Supplemental information for

Phosphorylation Dependent α-Synuclein Degradation Monitored by In-cell NMR

Wenwen Zheng\textsuperscript{a,b}, Zeting Zhang\textsuperscript{a}, Yansheng Ye\textsuperscript{a}, Qiong Wu\textsuperscript{a}, Maili Liu\textsuperscript{a} and Conggang Li\textsuperscript{a}

\textsuperscript{a} Key Laboratory of Magnetic Resonance in Biological Systems, State Key Laboratory of Magnetic Resonance and Atomic and Molecular Physics, National Center for Magnetic Resonance in Wuhan, Wuhan Institute of Physics and Mathematics, Chinese Academy of Sciences, Wuhan 430071, China
\textsuperscript{b} Graduate University of Chinese Academy of Sciences, Beijing 100049, China
\textsuperscript{†} These authors contribute equally to this work.

Corresponding Email Address: conggangli@wipm.ac.cn

Materials and Methods:
\textit{α-synuclein expression, purification, and phosphorylation.} α-Synuclein (α-syn) S129E, α-syn Y125E;Y133E mutants were constructed using site-directed mutagenesis employing complementary internal mutagenic primers and overlap extension PCR. α-syn and its variants were expressed and purified as described.\textsuperscript{1,2} Purity was assessed by SDS-PAGE. Purified proteins were lyophilized and stored at -80°C. Ser-129 phosphorylation was achieved by incubating α-syn and PLK3 (Life Technologies) in buffer containing 20 mM HEPES (pH7.4), 10 mM MgCl\textsubscript{2}, 1.0 mM adenosine triphosphate (ATP), and 2 mM dithiothreitol (DTT) for 10 h at 30°C. Phosphorylated protein was purified using a MonoQ column.

**Preparing Xenopus laevis oocytes and SK-N-SH cells for in-cell NMR.**
Oocytes were prepared as described.\textsuperscript{3} Briefly, ovary lobes were surgically removed to ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM MgCl\textsubscript{2}, 5 mM HEPES, pH 7.4). After washing, the lobes were digested with collagenase (2 mg/mL final concentration). Stage-VI oocytes were selected for microinjection. Protein (about 20 nL of a 5 mM solution) was injected via an IM-300 microinjector (Narishige Co. Ltd., Tokyo, Japan). About 150 oocytes in ND96 buffer containing 10% D\textsubscript{2}O were transferred to a Shigemi NMR tube.

Dephosphorylation and degradation of pS129 α-syn took place in the cells at the same time. So we monitor the dephosphorylation and degradation intervally using one sample, namely, acquiring \textsuperscript{15}N-\textsuperscript{1}H HSQC spectrum to monitor dephosphorylation firstly for 1h 20min, then turning to \textsuperscript{19}F spectrum to monitor degradation for 40min, and repeated the process for 5 times, the total experimental time is 10 hours. The supernatant was then assessed for leakage.

SK-N-SH cells were grown in complete medium, DMEM/high glucose (HyClone), supplemented with 10% (v/v) fetal bovine serum. Cells were harvested when cultures reached 80% confluence. For creating PD cell model, cells were treated with 0.5 mM 1-Methyl-4-phenylpyridinium iodide (MPP\textsuperscript{+}) (Sigma) for 24 h. Approximately 4-5\times10\textsuperscript{7} cells were harvested by using 0.25% trypsin/EDTA to detach cells from culture flasks and centrifuged at 200g for 10 mins at 25 °C.
Cell pellets were washed with 50 mL of phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄). Cell densities were quantified with a hemocytometer. Cells were mixed with protein (800 µM) in freshly prepared electroporation buffer (100 mM sodium phosphate, 5 mM KCl, 15 mM MgCl₂, 15 mM HEPES, 5 mM ATP, 5 mM reduced glutathione, pH 7.0) at 4x10⁶ cells/mL. The mixture (4x10⁶ cells in 100 µL) was transferred into electroporation cuvettes and electroporated into cells using an Amaxa Nucleofector 4D device (Lonza, CA-137 pulse program). Cells were pulsed three times with gentle mixing between pulses. After electroporation, cells were transferred to prepared cell culture dishes with 15 mL medium for recovery at 37 ºC, in 5% CO₂ for 5 h. Recovered cells were washed three times with PBS and harvested by trypsinization. After centrifugation at 200g for 10 min at 25 ºC, cells were re-suspended in 300 µL NMR buffer (90 mM glucose, 5 mM HEPES in DMEM with 10% D₂O) for NMR analysis. The protocol is similar to one previously described. Each in-cell NMR experiment required about 3 h of instrument time. The supernatant was then assessed for leakage.

**Cell lysates.** Oocytes were mechanically crushed on ice using a plunger and centrifuged at 15000g for 20 min at 4 ºC. SK-N-SH cells were harvested and washed twice with PBS. Cell pellets were sonicated on ice and centrifuged at 16000g for 10 min at 4 ºC. Lysates were frozen in N₂ (l) and lyophilized. Cell lysates (at a concentration of 10 g/L) containing 50 µM protein in total a volume of 250 µL were used for NMR analysis. To reveal the phosphorylation effect on α-synuclein degradation, a serine phosphatase inhibitor okadaic acid (OA, Sigma-Aldrich) dissolved in DMSO was added to lysates to slow down dephosphorylation of pS129-α-synuclein, with a final concentration of 1 µM. An equal volume (2.5 µl) DMSO was added to lysates as a control.

**Flow cytometry.** After in-cell NMR experiment, SK-N-SH cells were re-suspended in PBS supplemented with 10 µg/mL propidium iodide (Beyotime) to stain apoptotic and dead cells. After 30 min, cells were analyzed with a BD FACSMelody flow cytometer (BD Biosciences). Propidium iodide was excited at 488 nm. Emission was detected at 695/20 nm.

**Western-blotting.** α-syn (50 µM) was incubated with 0, 4, or 16 mg/mL of oocyte lysates for 12 h at 22 ºC. Samples were applied to 15% SDS gels and transferred to PVDF membranes, blocked with 5% (wt/wt) skim milk solution for 1.5 h and incubated with the α-syn primary antibody (SNCA Rabbit PolyAb, Proteintech; pS129 antibody, Abcam) for 1 h at 37 ºC. The membrane was washed with TBST buffer (150 mM NaCl, 20 mM Tris, 0.05% v/v Tween 20, pH 8.0), incubated with anti-rabbit IgG peroxidase secondary antibody (Biodragon), washed again with TBST buffer. Color was developed using the Enhanced HRP-DAB Chromogenic Substrate Kit (Tiangen).

**NMR.** Experiments were performed at 295.2 K on a 600 MHz spectrometer (Bruker) equipped with a H/F/(C, N) triple-resonance cryogenic probe, except for the ¹⁵N-¹H HSQC spectra in SK-N-SH cells, which were acquired on a 850 MHz spectrometer (Bruker) equipped with a H/C/N triple resonance cryogenic probe. The spectral width was 8417.51 Hz and 2188.27 Hz for ¹H and ¹⁵N, respectively. 128- and 2048- data points were acquired in t1 and t2, respectively. The number of acquisition was 32 for oocytes and 64 for SK-N-SH cells. ¹⁹F spectra were acquired on the 600 MHz spectrometer with a sweep width 11.31 kHz, a relaxation delay 2 s and an acquisition time 0.36 s, chemical shifts were referenced to trifluorotoluene at -63.72 ppm. The number of
acquisition was 1024 for oocytes and 4096 for SK-N-SH cells. Pulse field gradient diffusion experiments were performed on the 600 MHz spectrometer at 295.2K. The pulse program led to 2s with a sweep width 5.65 kHz, a relaxation delay 3.60 s and an acquisition time 1.45 s. Gradient strengths ranged from 1.0 to 48.8 G/cm. The diffusion coefficient is fitted as below:

\[ I = I_0 e^{-D \gamma ^2 g^2 \delta^2 (\lambda - \frac{\Delta}{3})} \]

Where \( I \) is the observed intensity, \( I_0 \) is the reference intensity (Signal without gradient field), \( D \) is the diffusion coefficient, \( \gamma \) is the gyro-magnetic ratio of the observed nucleus, \( g \) is the Gradient amplitude, \( \delta \) is the length of the gradient, \( \Delta \) is the diffusion time.

**Fluorescence Imaging.** The V3C α-syn variant was labeled using Alexa as described. \(^5\) Briefly, tris (2-carboxyethyl) phosphate and NaHCO\(_3\) were added in a 10-fold molar excess over 2 g/L protein, with shaking for 30 min at 25 °C. A 10-fold molar excess of Alexa Fluor 488 C5-maleimide (Invitrogen) was then added and incubated at 25 °C for 2 h. The modified protein was purified by gel filtration on a Superdex 100 column with 20% acetonitrile in PBS as an eluent. Purified protein was dialyzed against H\(_2\)O, lyophilized and stored at -80 °C. The labeled protein was microinjected into oocytes or electroporated into SK-N-SH cells. About 10 minutes after microinjection, oocytes began to be fixed with 4% (v/v) aqueous paraformaldehyde and dehydrated by using a 30% (wt/wt) sucrose solution. Frozen oocytes were sliced into 60 μm sections using a Thermo Fisher CryoSTAR NX50 microtome. Sections were imaged using the green channel of an Olympus IX73 microscope. For SK-N-SH cells, recovered electroporated cells were fixed in PBS containing 4 % (v/v) paraformaldehyde for 15 min. After washing with PBS, nuclei were stained with 150 μM Hoechst33342. Confocal images were acquired with a 25x water objective using an excitation wavelength of 405 nm and an emission wavelength of 488 nm on a Leica TCS SP8 laser-scanning microscope.

**Data Analysis.** NMR spectra were analyzed using Topspin 3.2. 8.0, 0.3 and 0.3-Hz exponential line-broadening for \(^{19}\)F, \(^1\)H and \(^{15}\)N were applied to free-induction decay before Fourier transformation, respectively. The degradation part was integrated from -135.1 to -135.4 ppm and from -135.9 to 136.2 ppm after baseline correction in the \(^{19}\)F spectra. The fraction of degradation is calculated using Eq S1:

\[
\text{Fraction degraded} = \frac{\text{Peaks area}_{(-135.1 \text{ to } -135.4 \text{ ppm})} + \text{Peaks area}_{(-135.9 \text{ to } -136.2 \text{ ppm})}}{\text{Peaks area}_{(-135.1 \text{ to } -136.2 \text{ ppm})}}
\]

The phosphorylated ser-129 peak was integrated from 8.74 to 8.95 ppm for \(^1\)H, from 117.6 to 119.1 ppm for \(^{15}\)N. The non-phosphorylated ser-129 peak was integrated from 8.23 to 8.44 ppm for \(^1\)H, from 116.0 to 117.5 ppm for \(^{15}\)N in the 2D \(^1\)H-\(^{15}\)N HSQC spectra. The fraction of phosphorylation is calculated using Eq S2:

\[
\text{Fraction phosphorylated} = \frac{\text{Peaks area}_{[\text{H}^1(8.74 \text{ to } 8.95 \text{ ppm}), \text{N}^{15}(117.6 \text{ to } 119.1 \text{ ppm})]}}{\text{Peaks area}_{[\text{H}^1(8.74 \text{ to } 8.95 \text{ ppm}), \text{N}^{15}(117.6 \text{ to } 119.1 \text{ ppm})]} + \text{Peaks area}_{[\text{H}^1(8.23 \text{ to } 8.44 \text{ ppm}), \text{N}^{15}(116.0 \text{ to } 117.5 \text{ ppm})]}}
\]
Figure S1. The $^1$H-$^{15}$N HSQC and $^{19}$F spectra of α-syn before and after S129 phosphorylation (a) Overlaid $^1$H-$^{15}$N HSQC spectra of α-syn (green) and pS129 α-syn (red) in buffer. (b) Overlaid $^{19}$F spectra of α-syn (green) and pS129 α-syn (red) in buffer.

Figure S2. Single-site $^{19}$F labeled α-syn were obtained by changing three tyrosine residues to phenylalanine each time, and only one tyrosine residue was labeled. $^{19}$F NMR spectra of wild type and single-site $^{19}$F labeled α-syn in buffer (a) and in cell lysates (b).
Figure S3. Quantification of \( \alpha \)-syn degradation using \( ^{19} \text{F} \) spectra and Western-blot analysis. (a) \( ^{19} \text{F} \) spectra of \( \alpha \)-syn degradation in oocyte lysates (calculated using Eq S1). (b) Western-blot of \( \alpha \)-syn degradation in oocyte lysates. (c) Quantification of \( \alpha \)-syn degradation using \( ^{19} \text{F} \) spectra and Western-blot, respectively.

Figure S4. The diffusion coefficients were measured by using \( ^{19} \text{F} \) NMR. (a) \( ^{19} \text{F} \) spectrum of \( \alpha \)-syn in lysates is divided into three parts, part 1 (from -135.1 to -135.4 ppm), part 2 (from -135.4 to -135.95 ppm) and part 3 (from -135.95 to -136.2 ppm). The diffusion coefficients of part 1 (b, \( 5.4 \times 10^{-10} \pm 4.1 \times 10^{-12} \text{ m}^2/\text{s} \)), part 2 (c, \( 7.3 \times 10^{-11} \pm 8.8 \times 10^{-13} \text{ m}^2/\text{s} \)) and part 3 (d, \( 8.8 \times 10^{-11} \pm 2.5 \times 10^{-12} \text{ m}^2/\text{s} \)) were shown, respectively.
Figure S5 Fluorescence image of injected phosphorylated (a), unphosphorylated (b) and phosphorylation mimetic (S129E, c) α-syn in oocytes.

Figure S6 Dephosphorylation and degradation of $^{15}$N-3FY pS129 α-syn in oocytes. (a) Overlaid $^1$H-$^{15}$N HSQC spectra of pS129 α-syn in buffer (red) and oocytes (blue). (b) $^{19}$F spectra of pS129 α-syn in buffer (top), oocyte lysates (middle) and oocytes (bottom) as a function of time.

Figure S7. Dephosphorylation and degradation of pS129 α-syn in oocyte lysates. (a) Dephosphorylation of pS129 α-syn in 10 g/L oocyte lysates, (b) degradation of pS129 α-syn (red) and α-syn (black) in 10 g/L oocyte lysates.
Figure S8. Phosphatase inhibitor Okadaic acid effects on the dephosphorylation of pS129, as well as the degradation of α-syn in oocyte lysates. (a) Time-resolved dephosphorylation of pS129 α-syn in the presence of Okadaic acid & DMSO (dimethyl sulfoxide) (black) and pS129 α-syn with DMSO (red, control). (b) Degradation of α-syn with DMSO & Okadaic acid (green) and α-syn with DMSO (blue, control) as a function of time. (c) Degradation of α-syn with Okadaic acid & DMSO (green) and pS129 α-syn with Okadaic acid and DMSO (black) as a function of time.

Figure S9 Dephosphorylation and degradation of Y125E;Y133E pS129 α-syn in oocytes. (a) $^{15}$N-$^1$H HSQC spectra of pS129 α-syn Y125E;Y133E in buffer (red) and oocytes after 9 h (blue). (b) Expanded region of spectra over time. (c) $^{19}$F spectra of pS129 α-syn Y125E;Y133E (left) and α-syn Y125E;Y133E (right) as a function of time in oocytes.
Figure S10. 1D $^1\text{H}-^{15}\text{N}$ HSQC spectra of pS129 α-syn in oocytes (a), SK-N-SH cells (b) (blue) and supernatant (red). Lack of resonances in spectra from the supernatants confirm that α-syn did not leak from the cells. (c) SK-N-SH viability quantified using flow cytometry, propidium iodide (PI) negative (left, 95.5%) and positive (right, 4.5%) Count: relative cell number. More than 95% of the cells are alive after the in-cell experiment.

Figure S11. Overlaid $^{15}\text{N}-^1\text{H}$ HSQC spectra of pS129 α-syn in buffer (red) and in SK-N-SH cells (blue).
Figure S12. Dephosphorylation and degradation of pS129 α-syn in SK-N-SH cell lysates. (a) Dephosphorylation of pS129 α-syn in 10 g/L SK-N-SH cell lysates. (b) Degradation of pS129 α-syn (red) and α-syn (black) in 10 g/L SK-N-SH cell lysates.

Supplement References: