Supplemental Materials

α-Synuclein binds TOM20 and inhibits mitochondrial protein import in Parkinson's disease

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Materials and Methods

Tom20-CTD expression and purification. DNA encoding the c-terminal domain of Tom20 (residues 49-145, Tom20-CTD) was incorporated into the bacterial expression vector pGEX-4T-1, for T7-driven expression with an N-terminal GST-purification tag. The vector was transformed into BL21-DE3 *E. coli* for protein expression. Tom20-CTD was expressed and purified as previously described (*1*). In lieu of purification of the Tom20-CTD-GST fusion protein, thrombin was added to the sample (1:500 vol/vol ratio of 10,000 units of thrombin stock in PBS) overnight with rocking while Tom20-CTD was still bound to the resin. The following day the supernatant was added to 1mL of washed benzamadine-agarose resin to remove thrombin. The resin was pelleted and the supernatant was collected, containing pure Tom20-CTD. Protein concentration was determined.

MTS peptide. The MTS peptide was synthesized, purified and sequenced by GenScript (Piscataway, NJ).

Transfection and selection of stably transfected cell lines. SH-SY5Y cells were transfected by nucleoporation according to the recommendation of the manufacturer (Lonza). Cells (2×10^6) were transfected with 3 µg of TOMM20 cDNA plasmid DNA (Origene # RC21074) and grown in neomycin selective media containing 400 ug/ml of Geneticin (Gibco). A neomycin-resistant control cell line was derived by transfecting SH-SY5Y cells with empty plasmid DNA (pCMV6-Entry [Origene # PS100001]). Cells were grown 6-8 weeks in selective media, at which time stably transfected clones were isolated and expanded. Western blotting and quantitative immunofluorescence showed that the cell line transfected with TOMM20 cDNA expressed 2-3-fold higher levels of TOM20 protein than wildtype or mock transfected cells. For the experiments reported here, a single stable, overexpressing and mock-transfected line was used; however, identical results were obtained in transient transfection experiments.

Fluorescence Binding Assay. Tryptophan fluorescence binding assays were performed using a Y39W mutation of α-syn. (Tom20-CTD contains no tryptophan residues.) α-Synuclein (in all modified forms) was diluted to a final concentration of 10µM at a final volume of 400µL. Increasing concentrations of purified Tom20-CTD were added (0-20µM). Fluorescence measurements were taken on a Shimadzu RF-5301PC spectrophotometer, with excitation at 280nm and measurement of the tryptophan emission spectrum from 300-400nm. The emission maximum at each titration point was plotted versus the concentration of Tom20-CTD and fit to a 1-site binding model using GraphPad Prism software.

Mitochondrial protein import assays. ***Imaging of mtGFP in SH-SY5Y cells:*** The cells were transfected with MTS-GFP DNA using Lipofectamine (24 h) in multi-well plates, then treated for 24 h with various species of α-synuclein (200 nM). For each batch of transfected cells, all treatments were carried out simultaneously, so transfection efficiency, toxicity or baseline mitochondrial mass did not vary between experimental conditions. Following fixation, mitochondrial ROIs were defined by MitoTracker or immunolocalization of VDAC or another marker. FCCP (50 µM), applied after transfection, removed the driving force ($\Delta\Psi_m$) for import and was used to calibrate "zero import"; treatment with vehicle defined maximal (100%) import. Thus, in a given experiment, values were calibrated between 0 and 100% and normalized to the vehicle condition. Statistical comparisons were then made to monomer, which was not distinguishable statistically from vehicle. ***In vitro mitochondrial protein import:*** Mitochondria were isolated from rat brain or cultured cells following glass Dounce homogenization at 4˚C in isolation medium (225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mM EGTA, 1 mg/ml bovine serum albumin, pH 7.4). Mitochondria were purified by differential centrifugation and the final mitochondrial pellet was resuspended in 200µL of import buffer (250mM sucrose, 10mM HEPES, 10mM succinate, 1mM ATP, pH 7.4). Pellets were diluted to 1mg protein/ml, and 80 µg of the solution was added to eppendorf tubes on ice. Samples were spun at 12,000g for 10 minutes at 4˚C, and the mitochondrial pellet was resuspended in 47µL of import buffer plus 3µg/ml [final] of α-syn. Samples

were then incubated on ice for 30 minutes. Following incubation, 3µL of ^{35}S-radiolabeled *in vitro*-synthesized pOTC (pre-ornithine transcarbamylase) was added to the mitochondrial suspension. ^{35}S was incorporated into pOTC using the Promega *in vitro* translation kit (TNT quick coupled transcription/translation). Import of pOTC was carried out at 25˚C for 45 min. As a control, and for calibration, FCCP and oligomycin were used to collapse membrane potential and eliminate the driving force for import. Following import, 50µL of DIGE + SDS loading buffer was added to stop the import reaction. Samples were boiled in water for 5 min and 3µL of each sample was loaded and run on a 4-12% SDS-PAGE gel, transferred, and left to develop at -80˚C. Film was exposed to the transfer membrane, and band quantification was performed using GelAnalyzer software. The ratio (mature OTC:pre-OTC) in the vehicle condition defined maximal import (100%) and the FCCP + oligomycin condition defined "zero import". Thus, in a given experiment, values were calibrated between 0 and 100% and normalized to the vehicle condition.

Mitochondrial membrane potential. ΔΨm was measured in cultured SH-SY5Y cells and in mitochondria isolated from rat brain using the potentiometric dye, TMRM. Cells were treated with α-synuclein (or 50 µM FCCP as a control) as described above. Isolated mitochondria were treated for 30 min with α-synuclein (or 500 nM FCCP as a control) as described for the import assay above. For ΔΨm measurements, cells or isolated mitochondria were incubated for 60 minutes at 37 °C with 250 nM TMRM (Invitrogen) in standard Hepes-buffered salt solution, pH 7.4. Cells were washed 3 x 5 min with standard HBSS and placed on a DM IRM inverted microscope (Leica) fitted with Leica HCN PLAN BD 60X/100X oil immersion objectives. TMRM fluorescence was monitored in single cells or in mitochondrial clusters using excitation light provided by a 75W xenon lamp-based monochromator (Ushio, Japan). Emitted light was detected using a CCD camera (Orca; Hamamatsu, Shizouka, Japan). Cells were excited at 555 nm and emitted fluorescence was passed through a 605 ± 40 nm band-pass filter (Omega Optical). TMRM fluorescence was measured in 20-30 individual cells or mitochondrial clusters for each cover slip. Background fluorescence, determined from 3 or 4 cell/mitochondria-free regions of the cover slips, was subtracted from all signals prior to calculating the fluorescence intensities.

AAV2-mediated gene transfer. Details per Zharikov et al (*2*). Additionally, AAV2-CBA-α-synuclein and AAV2-CBA-eGFP vectors were obtained through the Michael J. Fox Foundation from the UNC Vector Core, diluted to 8.1 x 10^{12} vg/ml, and injected per Zharikov et al (*2*). Rats were euthanized 6 weeks after injection.

Human tissue. Paraffin-embedded midbrain sections were obtained from the University of Pittsburgh Brain Bank. All banked specimens have undergone standardized premortem neurological and post-mortem neuropathological assessment. Diagnoses were confirmed and staging performed by the study neuropathologist (CTC) by examination of H&E, alpha-synuclein, tau, silver and ubiquitin stains of key sections needed for Braak tangle (*3*) *and Braak PD stages (4)*. The study design was reviewed and approved by the University of Pittsburgh Committee for Oversight of Research Involving the Dead. Midbrain sections from 5 PD/PDD patients and 4 control subjects, matched for age and postmortem intervals, were used for analysis. To eliminate endogenous fluorescence, human tissue was pre-treated with an autofluorescence eliminating reagent according to the manufacturer's instructions (Chemicon, Temecula, CA).

Other methods. Assays for ROS and oxidative damage (*5-7*), mitochondrial respiration (*5*), as we described previously.

References

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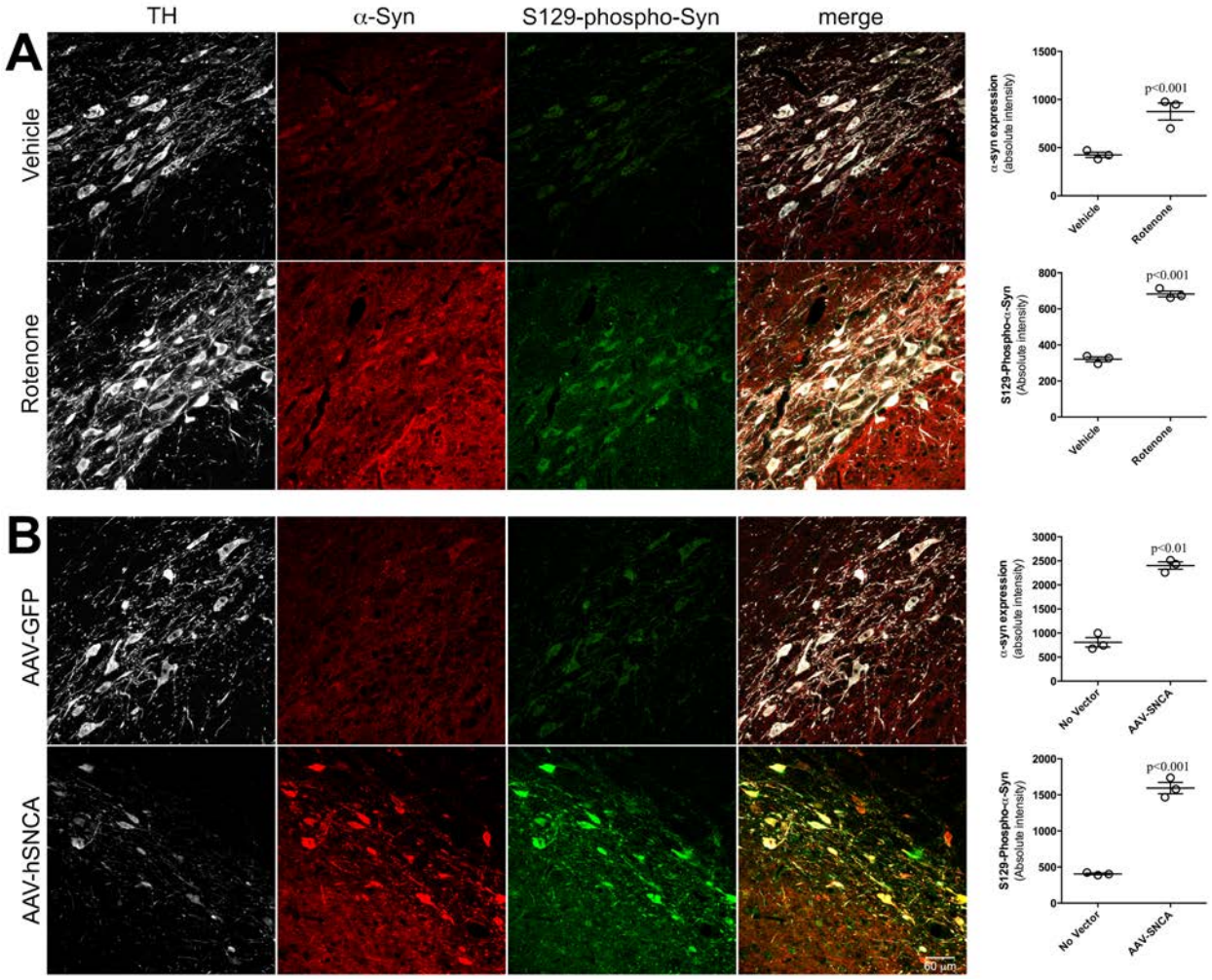

Figure S1. **(A)** Rotenone treatment increases both total levels of α-synuclein and S129-phospho-α-synuclein in substantia nigra. Scatter plots quantify changes. Statistical comparison by 2-tailed unpaired t-test; N=3. (B) Unilateral transduction with AAV-SNCA also increases both total levels of α-synuclein and S129-phospho-α-synuclein in substantia nigra. Statistical comparison by 2-tailed paired t-test; N=3. Scale bar = 60 µm.

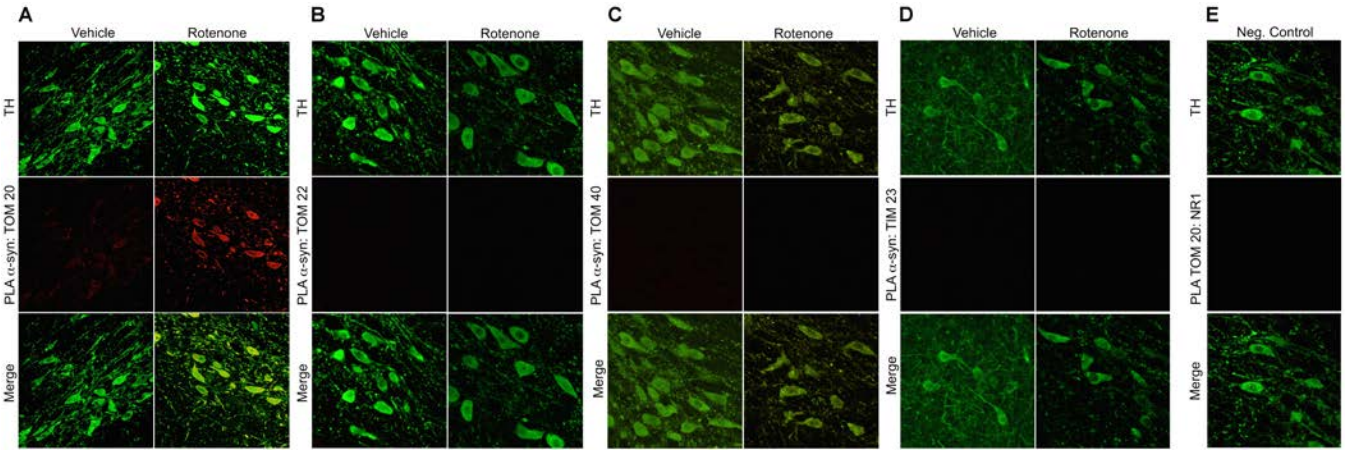

Figure S2. Positive and negative PL interactions with α-synuclein in substantia nigra pars compacta. **(A)** As described in figure 1, rotenone induced an interaction between α-synuclein and TOM20. However, there was no interaction detected between α-synuclein and TOM22 **(B)**, TOM40 **(C)** or TIM23 **(D)**. As a further negative control, we showed that there was no PL signal between TOM20 and the NR1 subunit of the NMDA receptor **(E)**.

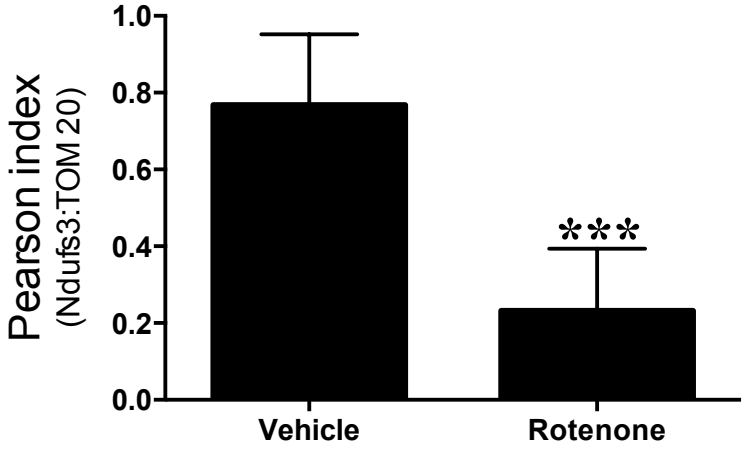

Figure S3. Rotenone induces a loss of mitochondrial localization of the nuclear-encoded, imported protein, Ndufs3. At least 100 cells were quantified per animal; N=3. ***p<0.0001; 2-tailed unpaired t-test.

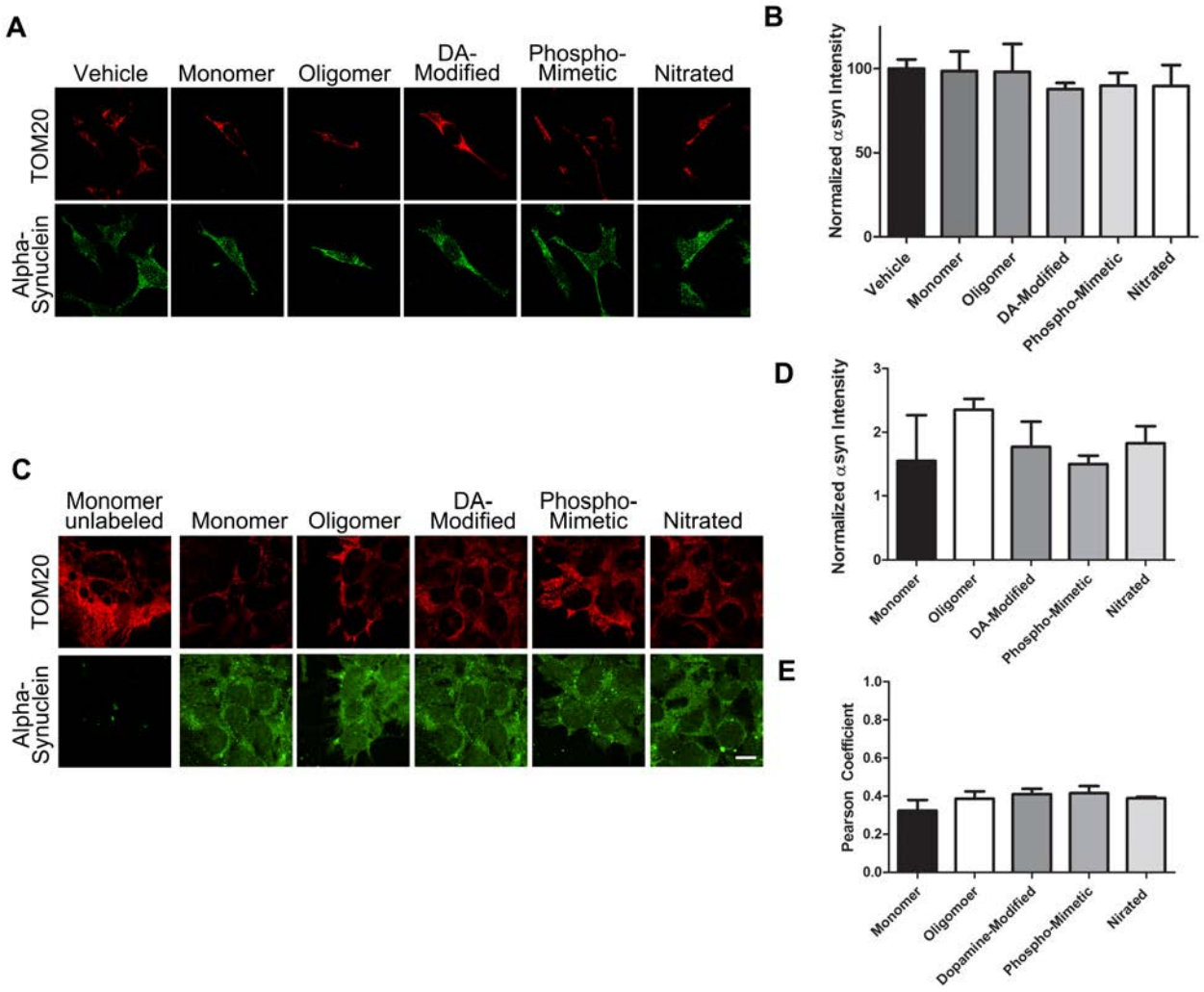

Figure S4. All species of α-synuclein used in this study enter cells to an equivalent extent, and when added at 200 nM, they do not change intracellular concentrations of α-synuclein or its localization. **(A)** SH-SY5Y cells were treated with (unlabeled) exogenous α-synuclein and total cellular α-synuclein was imaged at 24h by quantitative confocal immunofluorescence; TOM20 was also labeled. As quantified in **(B)**, addition of this concentration of exogenous α-synuclein did not have a measureable effect on cellular levels of α-synuclein. N=3. **(C)** α-Synuclein was labeled with rhodamine and then applied to SH-SY5Y cells at a concentration of 200 nM. When cells were imaged 24 h later, the amount of intracellular fluorescence was equivalent for all species of α-synuclein. **(D)** Quantification of 3 independent experiments illustrated in C. There were no differences in cellular penetration of the species of α-synuclein. **(E)** There was relatively little overall colocalization of α-synuclein with TOM20, and this did not vary between species of α-synuclein. Scale bar = 5 µm.

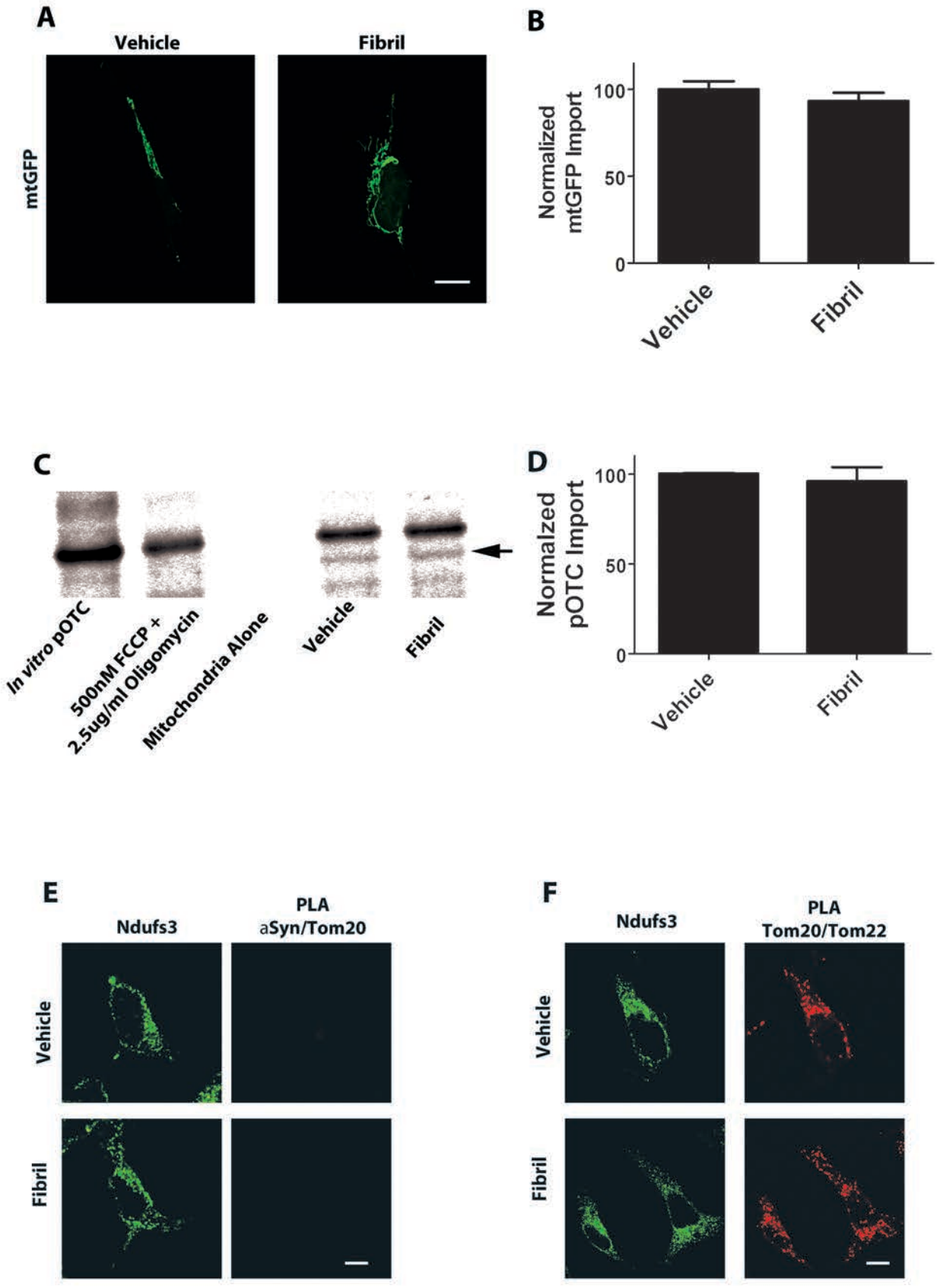

Figure S5. Fibrillar α-synuclein has no effect on *in situ* import of mtGFP in SH-SY5Y cells **(A, B)** or *in vitro* import of pOTC into isolated mitochondria *(arrow denotes mature imported OTC)* **(C, D)**, and it does not induce α-synuclein:TOM20 PL **(E)** or disrupt normal TOM20:TOM22 PL in HEK293 cells **(F)**. All experiments were performed at least 3 times. Scale bars = 5 µm.

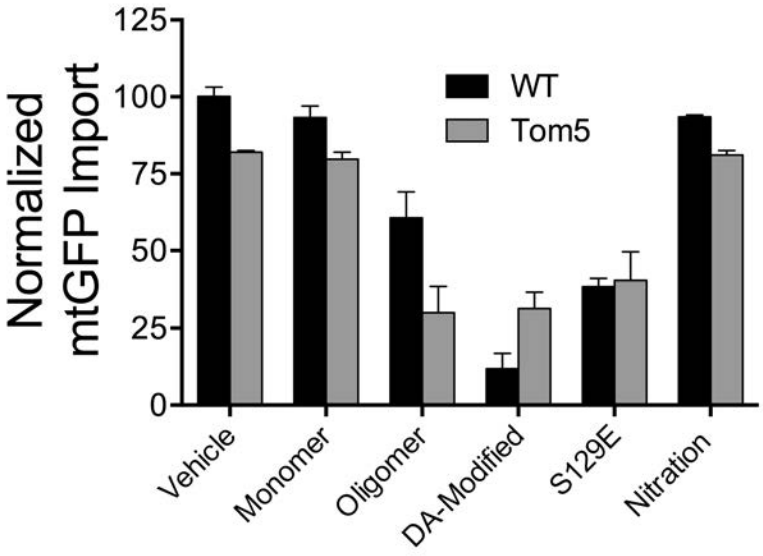

Figure S6. Transient overexpression of TOM5 by approximately 4-fold did not rescue loss of import mediated by oligomeric, dopamine-modified or S129E α-synuclein. (N=3) Compare to the effects of TOM20 overexpression in figure 3.

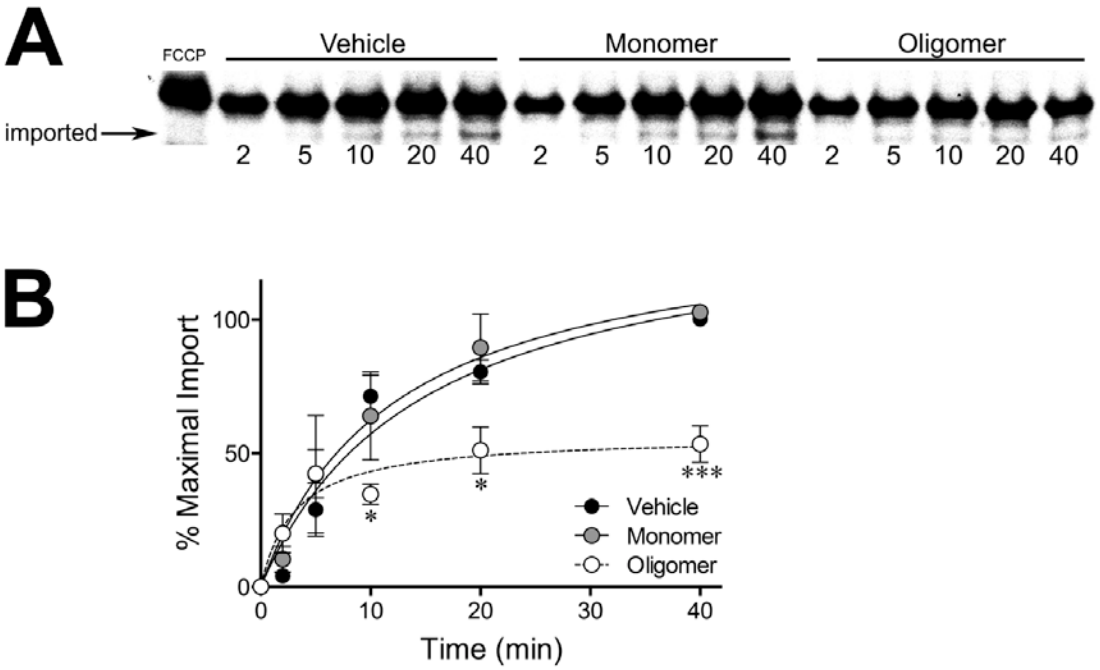

Figure S7. Time course of isolated brain mitochondrial protein import in the absence or presence of monomeric and oligomeric α-synuclein. Note that with vehicle or monomer treatment, from 2 – 40 minutes, there is a progressive increase in the intensity of the imported, mature OTC band. In the presence of oligomeric α-synuclein, import is reduced compared to monomer by 10 min and remains reduced for the duration of the experiment. *p<0.05, ***p<0.0001 by 2-way ANOVA. N=3.

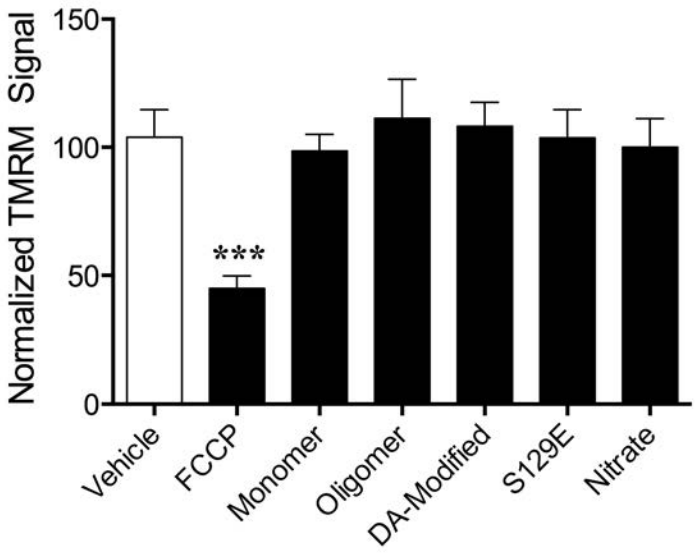

Figure S8. During the time course of *in vitro* protein import assays with isolated mitochondria, α-synuclein does not alter mitochondrial membrane potential as assessed with TMRM. ***p<0.0001; ANOVA; N=3.

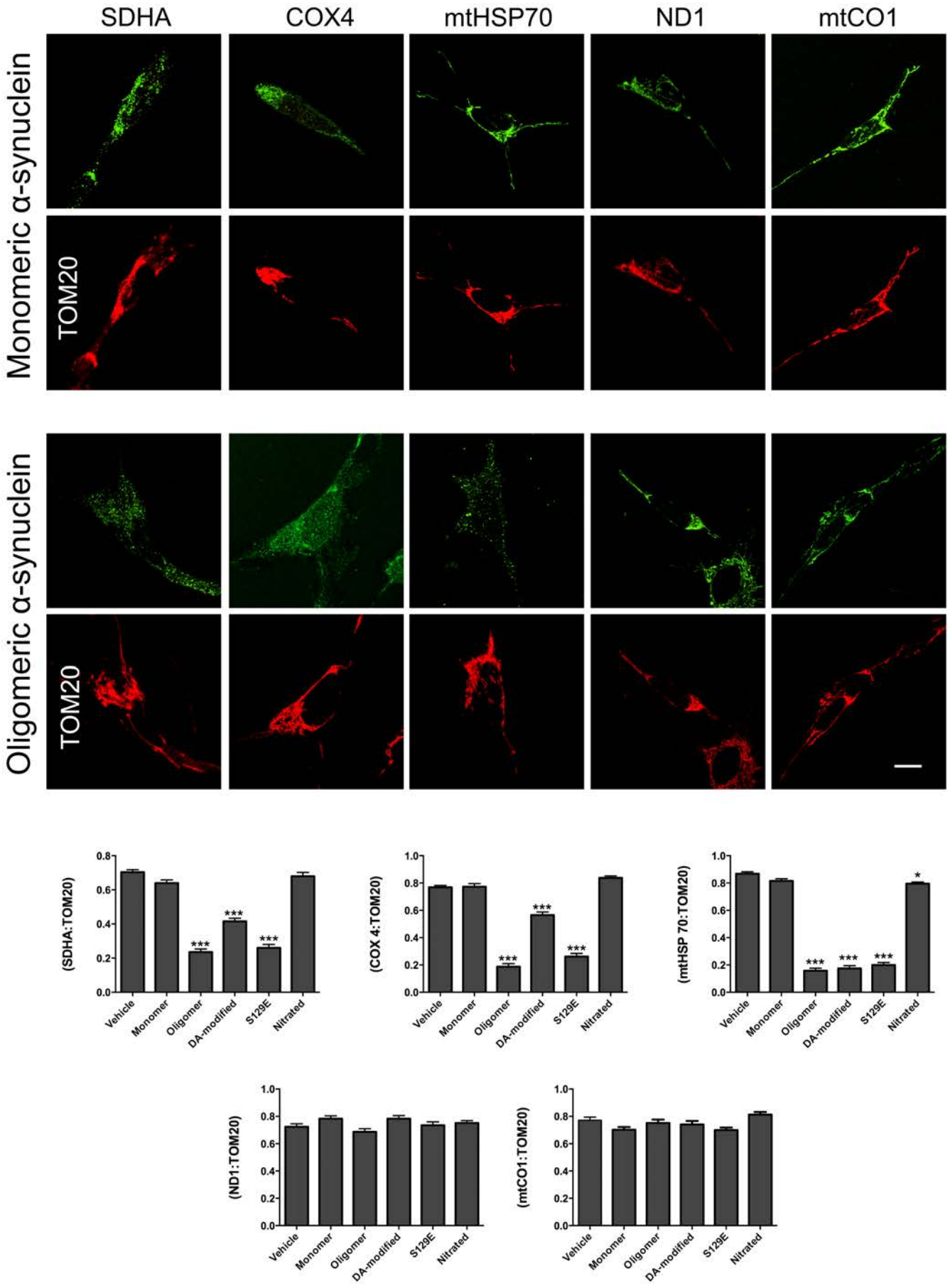

Figure S9. Import of nuclear-encoded, presequence containing proteins (SDHA, COX4, mtHSP70) is disrupted by oligomeric α-synuclein compared to monomeric α-synuclein. Mitochondrially-encoded proteins (ND1, mtCO1) are unaffected by

oligomeric α-synuclein. **(Top 2 rows)** SH-SY5Y cells were treated with monomeric α-synuclein for 24h and then stained for proteins of interest and for TOM20 (red). Note the colocalization of all proteins with TOM20 under these conditions. **(Middle panels)** SH-SY5Y cells were treated with oligomeric α-synuclein for 24h and then stained for proteins of interest and for TOM20 (red). Note that the nuclear-encoded proteins (SDHA, COX4, mtHSP70) have lost their mitochondrial localization and have acquired a more diffuse localization. **(Bar graphs)** Pearson coefficients of colocalization for each protein of interest after treatment with various species of α-synuclein. Note that the nuclear-encoded presequence-containing proteins were all affected by oligomeric, dopamine modified and S129E α-synuclein, but the mitochondrially-encoded proteins were not. Scale bar = 5 μm.

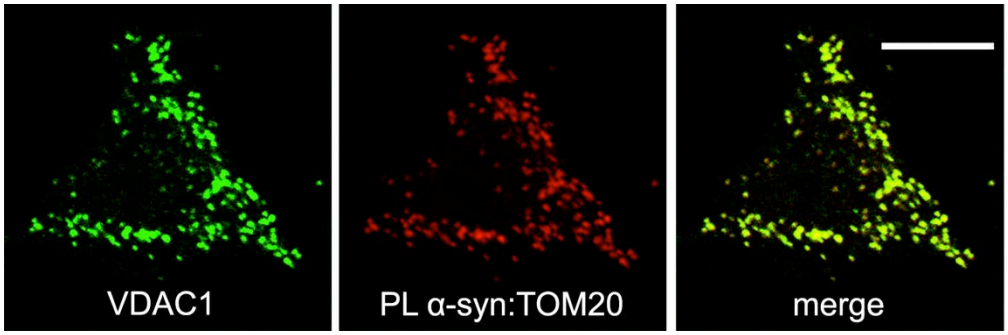

Figure S10. The α-synuclein:TOM20 PL signal colocalizes with mitochondria. After treatment of HEK293 cells for 24h with oligomeric α-synuclein, cells were processed for the PL assay and VDAC1 (voltage-dependent anion channel 1) immunofluorescence labeling. Note the perfect co-registration of the PL signal and the mitochondrial marker. (Scale bar = 5µm)

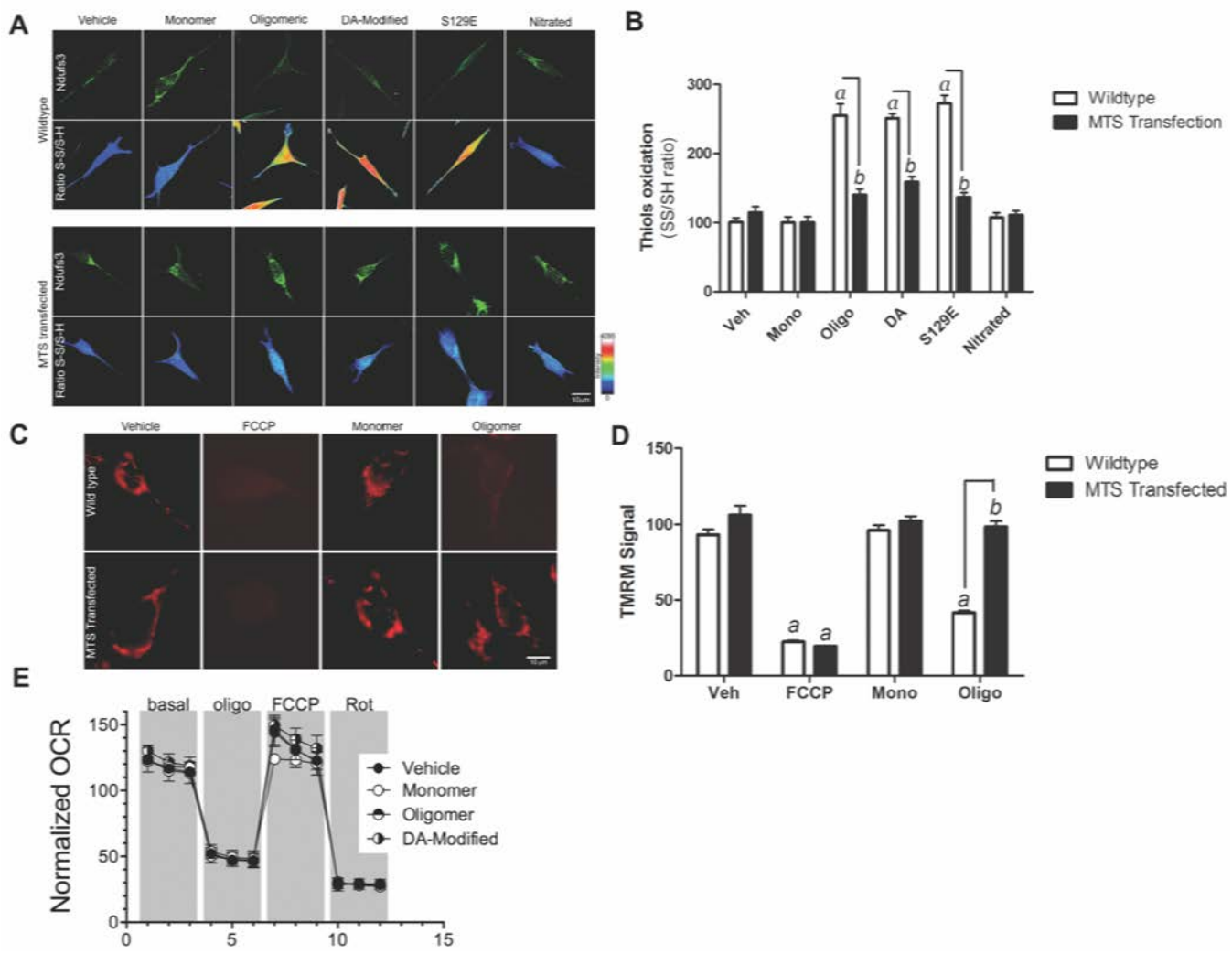

Figure S11. Downstream effects of α-synuclein on mitochondria are blocked by MTS overexpression. **(A)** In wildtype SH-SY5Y cells, a 24 h exposure to oligomeric, dopamine-modified or S129E α-synuclein induced oxidation of protein thiols; exposure to monomeric or nitrated α-synuclein did not. In SH-SY5Y cells overexpressing an MTS, α-synuclein did not induce oxidative stress. **(B)** Quantification of protein thiol oxidation in the experiments depicted in A. At least 100 cells were quantified per condition in each experiment. a, $p<0.001$ vs vehicle; b, $p<0.001$ vs mock transfected cells; 2-way ANOVA; N=3. **(C)** TMRM fluorescence in SH-SY5Y cells (as an index of mitochondrial membrane potential) is reduced by oligomeric but not monomeric α-synuclein. In SH-SY5Y cells overexpressing the MTS, oligomeric α-synuclein does not significantly impact mitochondrial membrane potential. **(D)** Quantification of the TMRM experiments. 30-50 cells were analyzed for each treatment in each of 3 independent experiments. a – $p<0.001$ vs. vehicle; b – $p< 0.001$ vs. wildtype cells; 2-way ANOVA; N=3. **(E)** In wildtype SH-SY5Y cells, a 24 h exposure to oligomeric or dopamine-modified α-synuclein reduced basal and FCCP-stimulated mitochondrial respiration, but monomeric α-synuclein was without effect, as shown in ***figure 7***. Here, in SH-SY5Y cells overexpressing the MTS, basal respiratory parameters were not altered, but the deleterious effects of oligomeric and dopamine-modified α-synuclein were prevented. N=3. Scale bars = 10 µm.

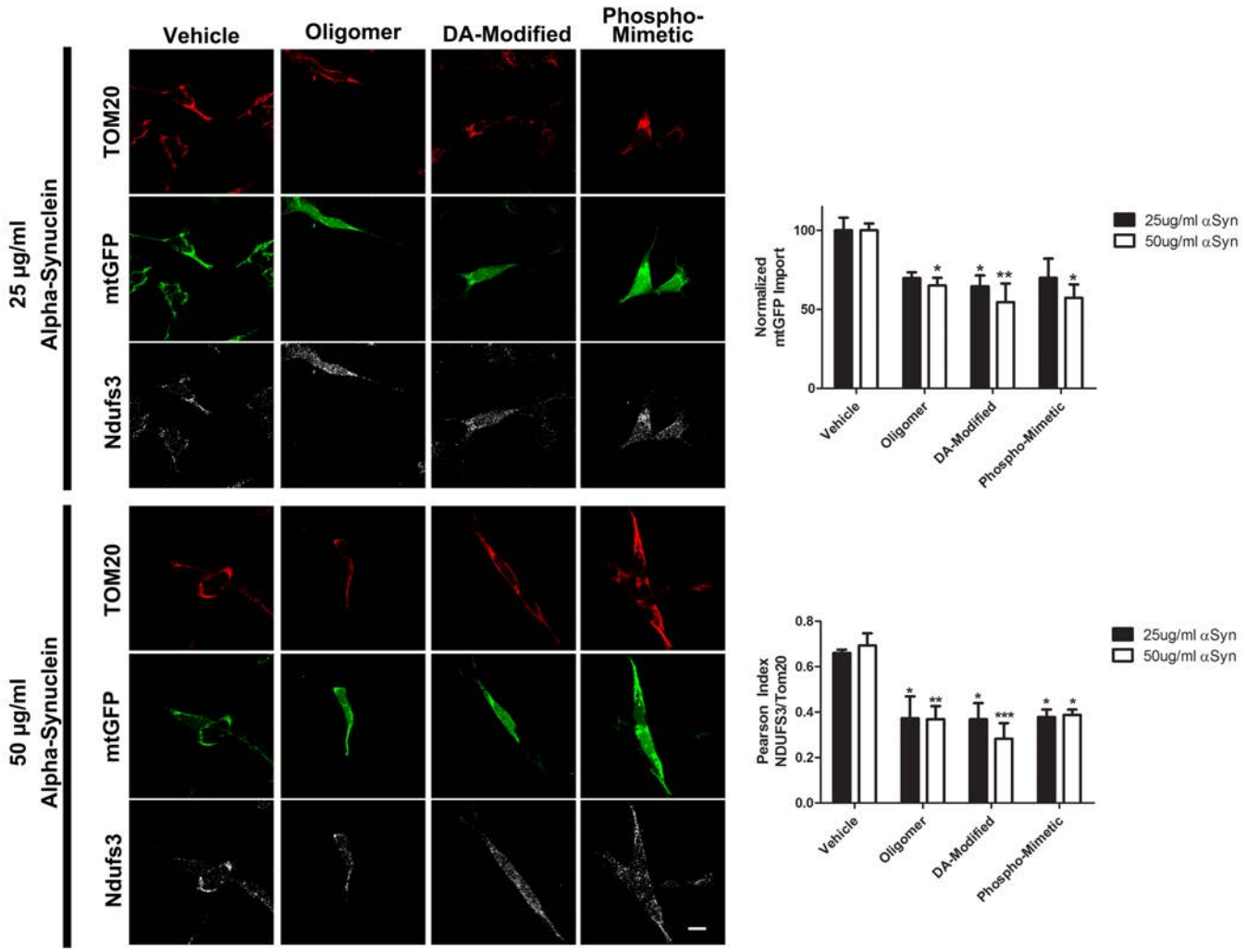

Figure S12. The protective effects of TOM20 overexpression on mitochondrial protein import can be overcome by increased concentrations of α-synuclein. As shown in ***figures 3 and 7***, modest overexpression of TOM20 (2 – 3-fold) protected against the effects of exogenous α-synuclein (200 nM) on mitochondrial protein import and its downstream effects. Here, cells were transfected with mtGFP and then treated with higher concentrations of α-synuclein (25 µg/ml or 1.7 µM and 50 µg/ml or 3.3 µM). Twenty four hours later the subcellular localizations of mtGFP and Ndufs3 were assessed. mtGFP import was assessed as described in Materials and Methods and Ndufs3 was assessed as colocalization with TOM20 (Pearson Index). The bar graphs reveal that, in contrast to 200 nM α-synuclein, the higher concentrations can overcome the protective effects of TOM20 overexpression (compare to figure 3). *p<0.05, **p<0.01, ***p<0.001 vs respective vehicle. 2- way ANOVA; N=3. Scale bar = 5 µm.

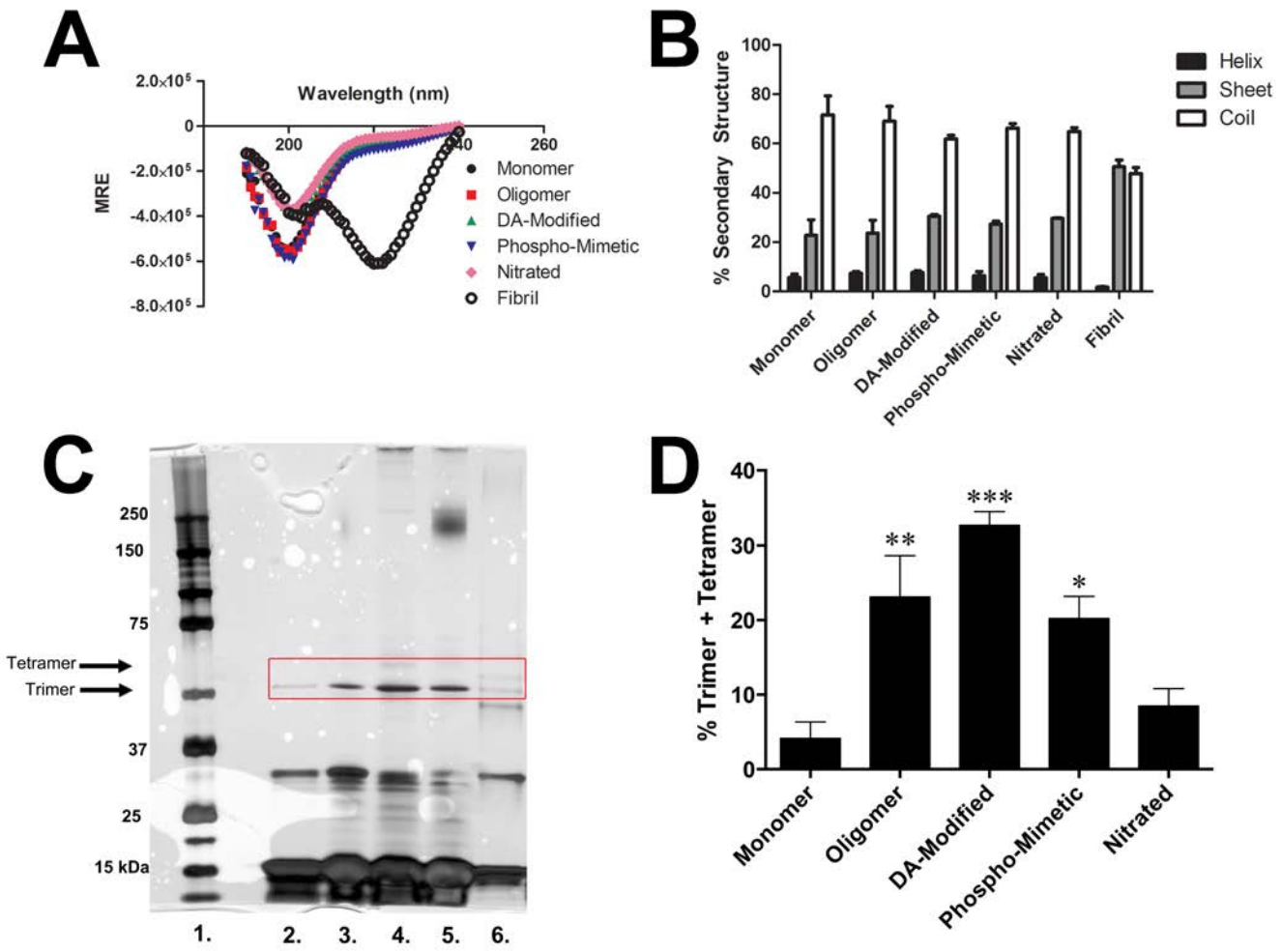

Figure S13. Structural analysis of the α-synuclein species used in this study. **(A)** Circular dichroism spectroscopy of the α-synuclein species used here. Other than the fibrils, other species displayed very similar spectra. **(B)** Quantification of the CD data. All of the species except the fibrils exist primarily in a random coil, with relatively small amounts of β-sheet and α-helix structure. As anticipated, the fibrils have more β-sheet structure than other species. **(C)** Silver stained SDS-PAGE gel of the α-synuclein species used here. Lane 1: MW ladder; lane 2: monomeric; lane 3: oligomeric; lane 4: dopamine-modified; lane 5: S129E; lane 6: nitrated. Red box outlines trimers and tetramers. **(D)** Quantification of the percent of total α-synuclein that is found as trimers and tetramers in a given lane. *p<0.05; **p<0.01; ***p<0.001 vs monomer by 1-way ANOVA. N=3.