c-Abl phosphorylates α-syn and regulates its degradation, implication for α-syn clearance and contribution to the pathogenesis of Parkinson's Disease

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Supplementary Figure Legends

Figure S1 Validation of α-syn Y39 phosphorylation site-specific antibody (pY39)

(A) Antibody specificity is demonstrated using peptide dot blot. 2µg of recombinant α-syn full length and α-syn peptides with various modifications were spiked on a dot blot and probed with the purified polyclonal anti-pY39 antibody raised in rabbits. The antibody only binds to α-syn peptide that is phosphorylated at residue Y39. Phosphorylation at other sites failed to show any detectable immunoreactivity.

(B) Antibody specificity is demonstrated using a blocking peptide on dot blot. 2µg of recombinant α-syn full length or α-syn phosphorylated on Y39 residue (pY39 α-syn) were spiked on a dot blot and probed with the purified polyclonal anti-pY39 α-syn antibody raised in rabbit pre-incubated with a blocking peptide (Ac-α-syn(33-45)-NH_2 pY39) or with a control peptide (Ac-α-syn(33-45)-NH_2). The specific blocking peptide abolished the ability of the anti-pY39 antibody to detect pY39-α-syn

(C) Antibody specificity was also investigated using WB analysis. HEK293T cells were transfected with WT α-syn or its mutants (Y39F, Y125F or Y39FY125F) together with plasmids encoding for Luciferase (LUC; negative control) or WT c-Abl or KD c-Abl (Kinase Dead mutant) or PP c-Abl (constitutively active form). Twenty-four hours post-transfection, cells were lysed and the proteins separated using SDS-PAGE and detected by WB analysis. The anti-pY39 α-syn antibody was able to detect at the expected size (15kDa) α-syn phosphorylation at residue Y39 only when WT c-Abl or PP c-Abl were overexpressed. It is noteworthy, that non-specific bands appear at higher molecular weight only when WT or PP c-Abl is overexpressed.

(D) Immunoblot analysis for pY39 α-syn in non tg and α-syn tg mice with the anti-pY39 α-syn antibody prior and after absorption with the specific pY39 α-syn blocking peptide demonstrating that the bands at 14 kDa are no longer detected. Total α-syn was demonstrated with the monoclonal antibody from BD biosciences. Actin is the loading reference control.

(E) Immunostaining of fronto-temporal cortex sections with the anti-pY39 α-syn antibody showing neuronal staining and immunoreactivity in α-syn tg mice but not in α-syn KO mice. Bar = 10 µm.

(F) Immunostaining with the anti-pY39 α-syn antibody in α-syn tg mice before and after absorption with the specific pY39 α-syn blocking peptide. Pre-incubation with the peptide completely abolishes the immunostaining of neurons. Bar = 10 µm.

(G) Specificity of the anti-pY39 α-syn antibody in primary cultures of hippocampal neurons. ICC analysis using the anti-pY39 antibody (1:250 dilution) shows a weak fluorescent signal corresponding to pY39 in WT neurons (upper panel), at an imaging threshold showing little signal in α-syn KO neurons (lower panel). Higher magnification images (insets) show that pY39 α-syn exhibits mostly cytosolic distribution in WT neurons. Hoechst 33342 was used to counterstain nuclei.

(H) Non-tg and α-syn tg mice immunoblots with anti-c-Abl before and after absorption with the c-Abl protein at 20 fold excess. Pre-incubation with the protein completely abolishes the detection of c-Abl by WB.

(I) Immunostaining with c-Abl antibody in α-syn tg mice before and after absorption with specific cAb1 protein. Pre-incubation with the protein completely abolishes the immunostaining of neurons. Bar = 10 µm.

Figure S2 *In vitro* kinase assays of α-syn phosphorylation by c-Abl

(A) Quantification of *in vitro* kinase assays comparing the phosphorylation of full-length WT α-Syn (gray bars) to recombinant full-length CrkII (black bars) by SH2-CD c-Abl, at different substrate concentrations. Phosphorylation was measured by scintillation counting of the incorporated γ-^{32}P-ATP as described in the methods section. Note that CrkII concentrations higher than 200 µM could not be tested due to limitations in the protein's solubility under the assay conditions.

(B) Expanded ^{1}H/^{15}N HSQC NMR spectrum of WT α-syn in the presence of c-Abl at t=0 (black peaks) and after t=5-6 hours of phosphorylation (red peaks) and performed at pH 7.4. The two framed peaks at the bottom of the spectrum correspond to Leu38 in the unphosphorylated protein (black peak labelled '#') and in the Y39-phosphorylated form (red peak labelled '*').

Figure S3 c-Abl induces α-syn phosphorylation on Y39 and Y125 residues in primary cultures of cortical neurons

(A) Brain homogenates from non-transgenic and mThy1-h α-syn WT transgenic (tg) mice from line 61 (48) (male, 6 m/o) were fractioned into membrane and cytosolic fractions and ran in SDS-PAGE gels, blotted and probed with antibodies against pY125, pY39, α-syn, c-Abl and actin as previously described (53). No positive signal for pY125 and pY39 α-syn was detected in the cytosolic fraction. Bands in the far right are recombinant proteins that served as positive controls and molecular weight markers.

(B) WT (top panels) or α-syn-KO (bottom panels) primary cortical neurons were infected with KD c-Abl lentivirus. Five days post-infection, neurons were immunostained with the appropriate antibodies and counterstained with DAPI to reveal the nucleus.

Figure S4 c-Abl induces α-syn phosphorylation on Y39 and Y125 residues in HeLa and M17 cells

(A) HeLa cells were transfected with WT α-syn or its mutants (Y39F, Y125F or Y39FY125F) together with plasmids coding for Luciferase (LUC; negative control) or WT c-Abl. Twenty-four hours post-transfection, cells were lysed and proteins separated by SDS-PAGE. α-syn phosphorylation status was assessed using phosphorylation site-specific antibody for Y39 or Y125 residues. α-syn and c-Abl expression were confirmed in an additional protein blot using specific antibodies. Actin was used as a loading control.

(B) M17 neuroblastoma cells were transfected with WT α-syn or its mutants (Y39F, Y125F, or Y39FY125F) together with plasmids coding Luciferase (LUC; negative control) or WT c-Abl. Twenty-four hours post-transfection, cells were lysed and proteins separated by SDS-PAGE. α-syn phosphorylation status was assessed using phosphorylation site-specific antibody for Y39 or Y125 residues. α-syn and c-Abl expression were confirmed in an additional protein blot using specific antibodies. Actin was used as a loading control.

(C) HeLa cells were transfected with WT α-syn together with plasmids coding for Luciferase or WT c-Abl. Twenty-four hours post-transfection the cells were immunostained with the appropriate antibodies and counterstained with DAPI to reveal the nucleus.

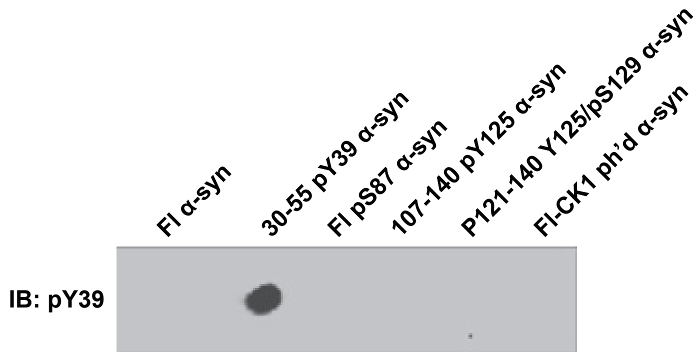
Figure S5 c-Abl drug inhibitors induce α-syn and c-Abl protein degradation but do not change their mRNA level

(A) Inhibition of c-Abl activity by Nilotinib, Imatinib or GNF-2 induces the degradation of α-syn and c-Abl in primary cortical neurons. Primary cultures of cortical neurons were treated with Nilotinib, Imatinib, GNF-2 or DMSO (negative control). After 16 h of treatment, cells were directly lysed in 2X Laemmli Buffer and the proteins were separated using SDS-PAGE and detected by WB analysis using the appropriate antibodies (left hand panel). Actin was used as a loading control. c-Abl and α-syn protein levels were evaluated by densitometry quantification (right hand panels). Band intensities were normalized in the following manner: (c-Abl/actin) or (α-syn/actin). Bars represent the mean ± SD of three independent experiments. * $p<0.05$ or ** $p<0.005$ (Student's t-test: DMSO vs. c-Abl drugs inhibitor).

(B) Inhibition of c-Abl activity by Nilotinib, Imatinib or GNF-2 does not change the mRNA levels of α-syn and c-Abl. Primary cultures of cortical neurons were treated with with Nilotinib, Imatinib, GNF-2 or DMSO (negative control). 16 h post-treatment, cells were lysed and mRNA extraction performed. Semi-quantitative RT-PCR using specific primers against c-Abl or α-syn followed by agarose gel electrophoresis (left hand panel) demonstrates that c-Abl and α-syn mRNA levels were not changed upon Nilotinib treatment. c-Abl and α-syn mRNA level were evaluated by densitometry quantification (right hand panel). The band intensities were normalized using the mRNA level of the housekeeping gene GAPDH: c-Abl/GAPDH or α-syn/GAPDH. The bars represent the mean ± SD of three independent experiments. $p>0.05$ (Student's t-test: DMSO vs. c-Abl drugs inhibitor).

Figure S1

A



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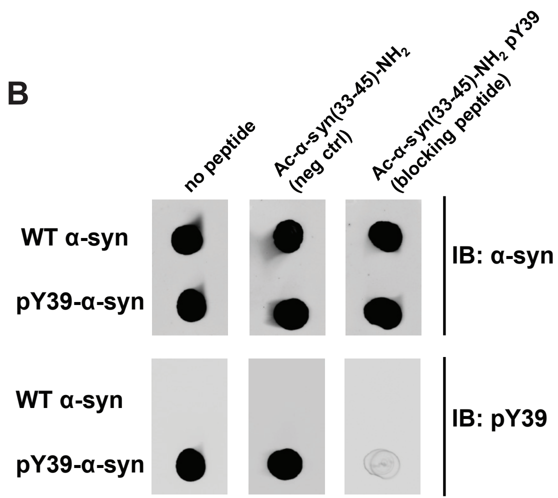

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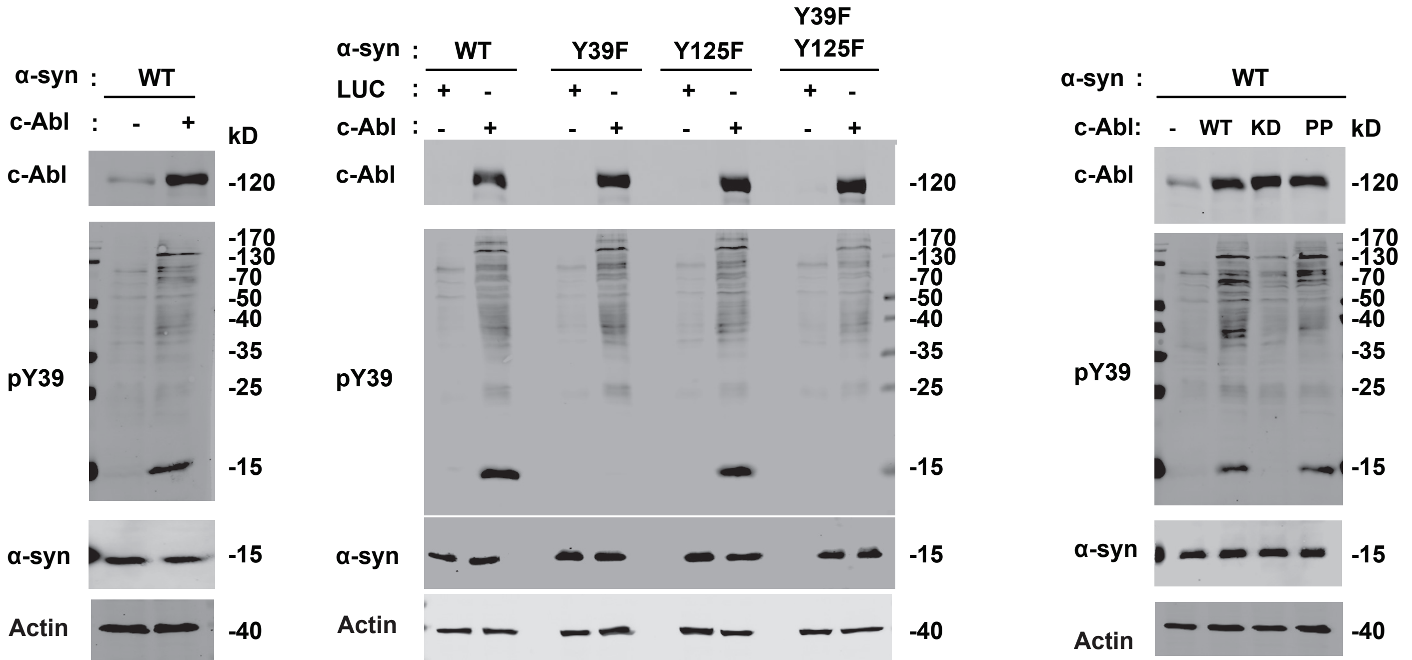

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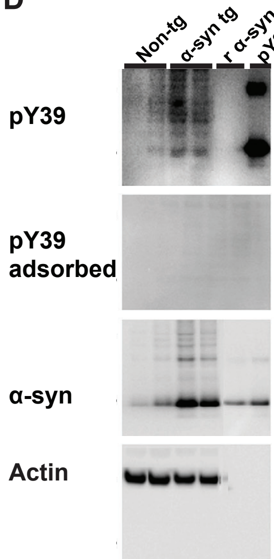

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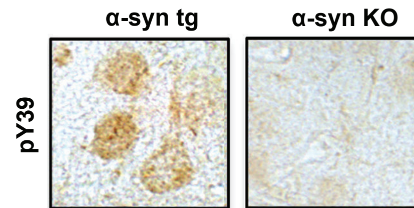

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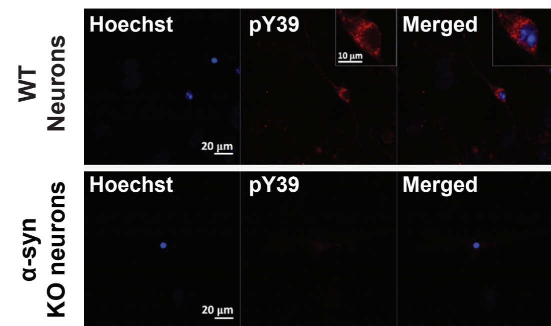

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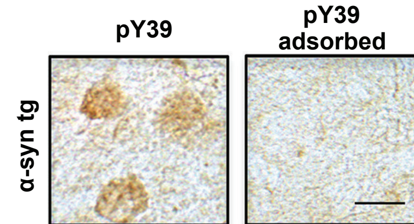

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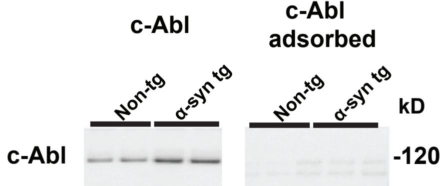

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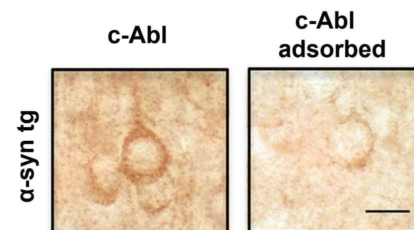

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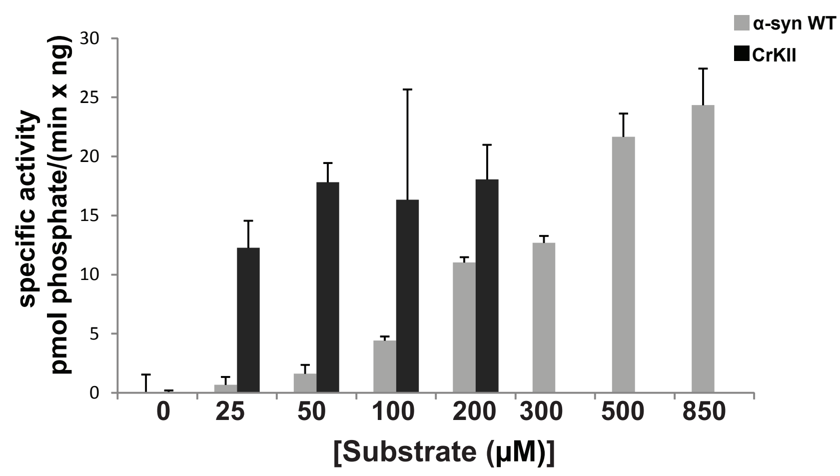

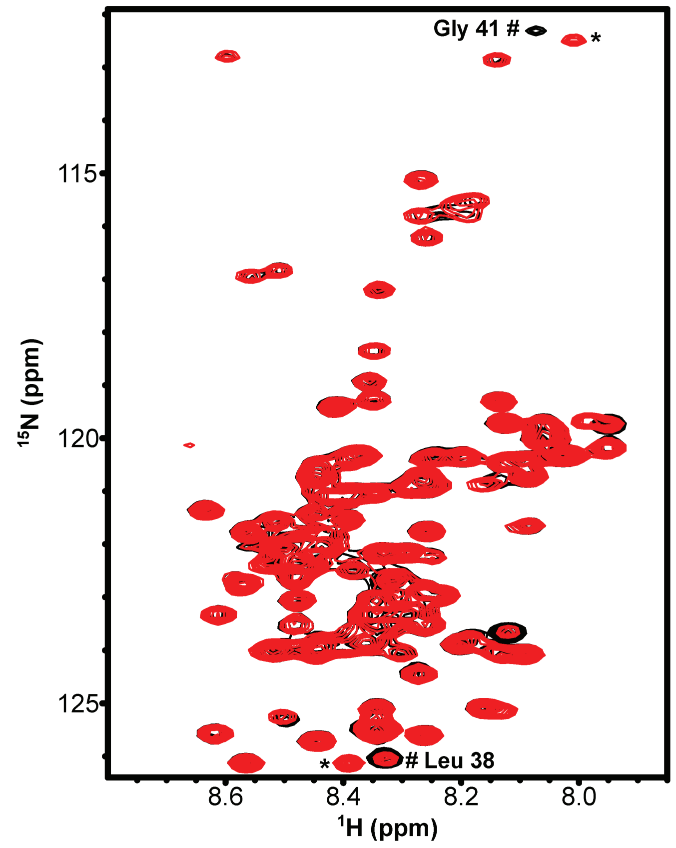

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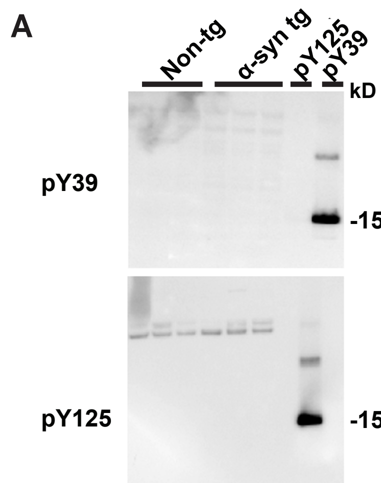


B

WT Cortical neurons

KD c-Abl | DAPI | c-Abl | α-syn | pY39 | Merged | 20 μm

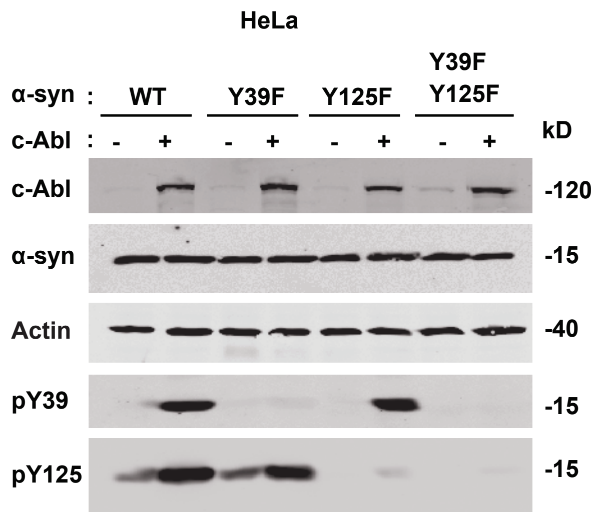
KD c-Abl | DAPI | c-Abl | α-syn | pY125 | Merged | 20 μm

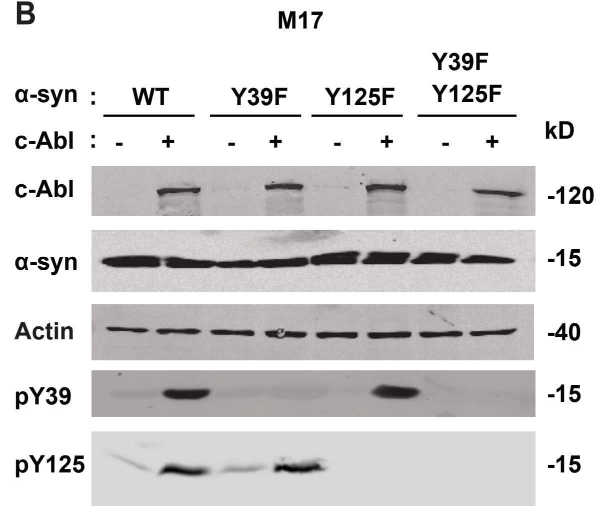
α-syn KO Cortical neurons

KD c-Abl | DAPI | c-Abl | α-syn | pY39 | Merged | 20 μm

KD c-Abl | DAPI | c-Abl | α-syn | pY125 | Merged | 20 μm

Figure S4





C

α-syn + LUC: DAPI, c-Abl, α-syn, pY39, Merged (10μm)

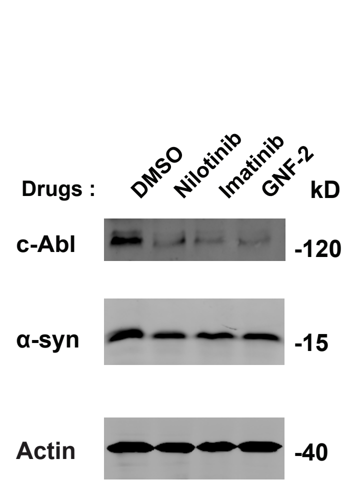
α-syn + c-Abl: DAPI, c-Abl, α-syn, pY39, Merged (10μm)

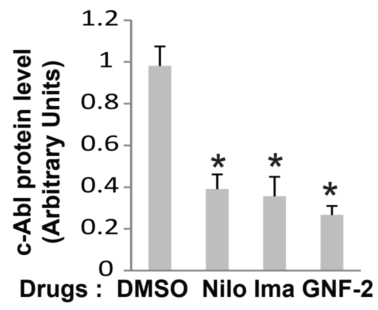
α-syn + LUC: DAPI, c-Abl, α-syn, pY125, Merged (10μm)

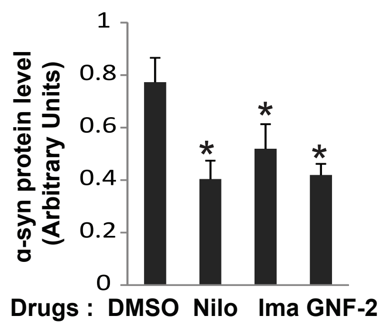
α-syn + c-Abl: DAPI, c-Abl, α-syn, pY125, Merged (10μm)

Figure S5

A







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