Supporting Information for

Elucidating the role of C-terminal post-translational modifications using protein semisynthesis strategies: α-synuclein phosphorylation at Tyrosine 125

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III. Semisynthesis and purification of WT α-syn T and pY125 α-syn
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I. Synthesis and characterization of α-syn C-terminal peptides

Peptide: α-Syn(A107C-140)
sequence: CPQEGILEDMPVDPDNEAYEMPSEEGYQDYEPEA
synthetic specifications: couplings used HBTU; double couplings performed at Cys107, Glu109, Met-116, Asp-119, and Met-127
yield: 25%
expected mass: 3886 Da

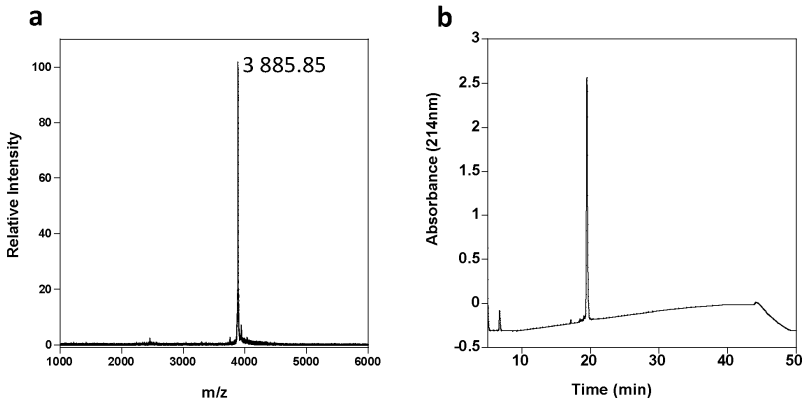

Figure S1. a) MALDI-TOF analysis (observed mass=3885.85Da) and b) RP-HPLC analysis of purified α-syn A107C-140 on a C_{18} analytical column (CosmoSil Protein-R, 4.6x250 mm, 5µm particle size) with a linear gradient of 0 to 80%B over 30min (A: water/0.1%TFA and B: acetonitrile/0.1% TFA).

Peptide: α-syn(A107C-140)_*p*Y125
sequence: CPQEGILEDMPVDPDNEA-*p*Y-EMPSEEGYQDYEPEA
synthetic specifications: see above
yield: 25%
expected mass: 3969 Da

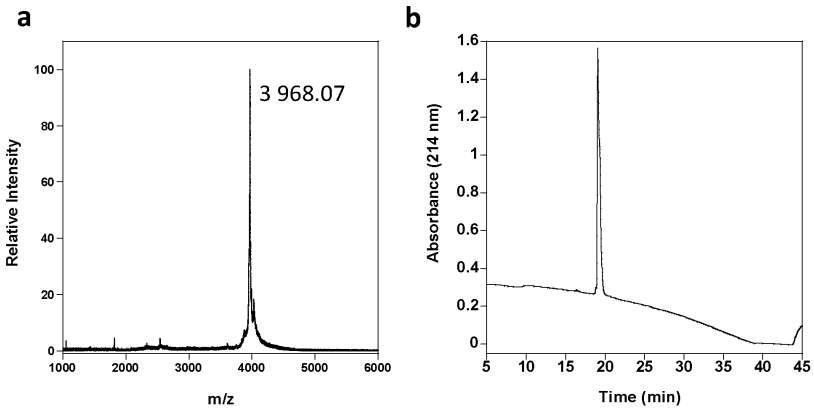

Figure S2. a) MALDI-TOF analysis (observed mass= 3968 Da). b) RP-HPLC analysis of purified α-syn A107C-140_*p*Y125 on a C_{18} analytical column (CosmoSil Protein-R, 4.6x250 mm, 5µm particle size) with a linear gradient of 0 to 80%B over 30min (A: water/0.1%TFA and B: acetonitrile/0.1% TFA).

II. Generation of ^{15}N-labeled α-syn(1-106)SR and non-labeled α-syn(1-106)SR

Non-labeled α-syn(1-106)SR

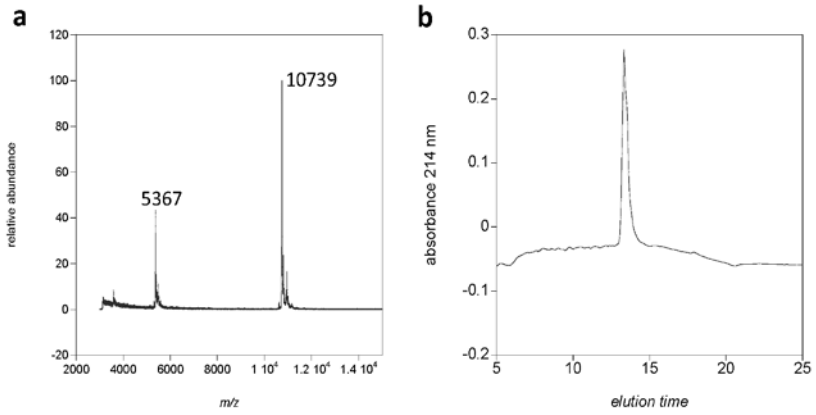

Figure S3. a) MALDI-TOF analysis (observed mass= 10739). The mass 5367 corresponds to the double-charged. b) RP-HPLC analysis of purified α-syn(1-106)SR on a C_4 analytical column (CosmoSil 5C_4) with a linear gradient of 0 to 80%B over 15min (A: water/0.1%TFA and B: acetonitrile/0.1% TFA).

^{15}N labeled α-syn (1-106)SR

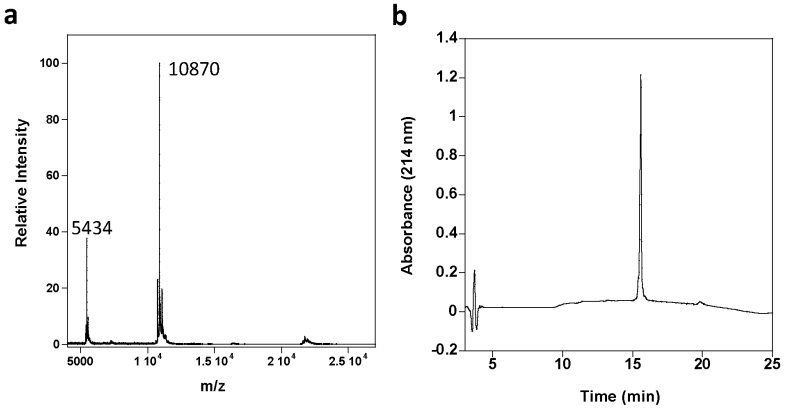

Figure S4. a) MALDI-TOF analysis (observed mass= 10870). The mass 5434 corresponds to the double-charged. b) RP-HPLC analysis of purified α-syn(1-106)SR ^{15}N labeled on a C_4 analytical column with a linear gradient of 0 to 80%B over 30min (A: water/0.1%TFA and B: acetonitrile/0.1% TFA)

III. Semisynthesis and purification of WT α-syn and pY125 α-syn

α-SynA107C wild-type

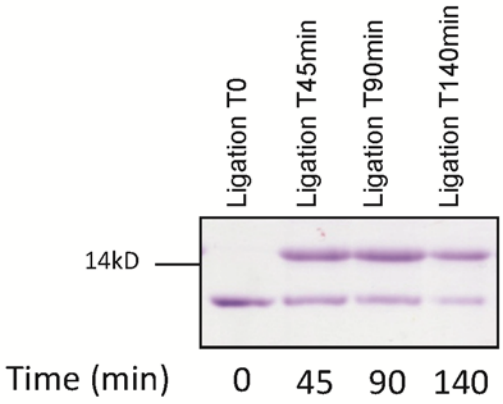

Figure S5. SDS-PAGE analysis of native chemical ligation reaction between α-syn(1-106)SR and α-syn(A107C-140).

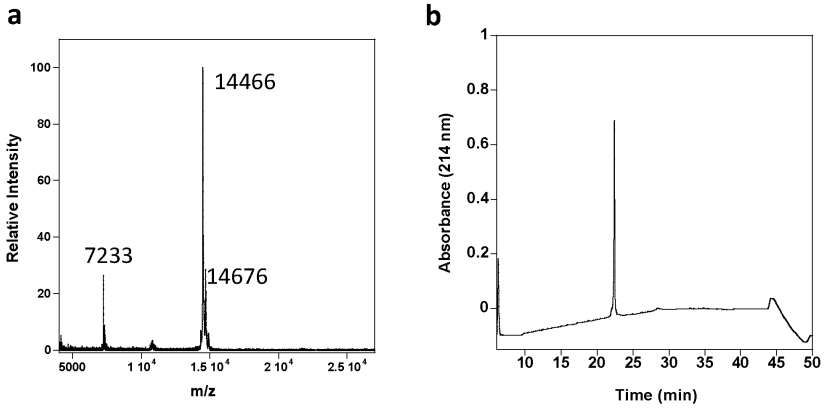

Figure S6. a) MALDI-TOF analysis of the desulfurized product (expected mass: 14461). The mass of 14676 corresponds to a sinapinic matrix adduct and the mass of 7233 to the double-charged. b) RP-HPLC of semi-synthetic WT α-syn on an analytical C18 with a linear gradient of 0 to 80%B over 30min (A: water/0.1%TFA and B: acetonitrile/0.1% TFA).

α-synA107C_*p*Y125

Figure S7. SDS-PAGE analysis of native chemical ligation reaction between α-syn(1-106)SR and α-syn(A107C-140_*p*Y125). Note: the ligation corresponding to the gel was done with two molar excess of α-syn(1-106)SR.

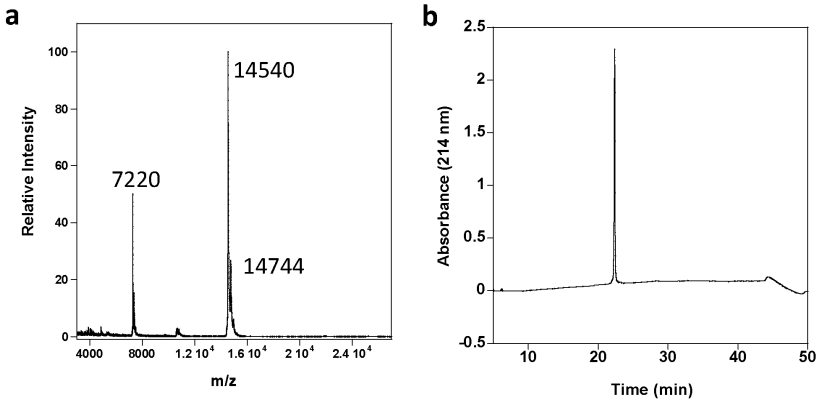

Figure S8. a) MALDI-TOF analysis of the desulfurized product (expected mass: 14541). The mass of 14744 corresponds to a sinapinic matrix adduct and the mass of 7220 to the double-charged. b) RP-HPLC of semi-synthetic pY125 α-syn on an analytical C18 with a linear gradient of 0 to 80%B over 30min (A: water/0.1%TFA and B: acetonitrile/0.1% TFA).

General scheme of ligation and purification

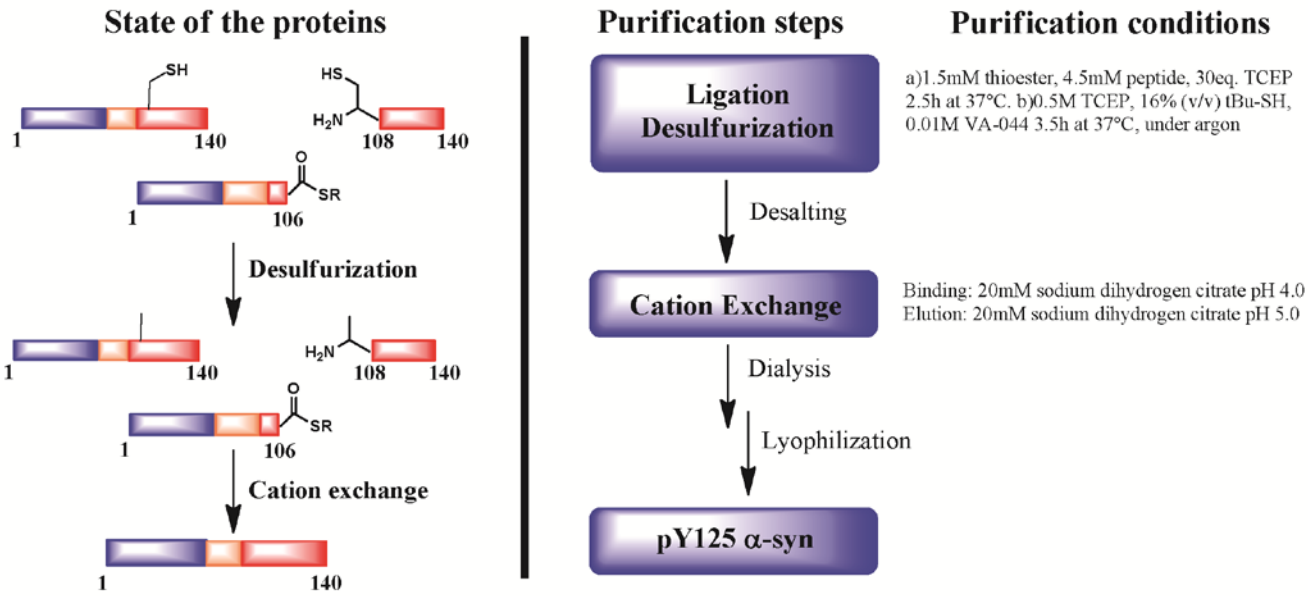

Figure S9. Scheme of the main steps for the generation of pY125 α-syn: ligation, desulfurization and purification by chromatographic methods.

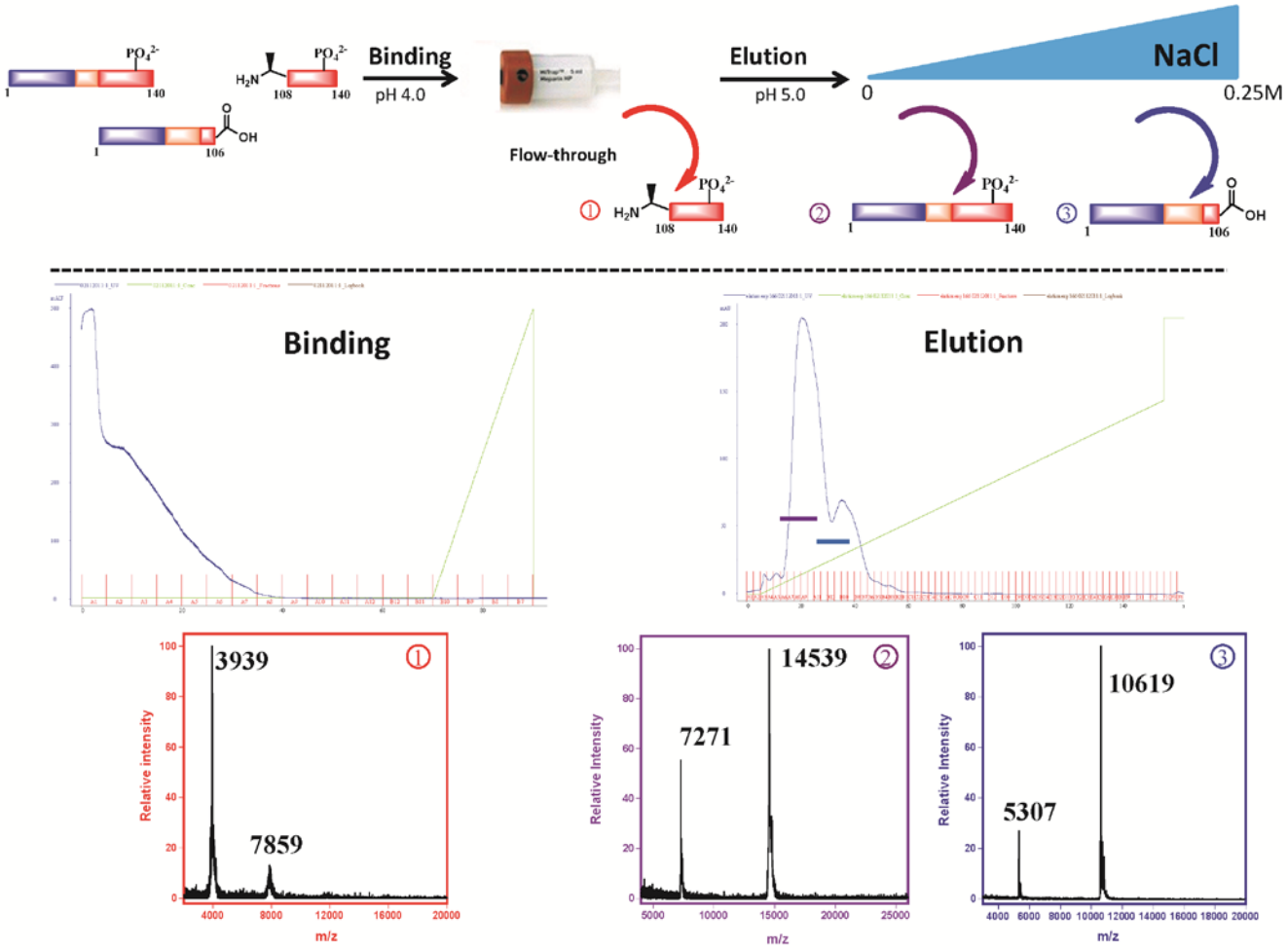

Figure S10. Scheme of the cation-exchange chromatography purification of pY125 α-syn.

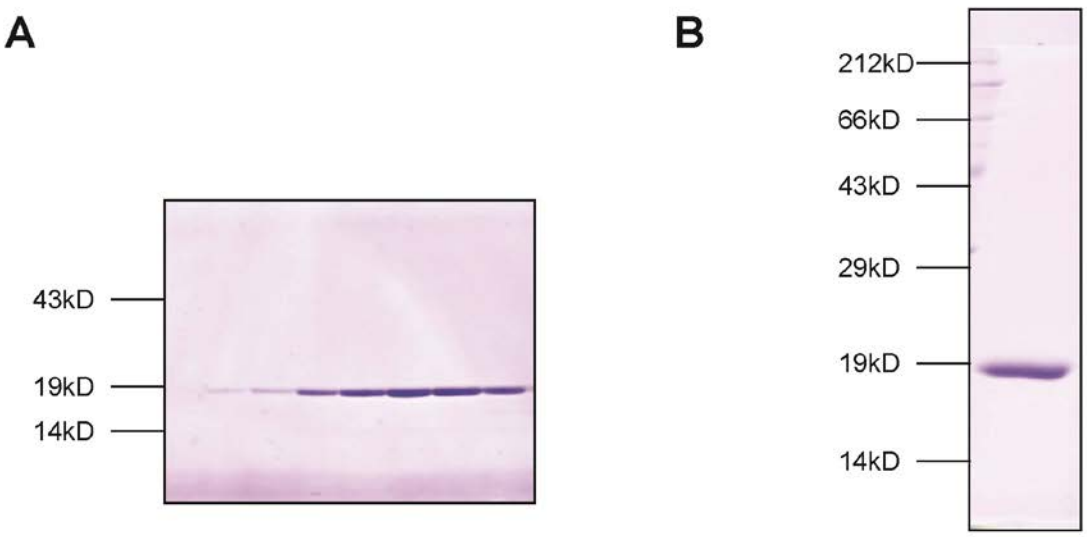

Figure S11. A: SDS-PAGE analysis of the fractions containing pY125 α-syn (eluting from the cation exchange column at pH 5). B: SDS-PAGE analysis of the pure lyophilized pY125 α-syn.

IV. Generation of α-syn A107C N^{15} labeled and Semisynthesis of α-syn A107C_*p*Y125 N^{15} labeled.

α-synA107C ^{15}N labeled

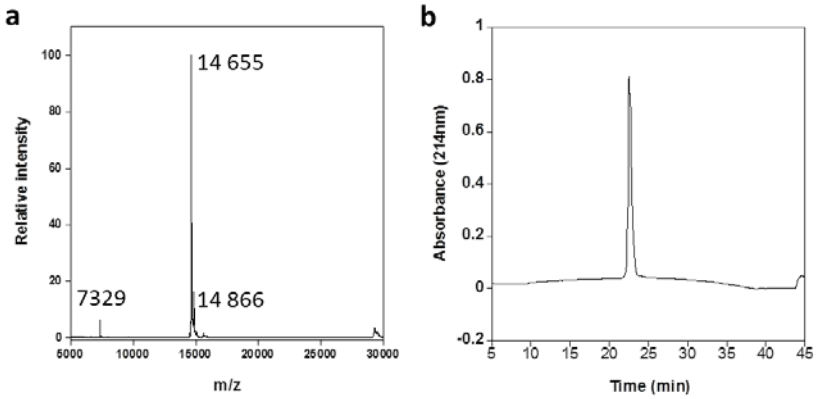

Figure S12. a) MALDI-TOF analysis of the protein (expected mass: 14659). The mass of 14866 corresponds to a sinapinic matrix adduct and the mass of 7329 to the double-charged. b) RP-HPLC of ^{15}N labeled α-syn A107C on an analytical Protein-R C_{18} column with a linear gradient of 0 to 80%B over 30min (A: water/0.1%TFA and B: acetonitrile/0.1% TFA).

α-synA107C_*p*Y125 ^{15}N labeled

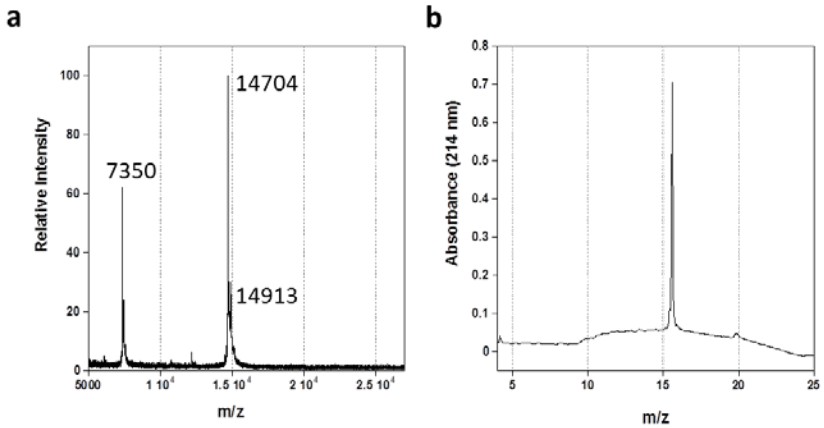

Figure S13. a) MALDI-TOF analysis of the ligated product (expected mass: 14701). The mass of 14913 corresponds to a sinapinic matrix adduct and the mass of 7350 to the double-charged. b) RP-HPLC of semi-synthetic pY125 α-syn ^{15}N labeled on an analytical Protein-R C_{18} column with a linear gradient of 0 to 80%B over 30min (A: water/0.1%TFA and B: acetonitrile/0.1% TFA).

V. TEM images of recombinant and semisynthetic WT α-syn

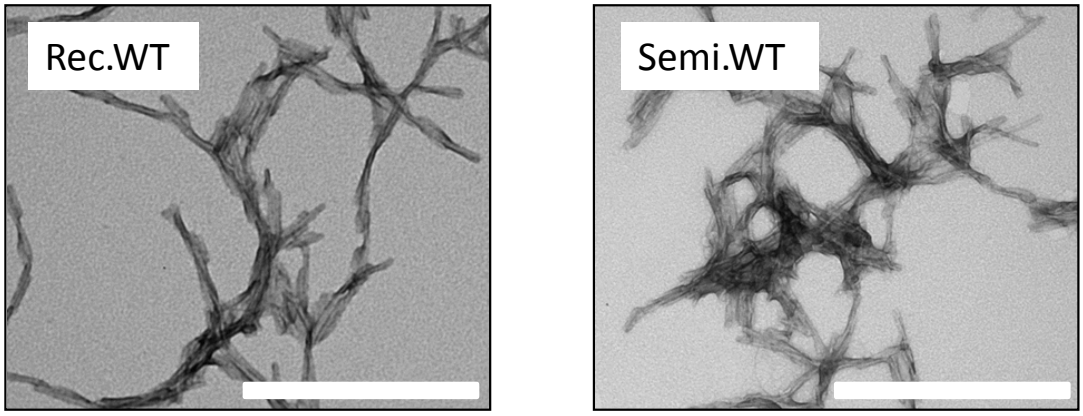

Figure S14. TEM images of recombinant WT and semisynthetic α-syn incubated for 7 days on a rotating wheel at 37°C. The images are representative of 3 independent experiments. The scale bars represent 200nm.

VI. Analysis of HEK and HeLa cells and mouse neurons treated with pervanadate: detection of non-specific bands

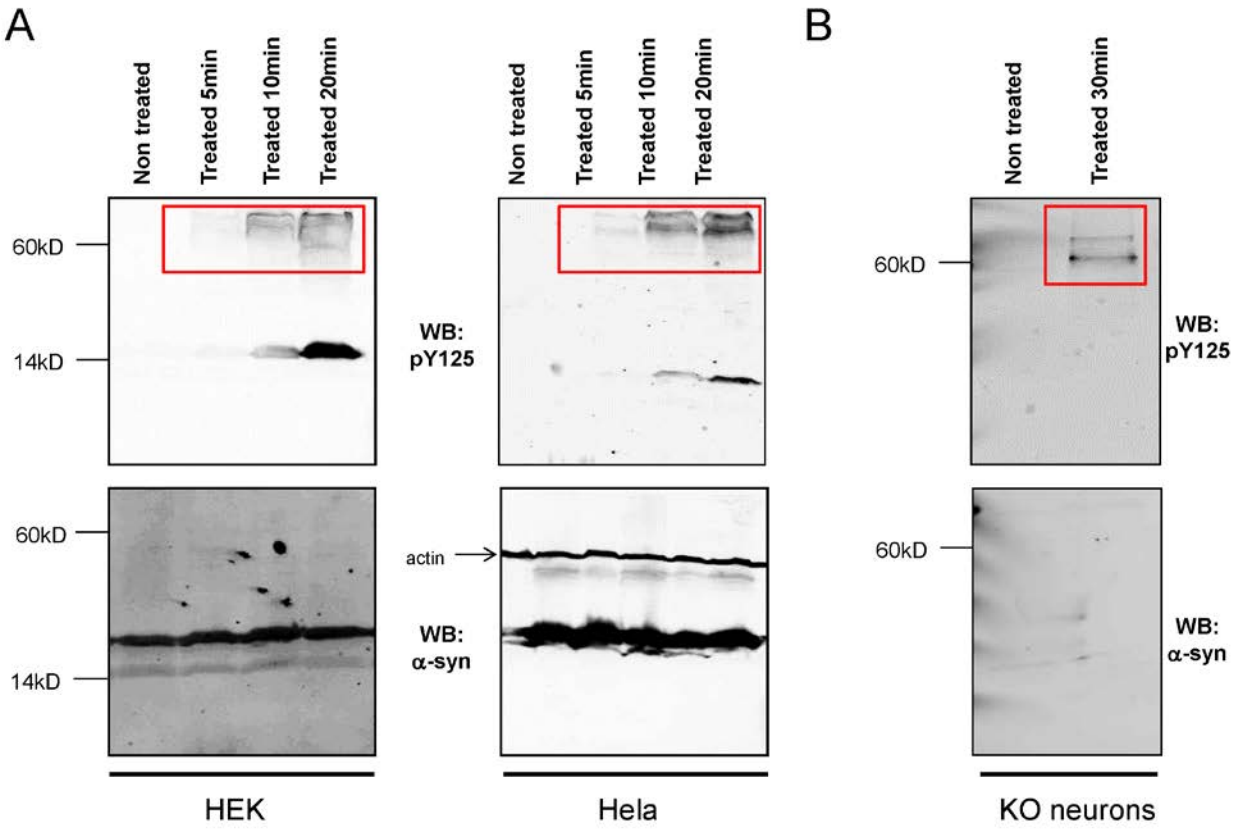

Figure S15. A. Immunoblots of HEK293 (left) and HeLa (right) cells over-expressing WT α-syn and treated with pervanadate for 5, 10 and 20min. Membranes were probed with WT α-syn (Biomol SA3400) and pY125 antibodies (BD Pharmigen). B. Immunoblots of mouse α-syn KO primary neurons treated with pervanadate for 30min. Membranes were probed with WT α-syn (BD transduction laboratory) and pY125 antibodies (BD Pharmigen).

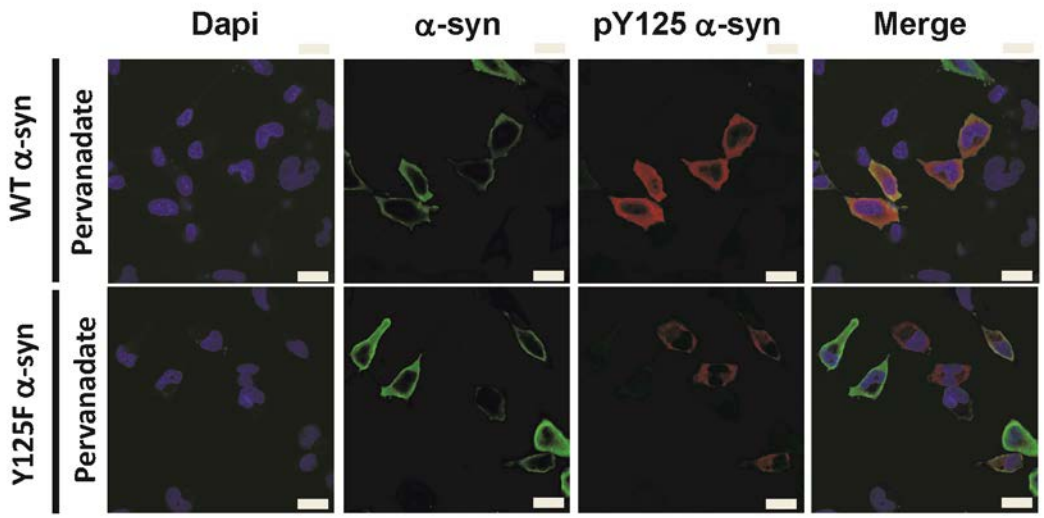

Figure S16. Confocal images of HeLa cells over-expressing WT or Y125F α-syn and treated with pervanadate for 30min. The cells were fixed and stained with anti-pY125 antibody (BD pharmigen) and anti-α-syn (abcam).

VII . Cross-talk between α-syn phosphorylation at S87 and Y125: *in vitro* phosphorylation assay

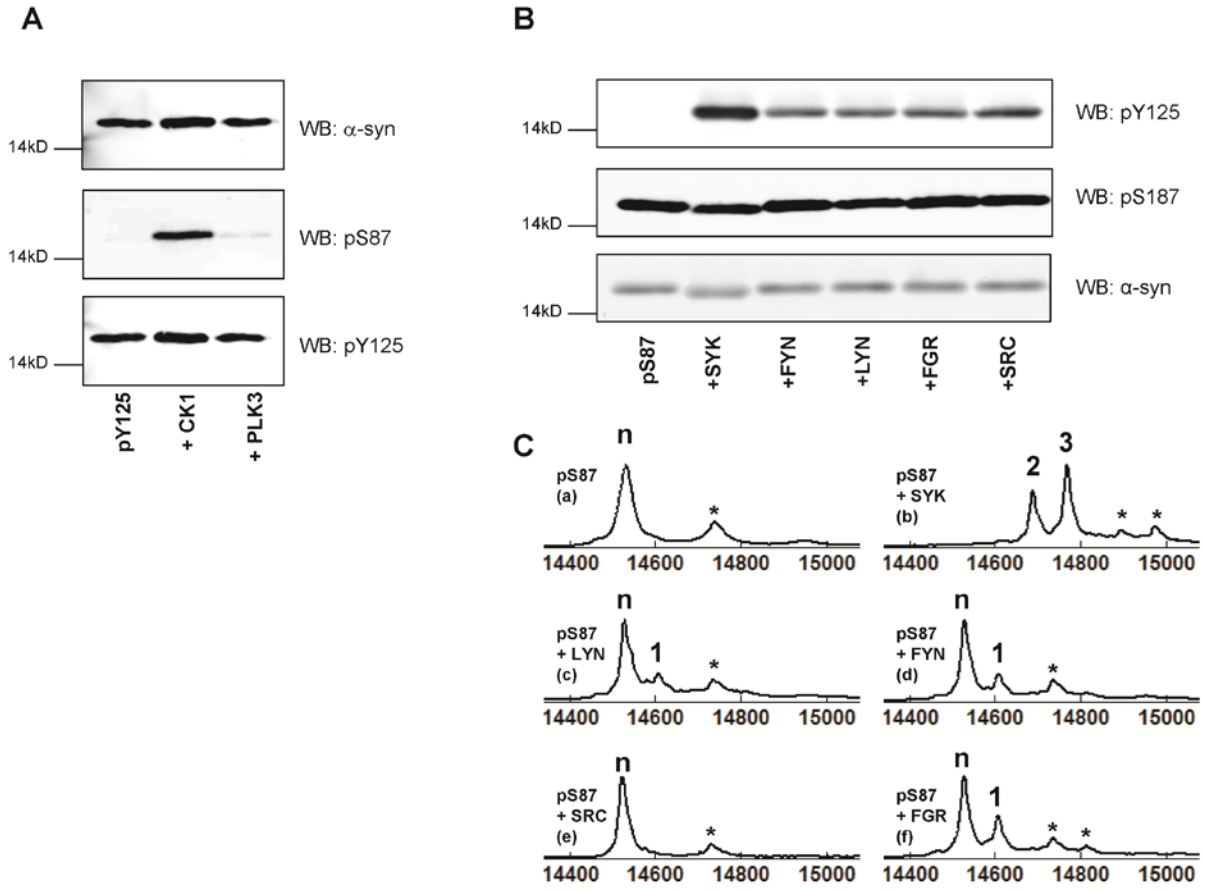

Figure S17. **A**: Immunoblots of pY125 α-syn phosphorylated using CK1 and PLK3. Membranes were probed using pS87, pY125, and α-syn antibodies. **B**: Immunoblots of pS87 α-syn phosphorylated using Syk, Lyn, Fyn, c-Src, and c-Fgr. Membranes were probed for pY125, pS87, and α-syn immunoreactivity. **D**: MALDI-TOF analysis of pS87 α-syn (a) phosphorylated by Syk (b), Lyn (c), Fyn (d), c-Src (e), and c-Fgr (f) after 14h of reaction. In all MALDI-TOF-MS spectra, the symbol 'n' indicates the starting material peak, while the numbers above other peaks correspond to the number of phosphorylation events, each detected by a +80 Da mass shift. The symbol '*' indicates a sinapinic acid matrix adduct.

Supporting information for the figure S17:

To obtain site-specific phosphorylation at S87, S129A α-syn was incubated with CKI until phosphorylation at S87 was complete, then pS87 α-syn was purified by reversed-phase HPLC, and incubated with the tyrosine kinases in the same conditions as in the experiment with pS129 α-syn. Using first the mutant S129A α-syn, we showed that the S129A mutation itself did not influence phosphorylation at Y125 (not shown). Using the purified pS87 α-syn, we observed that the behavior of pS87 α-syn is similar to that of pS129 α-syn and WT α-syn: phosphorylation at Y125 by Syk is very efficient as judged by Western Blot (Supplementary Figure S17 B) but again not specific as shown by MALDI-TOF MS (Supplementary Figure S17 C (b)). We noted, however, that pS87 α-syn might be more efficiently phosphorylated at Y125 by Fyn than pS129 α-syn, since a single phosphorylation event could be seen with pS87 α-syn but not pS129 α-syn. However, Western-Blot data does not support a large effect.

VIII- Complete references 5, 21, 30, 40 and 45

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