PHOSPHORYLATION AT S129, BUT NOT THE PHOSPHOMIMICS S129E/D INHIBITS THE FIBRILIZATION OF α-SYNUCLEIN

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SUPPLEMENTARY FIGURES

Fig.1. *In vitro* Superposition of a selected region of 1H -15N HSQC spectra of non-phosphorylated wt a-syn (blue) and phosphorylated wt α-syn (red). Selected resonance assignments are indicated with residue umbers.

Fig. 2. In vitro phosphorylation of α-syn by CK2. Previous studies reported that CK2-mediated phosphorylation of α-syn occurs mainly at S129 (Fujiwara et al., 2002; Okochi et al., 2000), whereas phosphorylation by CK1 occurs at both S87 and S129 (Okochi et al., 2000). Although we were able to confirm that CK2 phosphorylated α-syn at S129, we consistently observed that the yield of this reaction is extremely low (≤5% at best, by RP-HPLC and MALDI-TOF). Given the low yield observed with CK2, we explored the possibility of using CK1 to prepare S129-P. Generally, greater than 40% of α-syn is phosphorylated within the first 24 h of incubation with CK1. (a) CK2-mediated phosphorylation of S129 as detected in silverstained SDS-PAGE gel. (b) Phosphorylation of recombinant WT α-syn with CK2 results in a band that is immunoreactive to the anti-phospho-S129 antibody (1:5000), which can be detected as early as the first 8 h of incubation and increases only slightly during the next 24 h of incubation (c) RP-HPLC chromatograms of WT α-syn samples after phosphorylation for 24 h with CK2. (d) Kinetic of CK2 phosphorylation of α-syn WT at S129 monitored by MALDI-MS. Phosphorylation at S129 was monitored on aliquots of a tryptic digest of the incubation mixture sequentially deposited on the target using a dried droplet sample preparation with DHB/PA matrix. The 4280-4380 m/z inset cover the pseudomolecular region of C-terminal peptide [103-140] at t 0, 8, 12 and 24 hours. The CK2 phosphorylation reaction reaches a plateau after 8h00 of incubation time. Only a small proportion of Ser129 is phosphorylated by CK2 even after 24h00. Several attempts to increase the yield of CK2 phosphorylation at S129 by varying the reaction conditions failed, suggesting that recombinant α-syn is not efficiently phosphorylated by CK2 *in vitro*. Similar observations were later confirmed by other groups *(T. Iwatsubo & T. Chilcote, personal communication).*

Fig. 3. Purification and Characterization of phosphorylated S87A α-syn. CK1-mediated phosphorylation of S87A results in the appearance of two peaks in the HPLC chromatogram (a). The first minor peak (2-5% of the total protein, depending on the incubation time) corresponds to the diphosphorylated form of α-syn, whereas the second peak corresponds to the mono- and unphosphorylated forms of the S87A. However, we were not able to separate S87A from S87A/S129-P by RP-HPLC, due to the co-elution of the monophosphorylated S129 species (S87A/S129-P) and unphosphorylated S87A. Therefore, to minimize the amount of unphosphorylated species, we optimized the phospohrylation conditions to achieve nearly complete phosphorylation of S87A α-syn at S129 using CK1. S87A α-syn monophosphorylated at S129 was separated by RP-HPLC and the level of phosphorylation and the purity of the samples were verified by dot blot analysis using two individual anti-α-syn antibodies and the phosphorylation-specific antibodies (b), anti-phospho-S129 and anti-phospho-S87 and MALDI-TOF analysis (c). MALDI-TOF analysis of the peak corresponding to monophosphorylated S87A demonstrated the successful separation of the S87A/S129-P species.

Fig. 4. CK1-mediated phosphorylation of WT and S87A α-syn. 24 h post phosphorylation, WT α-syn is phosphorylated at S87 and S129 as indicated by the dot blot analysis employing the anti-PhSer87 (1:100) and anti-PhSer129 (1:5000) antibodies, while S87A is only phosphorylated at S129. All proteins were detected by the anti-α-syn antibody (211) (1: 500).

Fig. 5. Normalized weighted average $^{1}H/^{15}N$ chemical shift differences between different α-syn mutants. (a) WT α-syn compared to S87A α-syn. (b) S129E α -syn compared to WT α-syn. The domain organization of α-syn is shown on the top: basic N-terminal domain (red), hydrophobic NAC region (yellow), acidic C-terminal domain (blue) and the six repeats (green).

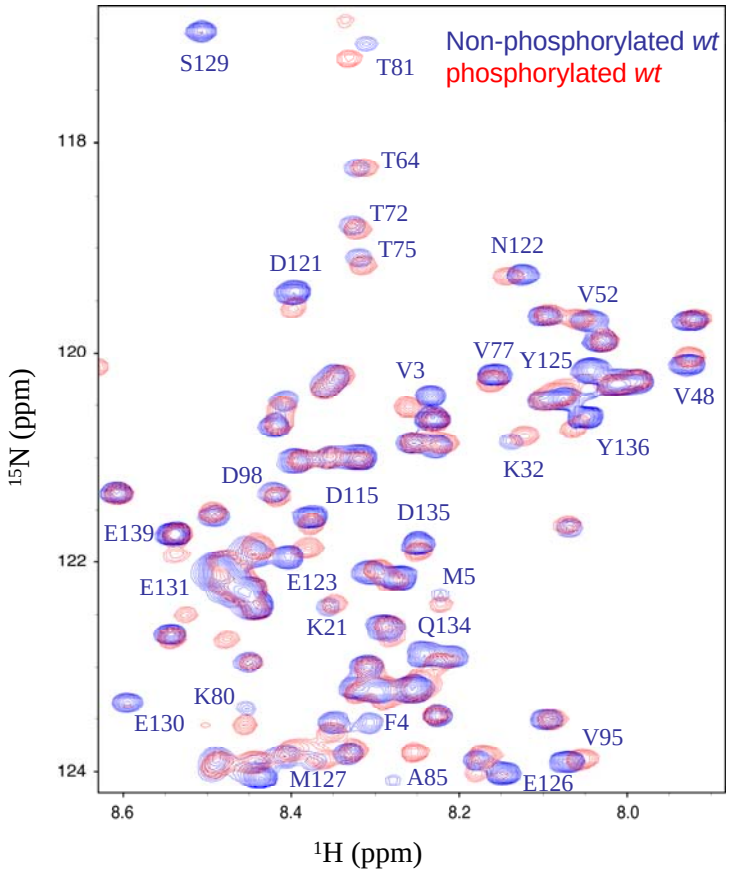
Fig. 6. Comparison of steady-state heteronuclear ^{15}N [^{1}H}-NOEs in unphosphorylated WT (black), unphosphorylated S87A (blue) and phosphorylated S87A (grey) α-syn at 15 °C. ^{15}N [1H]-NOE values are reported as the ratio of peak heights in paired spectra collected with and without an initial period (4 s) of proton saturation during the 5-s recycle delay. In (a) the data of only two of the three proteins are shown.

Fig. 7. Comparison of $^{3}J(HN,H\alpha)$ scalar couplings observed in S129D α-syn (grey) and in WT α-syn (black).

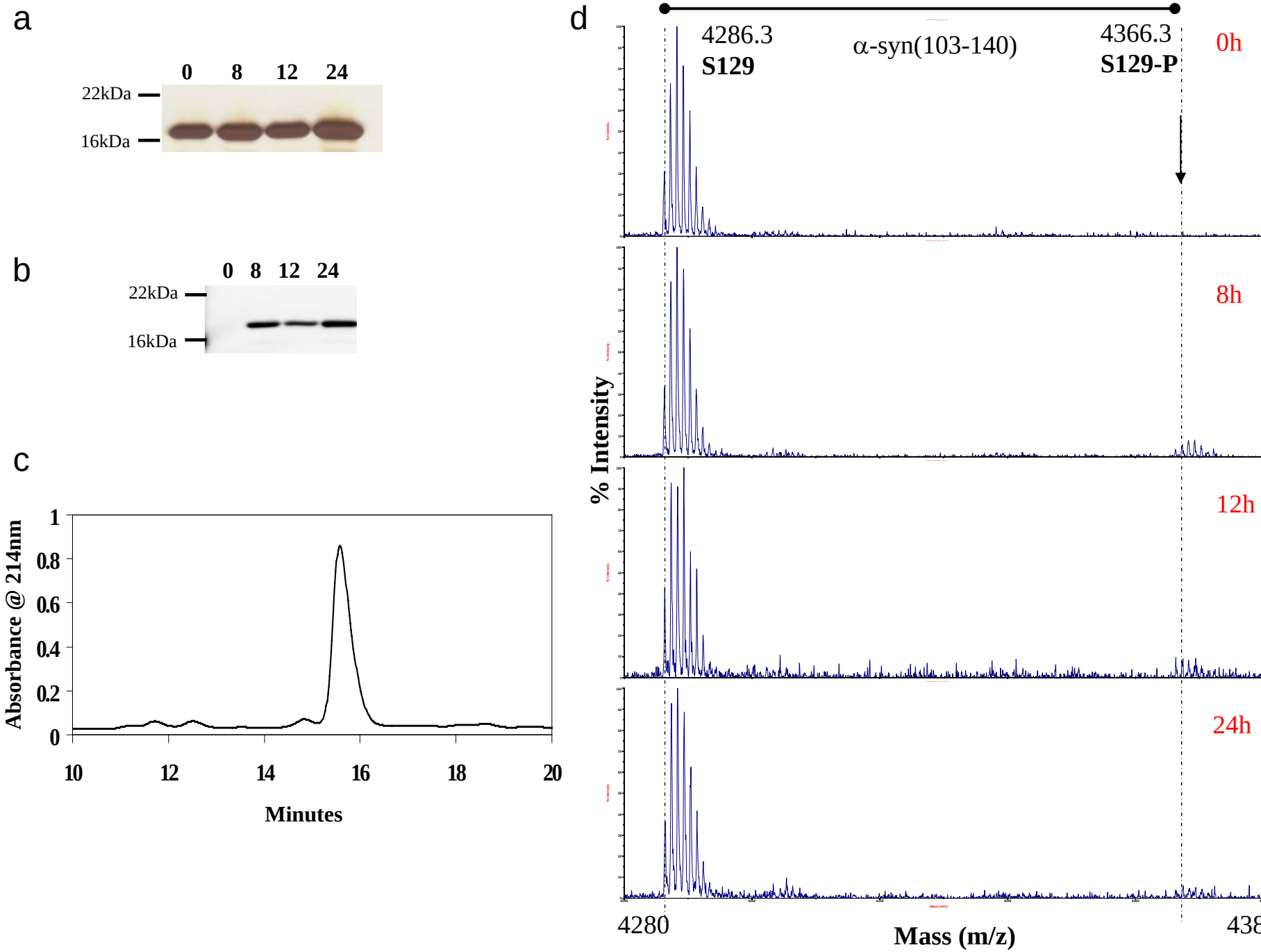
Fig. 8. S87A/S129-P α-syn aggregates after long incubation period. (a) 12% SDS gel of the S87A/S129-P α-syn sample after 20 days of aggregation. The sample was centrifuged for 30 min at 18000 g and the fibrillar pellet (P) was separated from the monomeric supernatant (S) and equal volumes of each were run on a 12%SDS gel. The pellet migrates with smearing indicating the presence of high molecular weight α-syn species. (b) TEM images of S87A/S129-P α-syn after 4 days or 20 days of aggregation.

Fig. 9. S87A/S129-P interacts with S87A and inhibits its aggregation in a does-dependent manner. S87A α-syn (70 μM) samples were mixed with the appropriate volume of 70 μM S87A/S129-P to generate samples containing 5% and 20% of the latter protein. (a) Coomassie stained 12% SDS gel of the proteins before aggregation for assessing the quality of the starting material. (7.23 μg of α-syn /well). (b) Coomassie stained gel of the samples after 96 h of aggregation. (c) Graph representing the area of monomeric peak after 96 h of aggregation at 37°C.

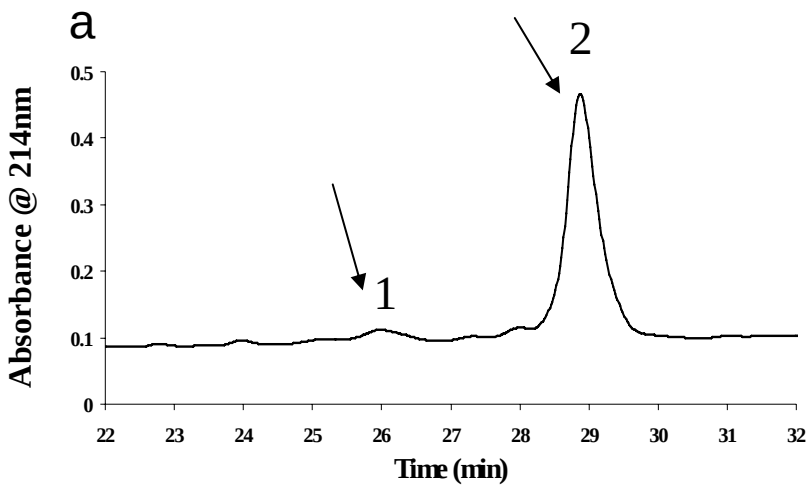
Supplementary Fig. 1

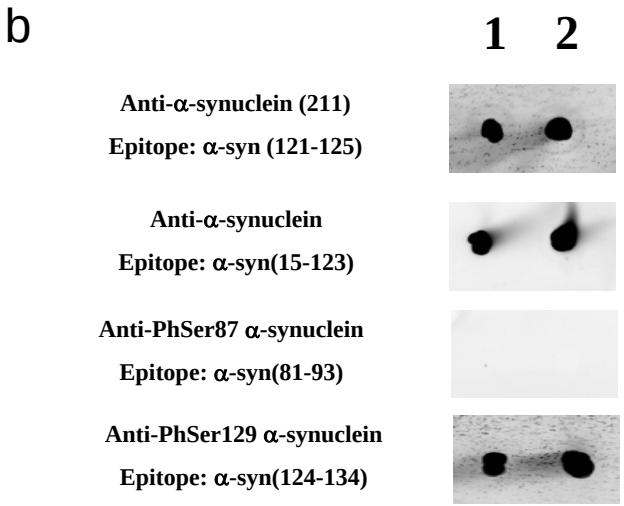


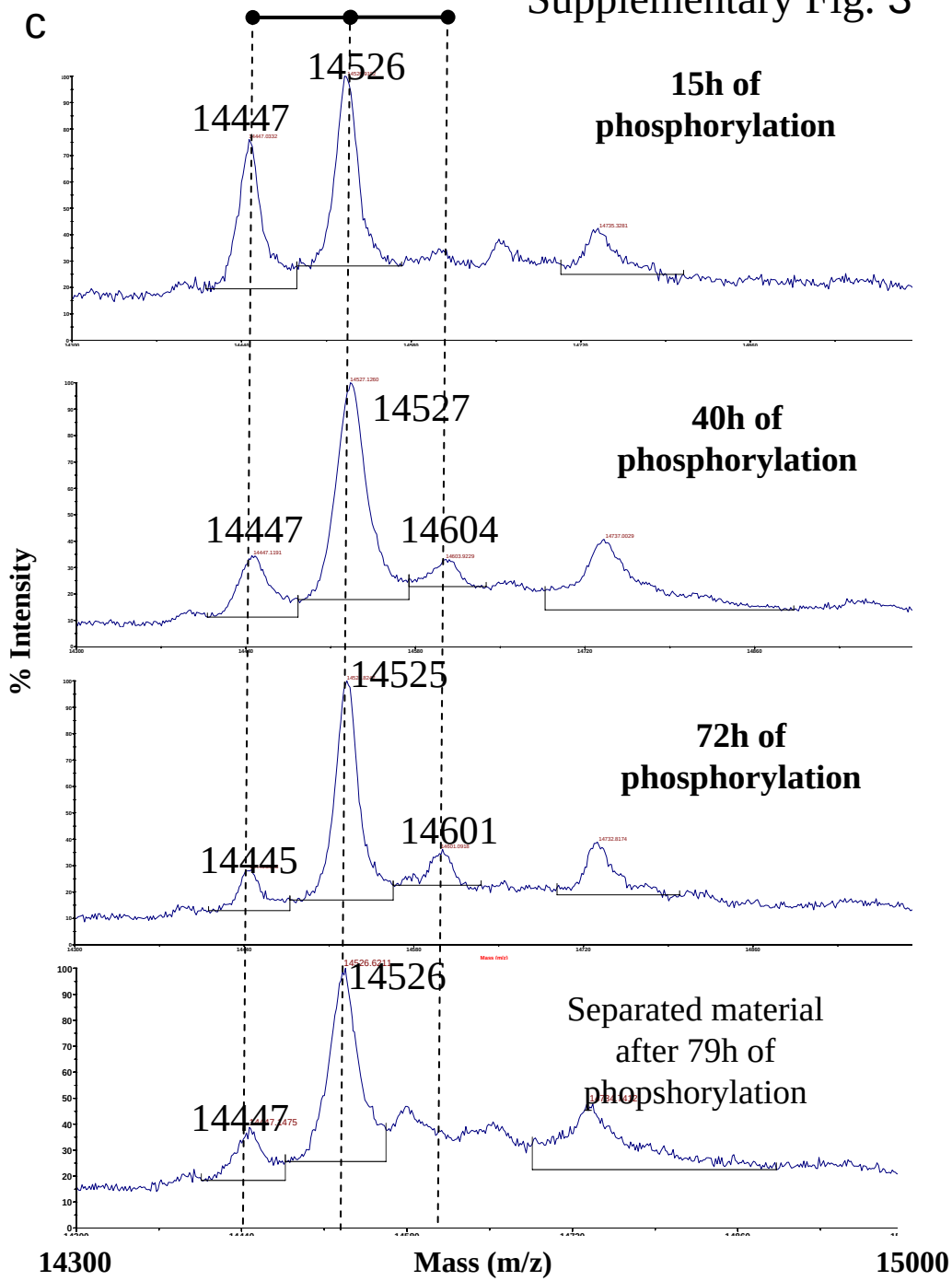
Supplementary Fig. 2



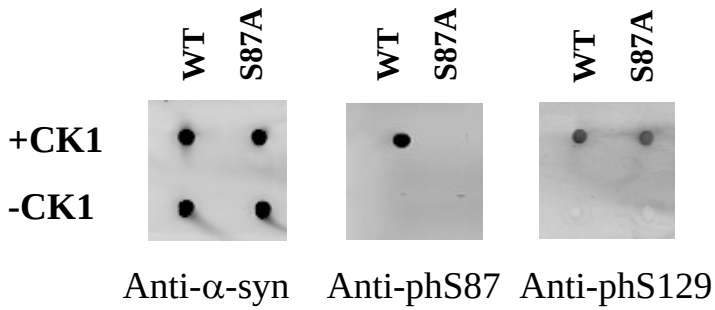
Supplementary Fig. 3







Supplementary Fig. 4



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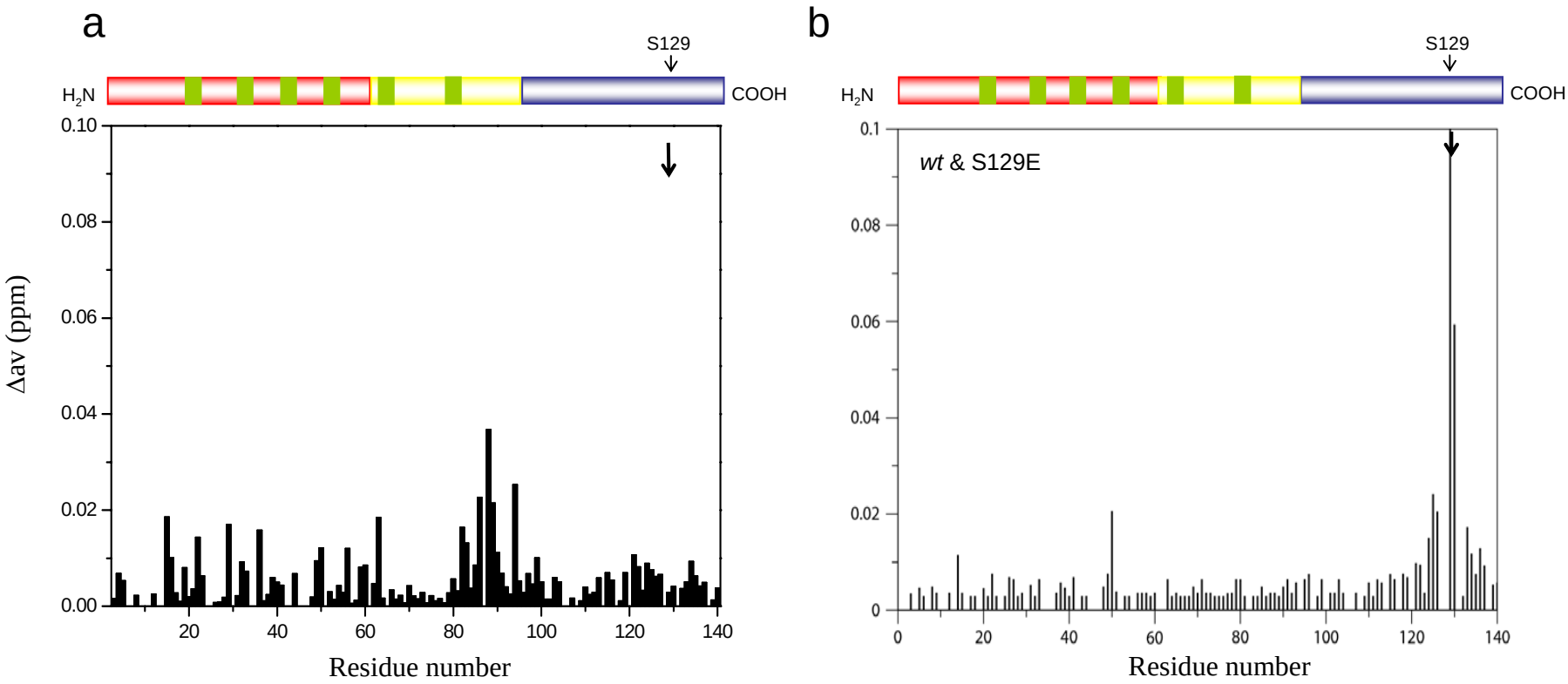

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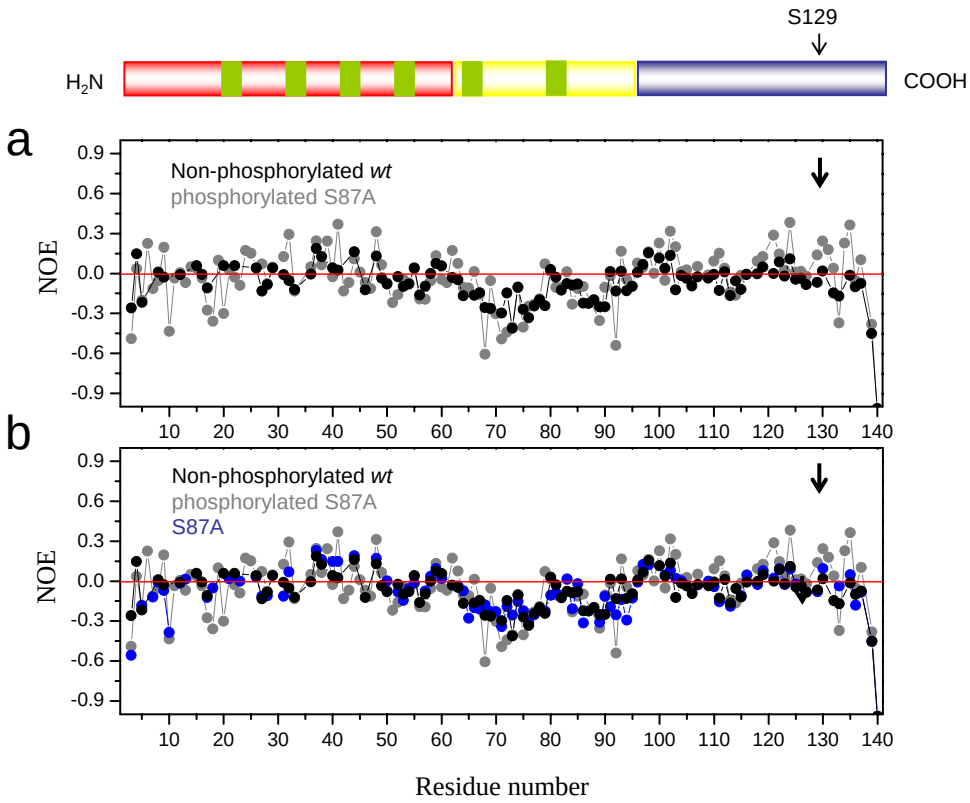

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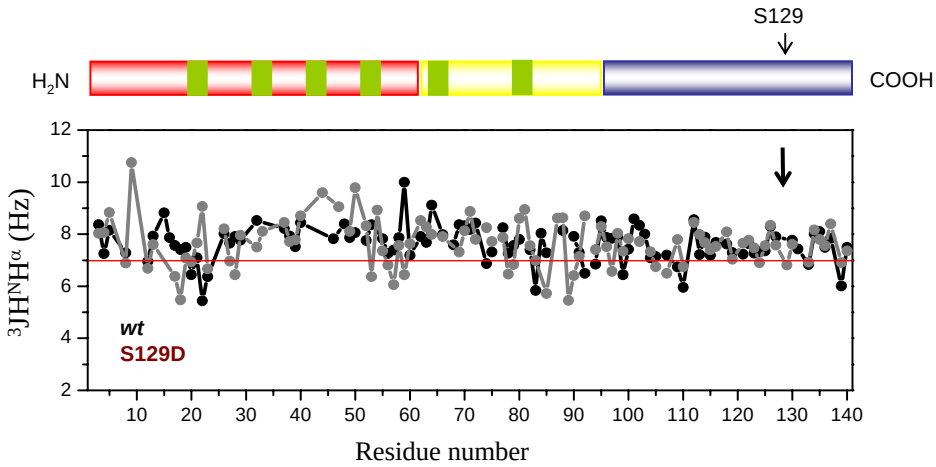


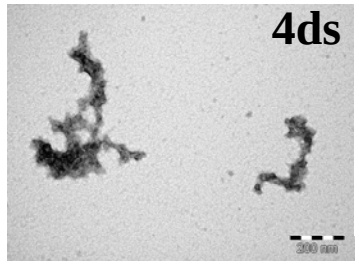
Supplementary Fig. 8

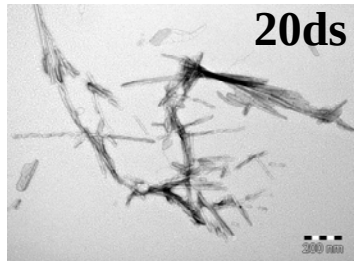
a

S: supernatant

P: pellet

b





Supplementary Fig. 9