incubation with NAMI-A (0–1000 µM). α-Syn (20 µM) was incubated in 20 mM phosphate buffer (pH 7.4, 37°C, 2 h). Curves show mean intensities ± s.d. (n = 3 independent experiments). (B) Quenching efficiency (F/F_0) vs. NAMI-A concentration for each variant, where F_o and F represent the fluorescence intensity in the absence and presence of NAMI-A, respectively. Fluorescence intensities (λ_{ex} = 280 nm, λ_{em} = 310 nm) were normalized to untreated controls. Data points represent mean ± s.d. (n = 3).

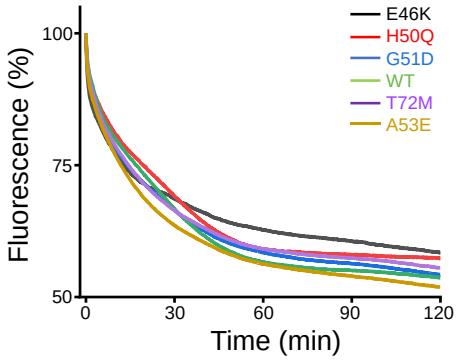


Figure S4. Reaction kinetics of α-Syn variants with NAMI-A. The time-dependent fluorescence quench was monitored on α-Syn variants (20 μM) in the reaction with NAMI-A (100 μM) at 37°C.

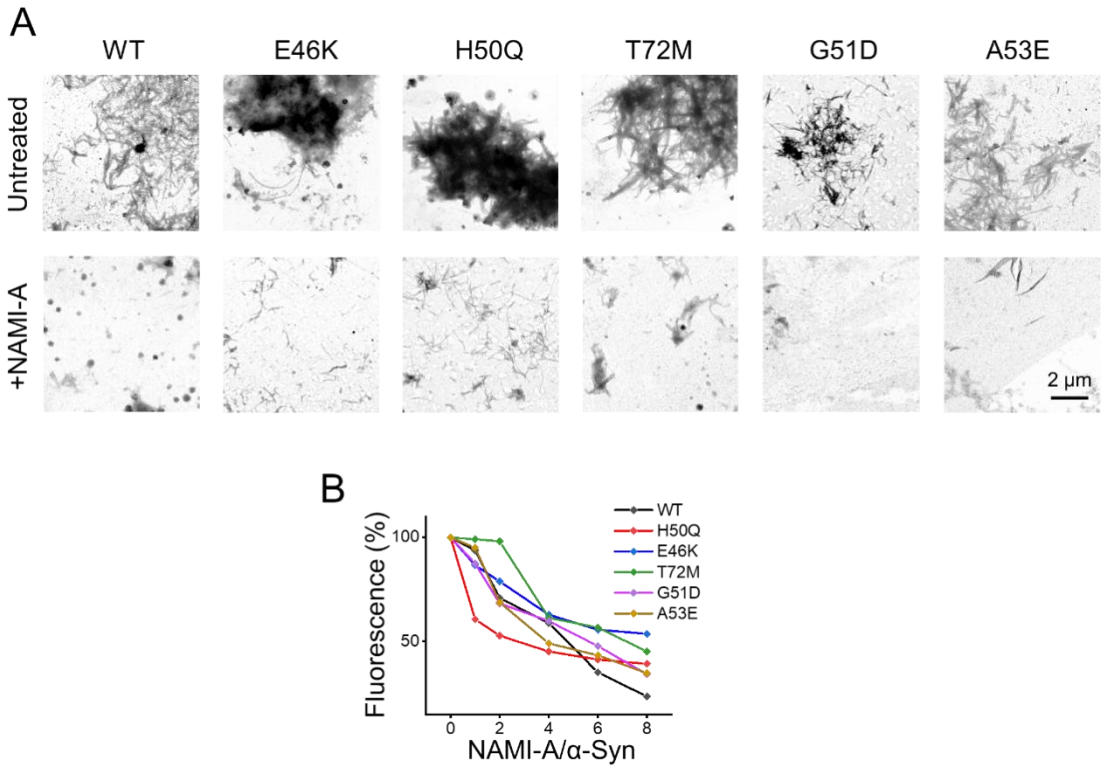


Figure S5. NAMI-A mediates degradation of preformed α-Syn aggregates. (A) TEM images of α-Syn (70 µM) incubated for 36 h alone (top) or followed by an additional 24 h incubation with 5 molar equivalents of NAMI-A at 37°C under 900 rpm (bottom). (B) Normalized ThT fluorescence analysis of β-sheet content in preformed oligomers (20 µM) of wildtype and variant α-Syn after 24 h incubation with NAMI-A at indicated molar ratios (37°C).

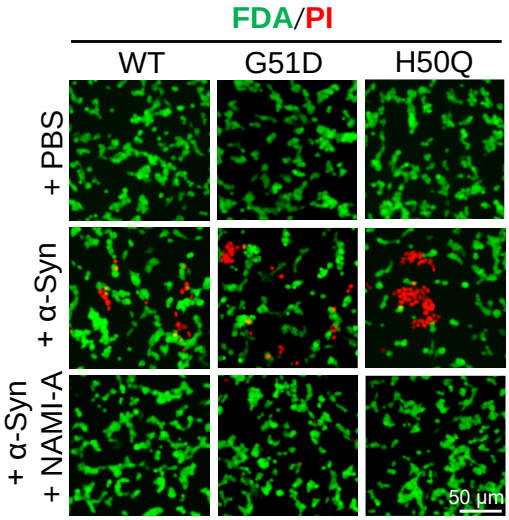


Figure S6. NAMI-A alleviates α-Syn-induced cytotoxicity analyzed *via* live/dead cell assay. SH-SY5Y cells were treated with pre-formed oligomers of wild-type (WT), G51D, or H50Q α-Syn (1 μM) for 6 h in the presence or absence of NAMI-A (5 μM). Cells were then stained with fluorescein diacetate (FDA, green) for live cells and propidium iodide (PI, red) for dead cells before fluorescence imaging. Scale bar: 50 μm.

Table S1. Assignment of peaks in the ESI-MS spectra of Figure 3A

Peak	Composition	Molecule Formula	m/z (charge)	
			Measured	Theoretical
a	α-Syn_{WT}	$C_{637}H_{1028}N_{170}O_{221}S_4$	1842.561 (+8)	1842.562 (+8)
a_1	α-Syn_{WT}(Ru)	$C_{637}H_{1028}N_{170}O_{221}S_4Ru$	1855.176 (+8)	1855.176 (+8)
a_2	α-Syn_{WT}[Ru(Im)]	$C_{640}H_{1032}N_{172}O_{221}S_4Ru$	1863.425 (+8)	1863.569 (+8)
a_3	α-Syn_{WT}[Ru(Im)H_2O]	$C_{640}H_{1034}N_{172}O_{222}S_4Ru$	1865.678 (+8)	1865.946 (+8)
b	α-Syn_{G51D}	$C_{639}H_{1030}N_{170}O_{223}S_4$	1849.802 (+8)	1849.816 (+8)
b_1	α-Syn_{G51D}(Ru)	$C_{639}H_{1030}N_{170}O_{223}S_4Ru$	1862.180 (+8)	1862.439 (+8)
b_2	α-Syn_{G51D}[Ru(Im)]	$C_{642}H_{1034}N_{172}O_{223}S_4Ru$	1870.686 (+8)	1870.640 (+8)
b_3	α-Syn_{G51D}[Ru(Im)H_2O]	$C_{642}H_{1036}N_{172}O_{224}S_4Ru$	1872.933 (+8)	1873.201 (+8)
c	α-Syn_{H50Q}	$C_{638}H_{1033}N_{171}O_{219}S_4$	1841.432 (+8)	1841.444 (+8)
c_1	α-Syn_{H50Q}(Ru)	$C_{638}H_{1033}N_{171}O_{219}S_4Ru$	1854.041 (+8)	1854.067 (+8)
c_2	α-Syn_{H50Q}[Ru(Im)]	$C_{641}H_{1037}N_{173}O_{219}S_4Ru$	1862.302 (+8)	1862.311 (+8)
c_3	α-Syn_{H50Q}[Ru(Im)H_2O]	$C_{641}H_{1039}N_{173}O_{220}S_4Ru$	1864.800 (+8)	1864.829 (+8)

Reference

(1) R. G. Kenny and C. J. Marmion, Toward multi-targeted platinum and ruthenium drugs—a new paradigm in cancer drug treatment regimens, *Chem. Rev.,* 2019, **119,** 1058-1137.

(2) K. Cao, Y. Zhu, Z. Hou, M. Liu, Y. Yang, H. Hu, Y. Dai, Y. Wang, S. Yuan and G. Huang, α-Synuclein as a target for metallo-anti-neurodegenerative agents, *Angew. Chem. Int. Ed.,* 2023, **62** , e202215360.

Raw gel electrophoresis images in Figure 1C and Figure S1C.

Figure 1C

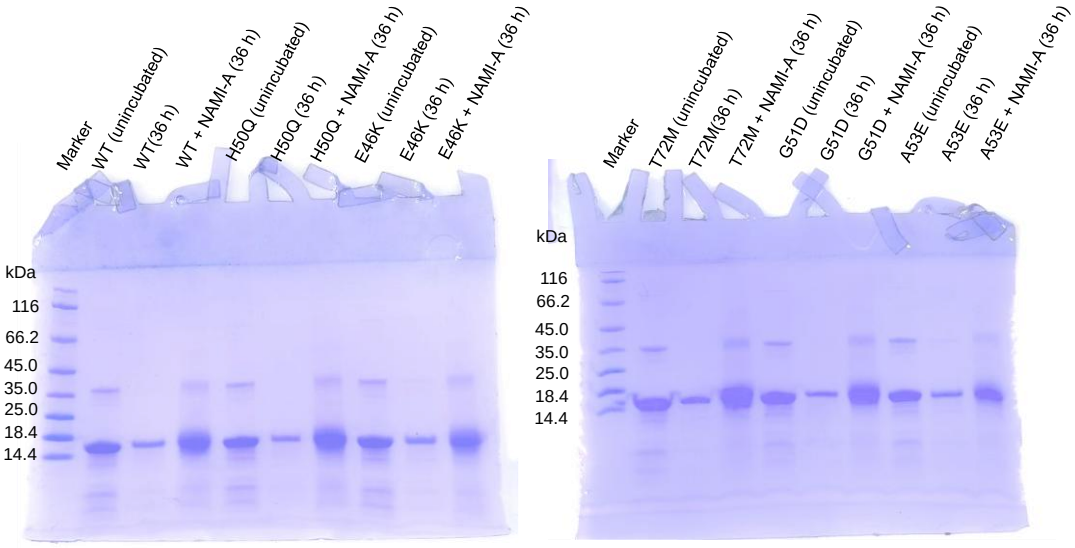


Figure S1C