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Efficient modification of alpha-synuclein Ser129 by Casein kinase 1 requires phosphorylation of Tyr125 as a priming event

Jonas Kosten^{§,‡}, Andres Binolfi^{§,‡}, Marchel Stuiver[§], Silvia Verzini^{§,¶}, Francois-Xavier Theillet[§], Beata Bekei[§], Marleen van Rossum[§], and Philipp Selenko^{§,*}

[§]In-Cell NMR Laboratory, Department of NMR-supported Structural Biology, Leibniz Institute of Molecular Pharmacology (FMP Berlin), Robert-Rössle Strasse 10, 13125 Berlin, Germany

[¶]Present address: Department of Biochemistry, University of Potsdam, 14476 Golm, Germany.

[‡]These authors contributed equally.

*To whom correspondence should be addressed: selenko@fmp-berlin.de

Abstract

S129-phosphorylated alpha-synuclein (α -syn) is abundantly found in Lewy-body inclusions of Parkinson's disease patients. Residues neighboring S129 also include the α -syn tyrosine phosphorylation sites Y125, Y133 and Y136. Here, we use time-resolved NMR spectroscopy to delineate atomic resolution insights into the modification behaviors of different serine and tyrosine kinases targeting these sites and show that Y125 phosphorylation constitutes a necessary priming event for the efficient modification of S129 by Casein kinase I, both in reconstituted kinase reactions and mammalian cell lysates. These results suggest that α -syn Y125 phosphorylation augments S129 modification under physiological *in vivo* conditions.

Keywords: Alpha-synuclein, phosphorylation, Casein-kinase 1, Parkinson's disease, NMR

Introduction

Aggregation of human α -syn into amyloid-rich Lewy bodies constitutes a hallmark of Parkinson's disease (PD) and other related synucleinopathies.¹ Whereas 90% of aggregated α -syn in neuronal inclusions is phosphorylated at S129 less than 4% of the soluble protein is modified,² which supports a possible link between α -syn S129 phosphorylation, protein aggregation and PD pathogenesis.^{2, 3} In intact neurons, S129 phosphorylation is thought to modulate dopamine uptake and to mediate the Rab8a interaction, blocking synaptic vesicle trafficking.⁴⁻⁶ Given this importance of α -syn S129 phosphorylation, several kinases have been identified that establish this modification *in vitro* and *in vivo*, including members of the Polo-like kinase family (Plk's) and Casein kinases I and II.⁷⁻¹²

In comparison to the modification of S129, phosphorylation of α -syn tyrosines is less well explored. Y125-phosphorylated α -syn is found in Lewy bodies,¹³ although no clear correlation between this modification and disease onset, or progression has been established.¹⁴ Possible roles of Y133 and Y136 phosphorylation are equally poorly understood.^{14, 15} By contrast, phosphorylation of α -syn Y39 contributes to cytotoxicity by impairing the cellular clearance of α -syn.¹⁶ All four tyrosines of α -syn, i.e., Y39, Y125, Y133 and Y136 are targeted by members of the Sarcoma (Src) family of kinases (SFKs) both *in vitro* and *in vivo*,¹⁷ with Fyn kinase being the most effective enzyme.¹⁸ Fyn preferentially targets Y125, whereas Y39, Y133 and Y136 constitute secondary phosphorylation sites.¹⁷ Other enzymes, such as the acidophilic tyrosine kinase Syk are reported to phosphorylate Y125, and, to a lesser extent, Y133 and Y136,¹⁹ whereas Y39 is thought to be the preferred *in vivo* substrate site for Abl kinases.¹⁶

α -Syn S129 and tyrosines Y125, Y133, Y136 are in close proximity, which raises the question whether crosstalk between these phosphorylation sites exists. Recent evidence suggests that phosphorylated Y125 and S129 occur together in Lewy bodies,¹³ however,

serine-tyrosine phosphorylation crosstalk has not been investigated before. The closeness of these C-terminal modification sites complicates any such analysis, especially when classical approaches of mutagenesis and immunodetection, or mass spectrometry are employed. Kinase activities may be lost upon sequence changes of substrate sites and antibody fidelity may be compromised on altered epitopes.²⁰ Similarly, the phosphorylation states of neighboring substrate sites are difficult to assess with mass spectrometry, because uniquely modified, proteolytic peptide fragments are not generated easily. To overcome these problems, we employed time-resolved high-resolution NMR spectroscopy to map and monitor α -syn serine-tyrosine phosphorylation in reconstituted kinase reactions and mammalian cell lysates. Our data show that different tyrosine kinases display clear substrate site preferences on α -syn and that phosphorylation of Y125 constitutes a necessary priming event for the efficient modification of S129 by Casein kinase I (CK1). These results suggest that modification of Y125 may increase the phosphorylation levels of S129 by the ubiquitously acting kinase CK1, thus strengthening the link between Y125-S129 phosphorylation and PD pathology.

Results and Discussion

We tested an extended set of receptor (RTKs)-, and non-receptor tyrosine kinases (non-RTKs) for their ability to phosphorylate N-terminally acetylated α -syn.²¹ After incubating 25 μ M of ¹⁵N isotope-labeled α -syn with 250 enzymatic units (U) of each enzyme for ~12 hours, we recorded two-dimensional (2D) ¹H-¹⁵N correlation spectra to determine the individual phosphorylation levels of Y39, Y125, Y133 and Y136 (**Fig. 1a** and **S1a**). Our experiments revealed that all tested kinases phosphorylated α -syn to different extents and that modification by non-RTKs was generally more efficient than phosphorylation by RTKs (**Fig. 1b**). Members of the Src family of kinases (SFKs), including Yes, Src, Fyn and Lyn, preferentially targeted Y125, whereas Y136 was the preferred substrate sites of focal adhesion kinase 1 (FAK1), but not of FAK2 and EphB4. RTKs displayed a slight preference for Y136 over

other sites, with the exceptions of Axl and Ron, which selectively modified Y39. Phosphorylation of Y125 with Fyn and Syk reached 100%, indicating that both kinases targeted this site with high efficiency, in line with previous reports.¹⁷⁻¹⁹ Compared to other α -syn tyrosines, Y39 phosphorylation was least efficient and only few kinases, namely Axl, Ron and Abl-1 and 2, modified this site specifically. Interestingly, active forms of Abl are up-regulated in response to dopamine-derived neurotoxins and in PD patients' brains.²² These results established that different tyrosine kinases target different α -syn sites with high specificity and efficiency *in vitro*. While some of them have clear implications in α -syn's biology, as outlined previously, the physiological relevance of others, especially with regard to possible roles in PD remains to be determined. Here, our results may provide initial points of reference for future functional studies. In addition, information about tyrosine kinase specificities and α -syn site preferences may be used to generate uniquely modified versions of the protein for biochemical analyses.²³

In a next step, we monitored α -syn tyrosine phosphorylation in a time-resolved fashion by recording consecutive sets of 2D ¹H-¹⁵N correlation experiments of reconstituted kinase reactions proceeding directly inside the NMR spectrometer (**Fig. 2a**). By plotting the changes of individual α -syn peak intensities against the reaction time, we established that Fyn phosphorylated Y125 faster than Y133, Y136 and Y39, whereas Syk phosphorylated Y125 and Y136 with comparable rates, in agreement with our previous results.¹⁷⁻¹⁹ Given that S129 is three residues away from Y125 and Y133, we asked whether modification crosstalk between these sites existed, and whether S129 modification affected the rates of tyrosine phosphorylation by Fyn and Syk? First, we pre-phosphorylated ¹⁵N isotope-labeled, N-terminally acetylated α -syn at S129 with Plk3 (**Fig. S1b**). Next, we reacted unmodified and S129-modified α -syn with 250 U of Fyn and Syk in time-resolved reaction setups to measure tyrosine phosphorylation rates (**Fig. 2a**). For the reaction with Fyn, we found no significant differences to unmodified α -syn, whereas Syk displayed a slight decrease in the rate of Y125

phosphorylation and an increase in the Y133 modification efficiency. Overall, S129 phosphorylation did not exert pronounced effects on the substrate site preferences and the general activities of both enzymes, in agreement with previous findings.²³ Next, we analyzed whether reciprocal tyrosine pre-phosphorylation influenced the modification of S129 by Plk3, CK1 and CK2, three kinases reported to phosphorylate this site.⁷⁻¹² We set up Plk3 reactions (250 U) with unmodified and tyrosine pre-modified α -syn substrates and found very similar S129 phosphorylation rates (**Fig. 2b**). We did not detect S129 phosphorylation of unmodified, or tyrosine-phosphorylated α -syn with 250-2000 U of CK2 (**Supp. Fig. 2a**), in agreement with recent *in vitro* studies showing that α -syn is a poor CK2 substrate.¹¹ Although CK1 has been used previously to phosphorylate α -syn at S87 and S129,^{11, 24} we did not observe phosphorylation of unmodified α -syn with 250 U of CK1 under our experimental conditions (**Fig. 2c**). This discrepancy likely arose from the low enzyme amounts that we used in our assay. Indeed, when we increased CK1 concentration to 2000 U we detected 10 % of S87 and S129 phosphorylation after 24 hours of incubation (**Supp. Fig. 2b**), suggesting that CK1 phosphorylated unmodified α -syn with low efficiency. In stark contrast, we detected virtually complete modification of S129 when we reacted tyrosine-phosphorylated α -syn with 250 U of CK1 (**Fig. 2c**), from which we inferred that modified tyrosines greatly enhanced CK1-mediated modification of S129.

Because CK1 typically requires ‘priming’ via a phosphorylated residue at the -3 position for efficient catalysis,²⁵ we asked whether phosphorylated Y125 (-4 position), or modified Y133 (+3 position) functioned as such a priming site for CK1 phosphorylation of S129? To answer this question, we individually replaced Y133 and Y125 with non-modifiable phenylalanines, leaving the respective other tyrosines intact, and pre-phosphorylated both α -syn mutants with Fyn (**Fig. 3a**). Upon subsequent incubation with CK1 (250 U), we observed efficient S129 phosphorylation of the Y133F mutant, containing the intact phospho-Y125 site, but not of the Y125F mutant with phospho-Y133, but lacking the N-terminal Y125 phospho-

site. These results suggested that Y125 phosphorylation functions as the priming event that enables the efficient modification of S129 by CK1 *in vitro*.

Next, we asked whether Y125 phosphorylation constituted a similarly important priming event for the modification of S129 by cellular CK1? In a first instance, we tested lysates from cultured mammalian A2780, K562, B65, SK-N-SH, SY-SY5Y and RCSN-3 cells for the presence of endogenous CK1, and members of the Plk family of kinases (**Fig. 3b**). Western blotting showed that CK1, Plk2 and partially also Plk3 are abundantly present in these cells. Upon the addition of ¹⁵N isotope-labeled, unmodified α -syn to these lysates, we did not detect S129 phosphorylation by NMR spectroscopy (data not shown). To investigate whether greater amounts of cellular kinases were needed to phosphorylate S129, we transiently over-expressed Plk3 in human A2780 cells (**Supp. Fig. 3**). Upon addition of unmodified α -syn to lysates from these cells, we observed rapid phosphorylation of S129, which confirmed that endogenous levels of CK1 and Plk's were not sufficient to establish S129 phosphorylation on unmodified α -syn under our experimental conditions. Next, we added tyrosine pre-phosphorylated α -syn to native cell lysates and recorded *in situ* NMR experiments on the resulting mixtures (**Fig. 3d and Supp. Fig. 4**). This time, we detected efficient *de novo* phosphorylation of S129 in all lysates, presumably by endogenous CK1. To test this idea, we performed similar reactions in the presence of increasing amounts of a CK1-specific kinase inhibitor. In support of our model, CK1 inhibition progressively diminished S129 phosphorylation in native A2780 cell lysates, which suggested that priming via Y125 phosphorylation was also required for the efficient phosphorylation of S129 by cellular CK1.

In summary, our results reveal that CK1 priming via tyrosine phosphorylation at the -4 position expands the repertoire of classical phospho-serine and -threonine priming sites at -3 positions.²⁵ Whether other examples of CK1 substrates with phospho-tyrosine priming sites exist remains to be determined. In the case of α -syn, phospho-Y125 mediated modification of

S129 by CK1 may be of particular interest because CK1 is constitutively active and abundantly expressed in many different cell types, including those of the brain.^{26, 27} In addition, co-localization of CK1 and α -syn aggregates in proteinaceous brain inclusions of transgenic α -syn mice and in *post mortem* structures of patients with Lewy-body dementia, and PD has been established.²⁴ Thus, transitions to the S129-phosphorylated form of α -syn may also result as a consequence of Y125 phosphorylation, a notion that has not yet received much attention. The presence of four post-translational phosphorylation sites within a narrow stretch of amino acids in the C-terminus of α -syn indicates a hotspot for regulated interactions. Indeed, many of the previously identified α -syn binding partners target this region. These include the dopamine transporter (DAT),²⁸ the GTPase Rab8a,⁵ and many others.⁶ Future investigations will determine how different serine-tyrosine phosphorylation states modulate these interactions.

Methods

NMR spectroscopy: End point and time-resolved NMR spectra of phosphorylation kinetics were recorded on 600 MHz and 750 MHz Bruker Avance spectrometers, equipped with cryogenically cooled triple resonance $^1\text{H}(^{13}\text{C}/^{15}\text{N})$ TCI probes, on α -syn samples dissolved in NMR buffer at pH 7.0. 2D ^1H - ^{15}N SOFAST-HMQC experiments²⁹ were recorded at 25° C. 2D NMR spectra were recorded with 1024 (^1H) and 256 (^{15}N) complex points, 44 scans and a recycling delay of 60 ms (~ 30 minutes acquisition time). Pulse sequences and its parameters were used as described previously.³⁰ To delineate phosphorylation rates, we measured cross-peak intensities and/or signal volumes of unambiguously assigned, well-resolved NMR cross-peaks reporting on the individual phosphorylation states of residues Y39, Y125, Y133, Y136 and S129 in the different time-resolved NMR spectra, as reported previously.³⁰ These were N122 and D121 for Y125 phosphorylation; E139 and A140 for Y136 phosphorylation; Y133 for Y133 phosphorylation;

L38, V40 and G41 for Y39 phosphorylation and S129 for S129 phosphorylation. Phosphorylation curves were fitted by a single exponential increase and plotted using GraphPad Prism 5.0. Error bars in the time-resolved reaction profiles represent the relative experimental noise of the individual NMR signals used to calculate the site-specific phosphorylation states.

In vitro phosphorylation reactions: Tyrosine kinases were purchased from SignalChem unless stated otherwise. CK1- δ and CK2 were purchased from New England Biolabs. Recombinant Fyn kinase was also obtained recombinantly, as a kind gift of Benno Kuroepka, FMP Berlin, and we determined that 10 μ L of this preparation corresponded to 250 U of commercial Fyn (SignalChem). Plk3 was purchased from Millipore. Unless otherwise specified, *in vitro* phosphorylation reactions were carried out in duplicates at 25° C with 250 U (1U = 1 nmol/min/mg) of the respective kinases and 25 μ M of α -syn in NMR buffer at pH 7.0, supplemented with 1 mM ATP (Fermentas) and 5 mM MgCl₂. MnCl₂ or DTT was also added in some reactions according to suppliers' instructions. Final reaction volumes were 150 μ L in all cases.

Transient Plk3 over-expression: Adherent A2780 cells were transfected with either pEGFP-C1 (mock, Clontech) or PLK3 1-344-pEGFP, encoding the kinase domain of human Plk3 fused to GFP (Addgene plasmid 23266),³¹ by using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. 22 hours after transfection, cells were detached and collected by mild trypsin treatment, washed twice in PBS and resuspended in NMR buffer at pH 7.0 containing 1X protease inhibitors (complete EDTA-free, Roche) 1X phosphatase inhibitor cocktails 2 and 3 (Sigma). Cells were centrifuged for 5 min. at 1000 rpm and the supernatant was discarded. The wet cell pellet was lysed as described below.

Phosphorylation reactions in mammalian cell lysates: Cells were grown to 80% confluence, detached and collected by mild trypsin treatment, washed twice with PBS and

resuspended in NMR buffer at pH 7.0 containing 1X protease inhibitors (complete EDTA-free, Roche). Cells were centrifuged for 5 min. at 1000 rpm and the supernatant was discarded. The wet cell pellet was lysed by 5 rounds of freeze-thaw using a dry ice/ethanol bath, after which the soluble fractions were obtained by a 10 min spin at 16,000 g at 4° C. Protein concentrations were determined using a Bradford assay (Bio-Rad). Extracts were used immediately after preparation or aliquoted and snap-frozen in liquid nitrogen and stored at -80° C until further use. Freeze-thaw cycles were avoided in order to preserve enzymatic activities.³⁰ Samples for Western Blotting were boiled for 5 minutes in 1X Laemmli buffer (Bio-Rad). Mammalian cell lines were: A2780 (human, ovarian, Sigma-Aldrich), K562 (human, leukemia, DSMZ), B65 (rat, neuroblastoma, Sigma Aldrich), SH-SY5Y (human, neuroblastoma, provided by Jan Bieschke, MDC-Berlin), RCSN-3 (rat, *substantia nigra*, Pablo Caviedes, University of Chile), SK-N-SH (human, neuroblastoma, Sigma-Aldrich). For phosphorylation reactions, extracts were adjusted to the desired total protein concentration in NMR buffer, supplemented with phosphatase inhibitor cocktails 2 and 3 (Sigma), 1 mM ATP and 5 mM MgCl₂ (final reaction volume, 150 μL). Reactions were carried out in duplicates at 25° C by adding 25 μM of isotope-labeled α-syn directly into the different cell lysates and immediately starting the NMR experiments. For the CK1 inhibition experiment, we added the indicated amounts of XX CK1 inhibitor (company) to A2780 cell lysates.

Supporting Information

Experimental details for NMR resonance assignments of tyrosine phosphorylated α-syn and immunoblotting. NMR spectra of unmodified and serine-tyrosine phosphorylated α-syn, phosphorylation of S129 by CK1 and CK2, *de novo* S129 phosphorylation in lysates of A2780 cells transiently over-expressing Plk3 or supplemented with a CK1 inhibitor. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Author Information

Address correspondence to: selenko@fmp-berlin.de.

[¶]Present address: Department of Biochemistry, University of Potsdam, 14476 Golm, Germany.

Author contributions

JK, AB and PS conceived and executed experiments, analyzed results and wrote the manuscript. MS, SV, F-XT, BB and MvR contributed reagents and experiments.

[‡]These authors contributed equally.

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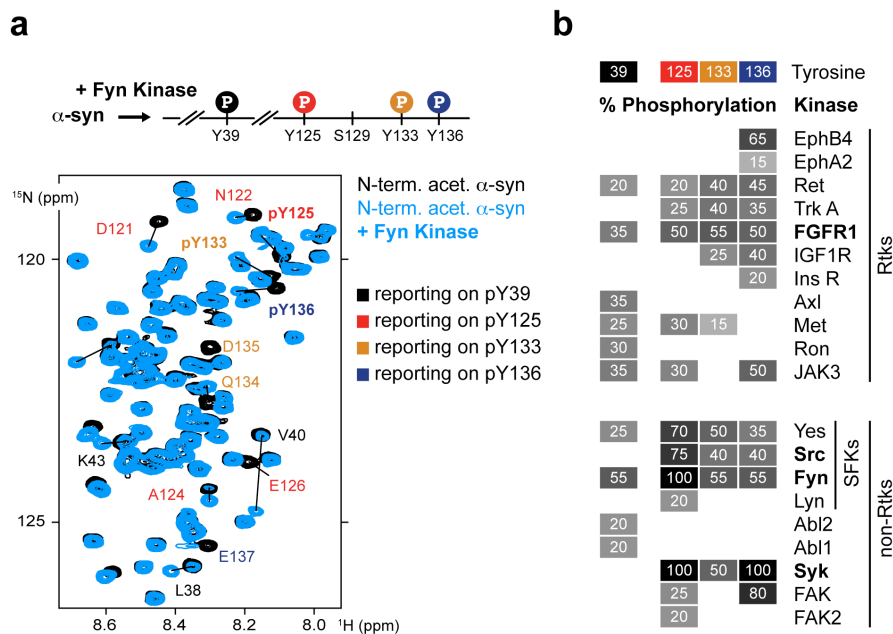


Figure 1. *In vitro* phosphorylation of α -syn tyrosines (a) Schematic representation of α -syn phosphorylation by FynK (top) and selected view of the 2D ^1H - ^{15}N HSCQ spectra of N-terminally acetylated, but otherwise unmodified α -syn (black) and of tyrosine-phosphorylated α -syn (blue) (bottom). Labels indicate the chemical shift changes characteristic for the individual tyrosine phosphorylation sites. (b) Phosphorylation of α -syn by different RTKs and non-RTKs (250 U). Percentages of site-specific phosphorylation levels were quantified based on NMR cross-peak intensities after ~12 hours of kinase incubation. Kinases are ordered according to their evolutionary distances. Experiments were performed on 25 μM α -syn in NMR buffer at 25 $^\circ\text{C}$ and pH 7.0.

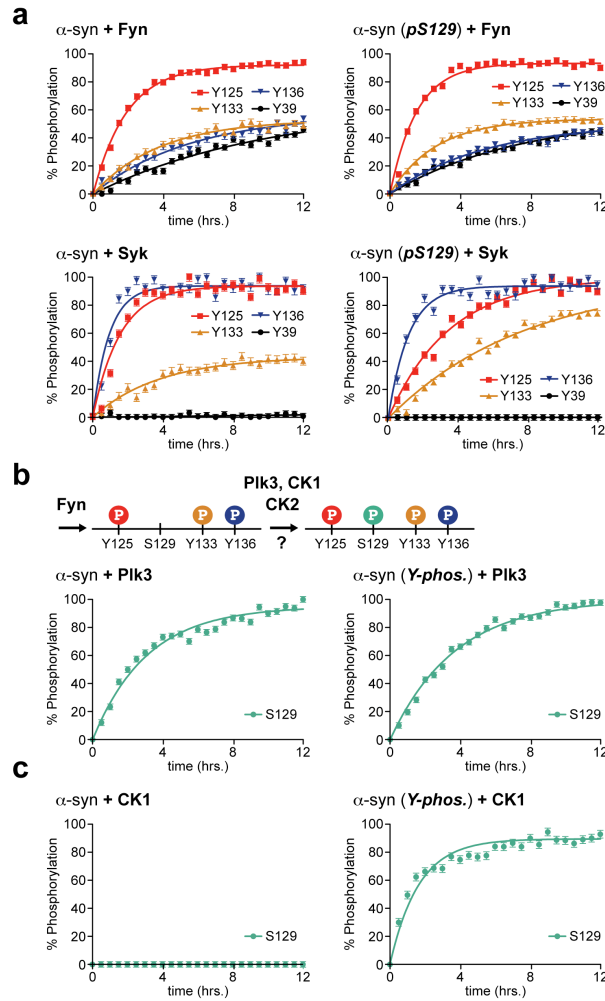


Figure 2. α -syn serine-tyrosine phosphorylation crosstalk (a) Time-resolved modification rates of individual α -syn tyrosines in reconstituted phosphorylation reactions with 250 U of Fyn (top) and Syk (bottom). Unmodified (left) or S129-phosphorylated α -syn (right) serves as the substrate. (b and c) Time-resolved S129 phosphorylation rates of unmodified (left), or tyrosine-phosphorylated α -syn (right), with Plk3 and CK1 (250 U). Experiments were performed on 25 μ M α -syn samples in NMR buffer at 25 $^{\circ}$ C and pH 7.0.

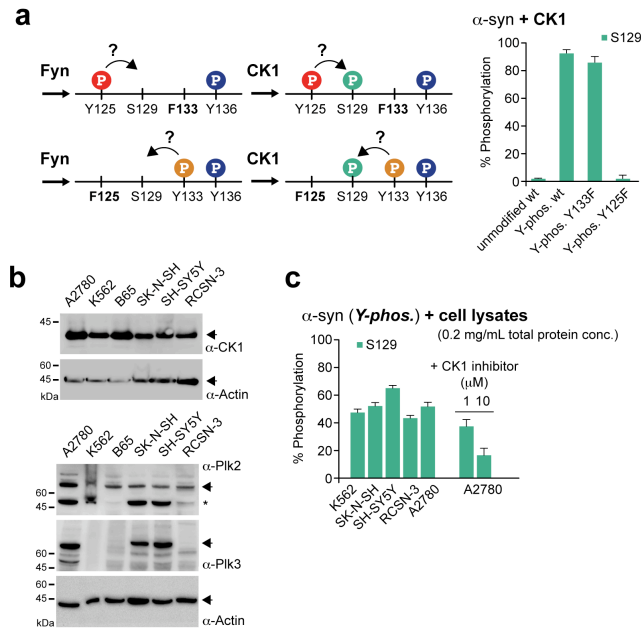


Figure 3. α -syn S129 phosphorylation by cellular CK1 (a) α -syn Y125F, and Y133F mutants confirm the CK1 priming effect via Y125 phosphorylation. CK1-mediated S129 phosphorylation levels of wild-type (wt) and mutant α -syn. (b) Western blots of endogenous CK1 and Plk2, Plk3 in lysates of cultured mammalian A2780, K562, B65, SK-N-SH, SY-SY5Y and RCSN-3 cells (50 μ g of total protein per lane). Actin serves as the loading control. (c) *De novo* S129 phosphorylation of tyrosine-phosphorylated α -syn after incubation in cell lysates with phosphatase inhibitors for 15 hours, including in A2780 lysates with increasing amounts of a CK1 inhibitor (*far right*). Experiments were performed on 25 μ M α -syn samples in NMR buffer at 25 $^{\circ}$ C and pH 7.0.

Supplementary information for:

**Efficient modification of α -synuclein Ser129 by Casein
kinase 1 requires phosphorylation of Tyr125 as a priming
event**

Jonas Kosten^{§,‡}, Andres Binolfi^{§,‡}, Marchel Stuiver[§], Silvia Verzini^{§,¶}, Francois-Xavier
Theillet[§], Beata Bekei[§], Marleen van Rossum[§], and Philipp Selenko^{§,*}

[§]In-Cell NMR Laboratory, Department of NMR-supported Structural Biology, Leibniz Institute of
Molecular Pharmacology (FMP Berlin), Robert-Rössle Strasse 10, 13125 Berlin, Germany

[¶]Present address: Department of Biochemistry, University of Potsdam, 14476 Golm, Germany.

[‡]These authors contributed equally.

*To whom correspondence should be addressed: selenko@fmp-berlin.de

Supplementary methods

Protein expression and purification. Details ^{15}N and ^{15}N - ^{13}C isotopically enriched N-terminally acetylated α -syn as obtained by co-expressing plasmids encoding wild-type α -syn (pT-T7) and the yeast NatB acetylase complex¹ in *E. Coli* BL21 (DE3) Express. Cell cultures were induced at OD₆₀₀ 0.6 with 1 mM IPTG (Roth) and grown overnight at 30° C. Recombinant proteins were purified as previously described.^{2,3} Purity of protein samples was determined by SDS-PAGE. N-terminal acetylation was complete in all samples as determined by ^1H - ^{15}N hetero-nuclear NMR spectroscopy.³ 4,4-Dimethyl-4-silapentane-1-sulfonic acid (DSS), ^{15}N NH_4Cl and U- ^{13}C glucose were purchased from Sigma. Protein samples were used in 20 mM phosphate buffer, 150 mM NaCl and 10% D₂O (i.e. NMR buffer). Protein concentrations were determined spectrophotometrically by absorption measurements at 274 nm, with 5600 M⁻¹cm⁻¹ as the extinction coefficient (ϵ). Changes in molar absorption coefficients due to tyrosine phosphorylation were taken into account when quantifying the differently phosphorylated species.⁴ α -syn Y125F and Y133F mutants were generated by site-directed mutagenesis with the following primer pairs:

Y125F: Fw, 5'ggatcctgacaatgaggcctttgaaatgccttctgaggaagg3',

Rev: 5'cccttcctcagaaggcatttcaaaagcctcattgtcaggatcc3';

Y133F: Fw, 5'gccttctgaggaagggttcaagactatgaacctgaagcc3',

Rev: 5'ggcttcagggtcatagtcttgaacccttctcagaaggc3'

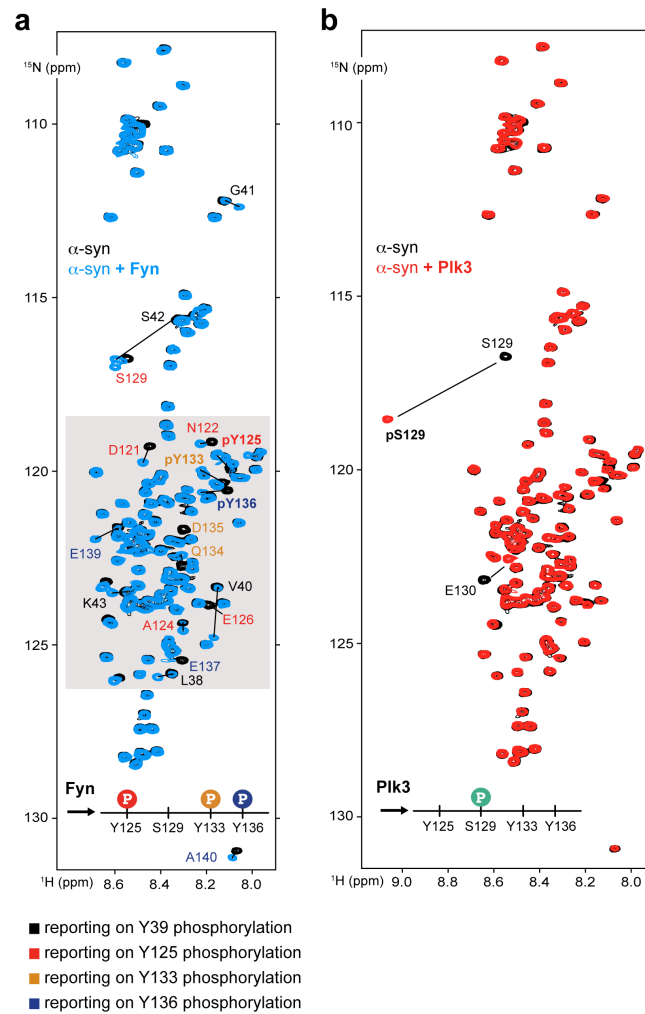
NMR resonance assignment of Y phosphorylated α -syn species. For the assignment of tyrosine phosphorylated N-terminally acetylated α -syn, 2x 300 μL of 150 μM ^{15}N , ^{13}C labeled protein were incubated twice with 625 enzymatic units (U) of Fyn kinase for 24 hours (NMR buffer, pH 7.5), pooled, adjusted to pH 6.4, and concentrated to 300 μL and 300 μM . Under

this large excess of added kinase we achieved full phosphorylation of all four tyrosines in α -syn. ^1H - ^{15}N HSQC experiments and triple resonance HNCACB (Bruker standard pulse sequences) were recorded at 15° C on a 900 MHz Bruker Avance spectrometer, equipped with a cryogenically cooled triple resonance ^1H ($^{13}\text{C}/^{15}\text{N}$) TCI probe. Parameters used were 1024 (^1H) and 256 (^{15}N) complex points and 16 scans for 2D ^1H - ^{15}N HSQC experiments, and 1024 (^1H), 72 (^{15}N) and 96 (^{13}C) complex points and 16 scans for 3D HNCACB experiments. NMR spectra were zero-filled to 4-times the number of real points and processed using a sine window function and baseline correction for all dimensions. Backbone assignments were performed with CARA (Computer-Aided Resonance Assignment, Institute for Molecular Biology and Biophysics, Zuerich Switzerland) starting from a previous assignment of unphosphorylated, N-terminally acetylated α -syn.⁵ We also relied on the use of the tyrosine point mutants, Y125F and Y133F to obtain *de novo* assignments for the different phosphorylated protein species. Acquisition, processing and visualization of NMR spectra were performed with TOPSPIN 3.1 (Bruker), SPARKY⁶ and iNMR.

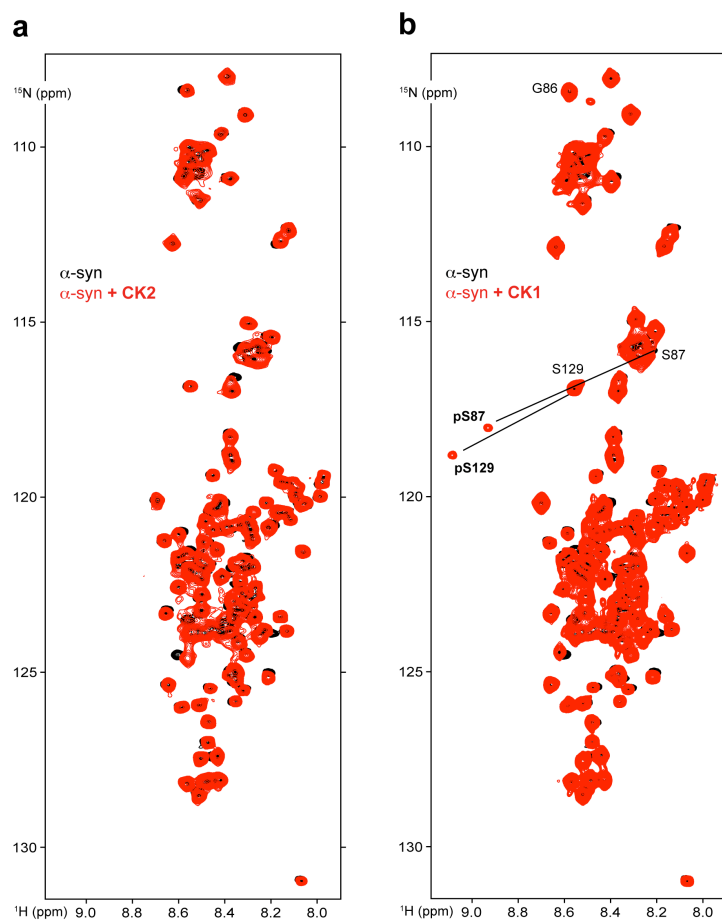
Immunoblotting. Samples were separated by SDS-PAGE using 4-20% gradient gels (Bio-Rad) and transferred onto PVDF membranes. Blots were blocked in 5% milk or 5% BSA in TBS-T for 1 hour at room temperature, before incubating them overnight at 4° C with the following primary antibodies: anti-GFP (1:1500, Abcam, ab290), anti-CK1 (1:500, NEB, 2655S), anti-Plk2 (1:500, Santa Cruz, sc-25421), anti-Plk3 (1:400, BD Pharmingen, 556518) or anti- β -Actin (1:5000, Abcam, ab6276). Membranes were incubated with HRP secondary antibodies for 1 hr. at room temperature, after which they were developed using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific). Luminescence was detected using a Bio-Rad Molecular Imager and ImageLab software.

Supplementary references

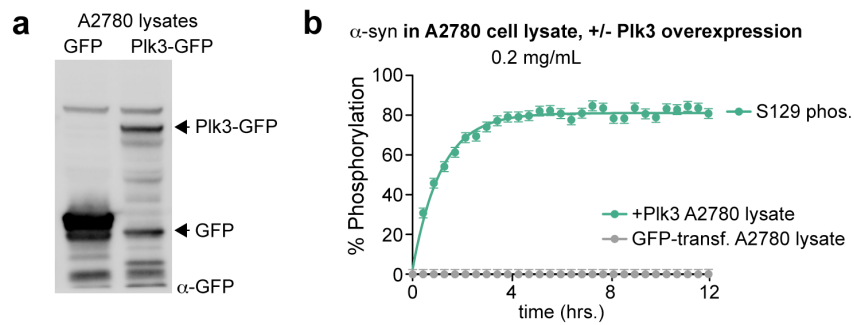
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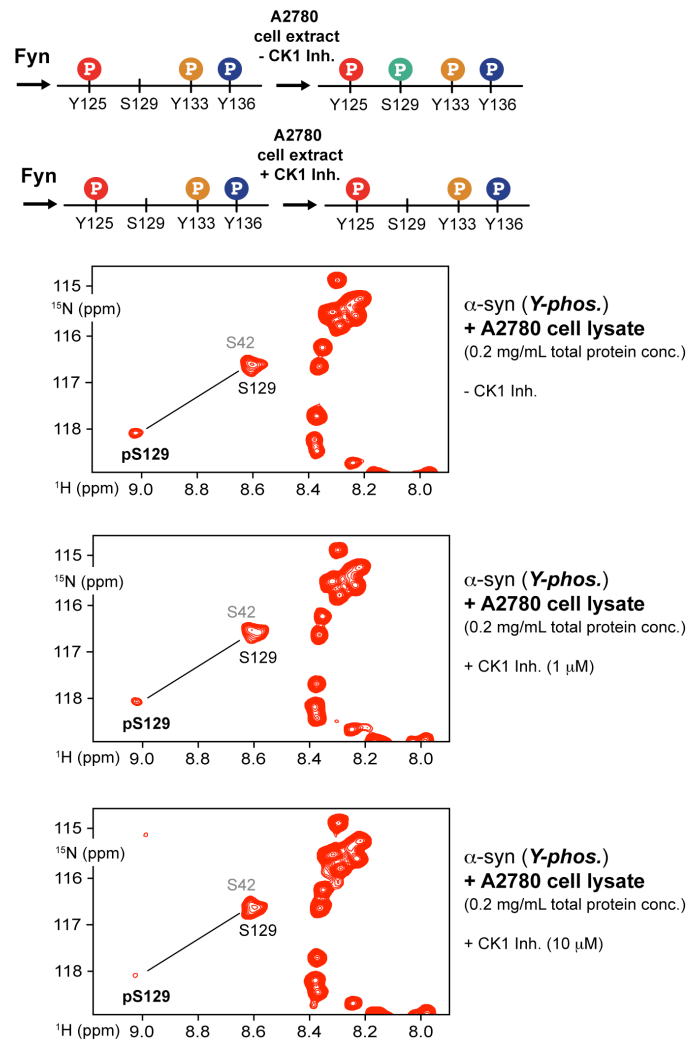
Supp. figure 1. NMR characteristics of α -syn serine and tyrosine phosphorylation. (a) 2D ^1H - ^{15}N HSCQ spectra of N-terminally acetylated, but otherwise unmodified α -syn (black) and tyrosine-phosphorylated α -syn (with 250 U recombinant Fyn, blue). Phosphorylation leads to characteristic chemical shift changes of the modified tyrosine residues. Additional resonance displacements are indicated. The gray shading depicts the region shown in figure 1b in the main text. (b) 2D ^1H - ^{15}N HSCQ spectra of N-terminally acetylated, but otherwise unmodified α -syn (black) and of S129-phosphorylated α -syn (with 250 U of recombinant Plk3, red). Phosphorylation leads to characteristic chemical shift changes of the modified serine. Additional resonance displacements are indicated. Experiments were performed on 25 mM α -syn samples in NMR buffer at 25°C and pH 7.0.



Supp. figure 2. *In vitro* phosphorylation of α -syn with CK1 and CK2. 2D ^1H - ^{15}N HSCQ spectra of N-terminally acetylated α -syn before (black) and after (red) incubation with 2000 units of CK2 (a) and CK1 (b) for 24 hs. Phosphorylation by CK1 leads to characteristic chemical shift changes of the modified serine 87 and 129. Additional resonance displacements are indicated. Integration of G86 and S129 signals in their free and phosphorylated protein species showed that the amounts of S87 and S129 was approx. 10%. Experiments were performed on 25 μM α -syn samples in NMR buffer at 25°C and pH 7.0.



Supp. figure 3. *De novo phosphorylation of S129 in cell extract.* (a) Western blots of transiently transfected A2780 cells over-expressing GFP (mock) and Plk3-GFP (50 mg of total protein per lane). (b) α -syn S129 phosphorylation in A2780 cell lysates containing transiently over-expressed Plk3 in the absence. Experiments were performed on 25 μ M α -syn samples in NMR buffer at 25°C and pH 7.0.



Supp. figure 4. Phosphorylation of S129 residue of α -syn by endogenous CK1. Serine region of the 2D ^1H - ^{15}N HSCQ spectra of N-terminally acetylated, Y-phosphorylated α -syn incubated with an A2780 mammalian cell extract for 12 hs in the absence of the CK1 inhibitor (top spectrum) and in the presence of 1 μM (medium spectrum) and 10 μM CK1 (bottom spectrum) inhibitor. Experiments were performed on 25 μM α -syn samples in NMR buffer at 25°C and pH 7.0.