PNAS RESEARCH ARTICLE | MEDICAL SCIENCES

Phosphorylation of α-synuclein at T64 results in distinct oligomers and exerts toxicity in models of Parkinson's disease

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Edited by Gregory Petsko, Brigham and Women's Hospital Center for Neurologic Diseases, Boston, MA; received August 26, 2022; accepted April 16, 2023

α-Synuclein accumulates in Lewy bodies, and this accumulation is a pathological hallmark of Parkinson's disease (PD). Previous studies have indicated a causal role of α-synuclein in the pathogenesis of PD. However, the molecular and cellular mechanisms of α-synuclein toxicity remain elusive. Here, we describe a novel phosphorylation site of α-synuclein at T64 and the detailed characteristics of this post-translational modification. T64 phosphorylation was enhanced in both PD models and human PD brains. T64D phosphomimetic mutation led to distinct oligomer formation, and the structure of the oligomer was similar to that of α-synuclein oligomer with A53T mutation. Such phosphomimetic mutation induced mitochondrial dysfunction, lysosomal disorder, and cell death in cells and neurodegeneration in vivo, indicating a pathogenic role of α-synuclein phosphorylation at T64 in PD.

Parkinson's disease | α-synuclein | zebrafish

Significance

We report a novelphosphorylation site of α-synuclein at T64 in Parkinson's disease (PD) model animals and human postmortem brains. This T64 phosphorylation modulates the properties of α-synuclein, and enhance distinct oligomer formation. T64 phosphomimetic mutant is highly toxic in cell cultures and in vivo zebrafish resulting in movement disorder and neurodegeneration. Our findings reveal new and important steps of α-synuclein toward gaining toxic functions in PD.

α-Synuclein accumulates in cytosolic inclusions referred to as Lewy bodies in sporadic Parkinson's disease (PD) (1), and its missense mutations (2–4), as well as gene dosage changes (5–7), can cause an autosomal dominant form of familial PD. Previous studies have indicated a causal role of α-synuclein in the pathogenesis of PD. However, the molecular and cellular mechanisms of α-synuclein toxicity remain unclear. An increasing number of studies have reported that α-synuclein is subjected to several post-translational modifications, including phosphorylation, acetylation, nitration, ubiquitination, and glycosylation. Among them, α-synuclein phosphorylation at S129 is one of the most studied modifications. Although S129 phosphorylation is infrequently observed in a physiological state, it is significantly elevated in the brains of patients with PD, suggesting a potential relationship with the disease (8–10). Several studies have examined the pathological roles of S129 phosphorylation (11–13), with a recent paper demonstrating its protective effects against the formation of α-synuclein fibrils and toxicity (14).

Here, we report a novel phosphorylation site of α-synuclein at T64 in PD model animals and human postmortem brains. We further examine the role of T64 phosphorylation in modulating the properties of α-synuclein and demonstrate the distinct oligomer formation induced by T64D phosphomimetic mutation. Such phosphomimetic mutant is highly toxic in cell cultures and in vivo zebrafish resulting in movement disorder and neurodegeneration. Our findings reveal new and important steps of α-synuclein toward gaining toxic functions in PD.

Results

Identification of α-Synuclein Phosphorylation at T64. We previously reported age- and α-synuclein-dependent degeneration of dopamine and noradrenaline neurons in the annual killifish *Nothobranchius furzeri* (15). We first explored the post-translational modification of endogenous α-synuclein of *N. furzeri* and compared it to that of *Oryzias latipes* (medaka), which is genetically close to *N. furzeri* but does not show age-dependent neurodegeneration of dopamine and noradrenaline neurons. α-Synuclein was purified by immunoprecipitation using fish brain lysates (*SI Appendix*, Fig. S1*A*), and the corresponding bands were excised and subjected to mass spectrometry. Among the identified phosphorylation sites in α-synuclein through tolerant screening, phosphorylation at four novel sites (T33, T44, T50 and S60) was identified in *N. furzeri*, while that was not identified in *O. latipes* (Fig. 1*A* and *SI Appendix*, Table S1). We utilized similar methods for human postmortem brains (nucleus basalis of Meynert and amygdala) pathologically diagnosed with PD (*SI Appendix*, Fig. S1*B*). Only one phosphorylation site at T64 (T64-P), which corresponds to S60-P of *N. furzeri* α-synuclein, could be detected in the

Author contributions: Hideaki Matsui, S.I., Hideki Matsui, M.T., S.-i.K., K.H., and O.O. designed research; Hideaki Matsui, S.I., Hideki Matsui, J.I., R.G., M. Hirose, T.Y., A. Koyama, T.K., N.M., S.H., M.Y., A.S., K.I., and M. Hasegawa performed research; Hideaki Matsui, Hideki Matsui, M.T., N.U., S.-i.K., R.T., K.H., and A. Kakita contributed new reagents/analytic tools; Hideaki Matsui, S.I., and Hideki Matsui analyzed data; and Hideaki Matsui and S.I. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2214652120/-/DCSupplemental.

Published May 30, 2023.

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human brain through the same method (Fig. 1*B* and *SI Appendix*, Fig. S1*C* and Table S1). We subsequently generated antibodies against phosphor-S60 (S60-P) of *N. furzeri* α-synuclein and phosphor-T64 (T64-P) of human α-synuclein (*SI Appendix*, Fig. S1 *D* and *E*). CIP-induced dephosphorylation experiments, knockout validation using α-synuclein-deficient *N. furzeri* brain samples, dot blot analysis using several phosphorylated peptides of human α-synuclein, and other in vitro experiments showed the specificity of these antibodies (Fig. 1 *C* and *D*, please also refer to *SI Appendix*, Fig.S3). We compared the levels of these phosphorylations by sequential extraction of soluble-insoluble proteins and Western blotting (16, 17). S60-P was increased in the TBS-insoluble SDS-soluble fraction of the brains of aged *N. furzeri* (at 5 mo) compared to those of young fish (at 1 mo) (Fig. 1*E* and *SI Appendix*, Fig. S1*F*). Antibodies against phosphor-T64 (T64-P) showed significant elevation of T64-P in the TBS-insoluble SDS-soluble fraction of the PD brain lysates (Fig. 1*F* and *SI Appendix*, Fig. S1*G*). While the monomeric form of T64-P α-synuclein was not evident in these experiments, immunoprecipitation using an anti-α-synuclein antibody (MJFR1) followed by Western blotting for T64-P α-synuclein indicated the presence of monomers or smaller forms in the PD brains (*SI Appendix*, Fig. S1*H*). Repeated freeze and thaw cycles also enabled the clear detection of the T64-P α-synuclein monomer band suggesting that each monomer binds strongly with each other in the oligomer (*SI Appendix*, Fig. S1*I*).

Taken together, S60-P of *N. furzeri* α-synuclein or its counterpart T64-P of human α-synuclein was increased in aging brains of *N. furzeri* or PD brains, respectively.

Characterization of α-Synuclein Phosphorylation at T64. Next, we tested whether the antibody against human T64-P α-synuclein is available for immunostaining using absorption experiments with T64-P α-synuclein peptide. In mouse PD model brains and human postmortem PD brains, the signals stained with T64-P α-synuclein antibody were significantly diminished with absorption treatment suggesting this antibody is specific to human T64-P α-synuclein and can be used in immunohistochemistry (*SI Appendix*, Fig. S2*A*).

α-Synuclein fibrils transfection induces the formation of α-synuclein aggregates (18). In SH-SY5Y cells transfected with α-synuclein fibrils, T64-P and S-129 α-synuclein signals were

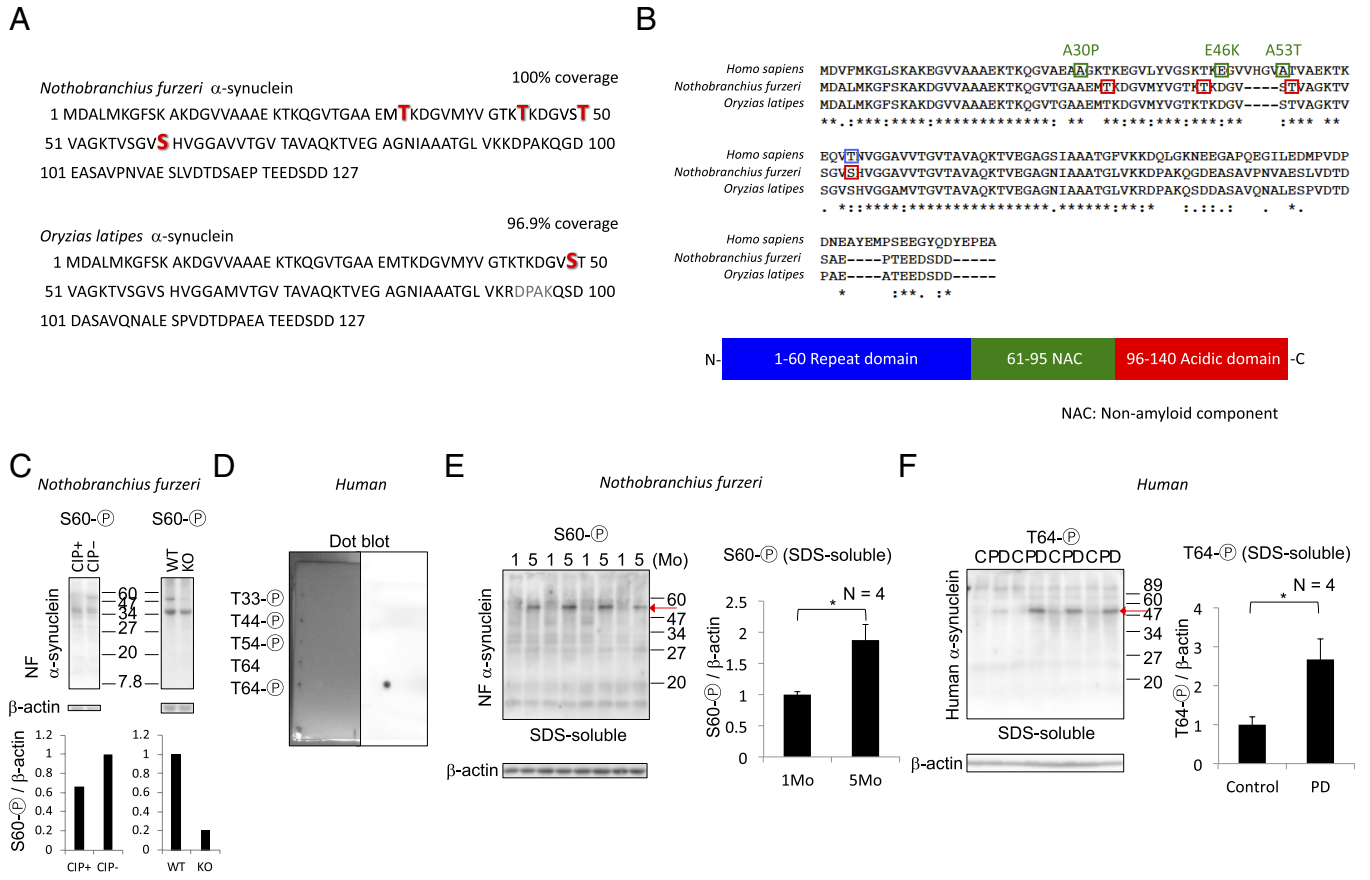

Fig. 1. Identification of a novel phosphorylation site of human α-synuclein at T64. (*A*) α-synuclein of *N. furzeri* or *O. latipes* brains were immunoprecipitated and subjected to liquid chromatography–tandem mass spectrometry. The figure indicates phosphorylation sites identified in *N. furzeri* α-synuclein or *O. latipes* α-synuclein through tolerant screening. Identified residues are indicated by bold red characters. Please also refer *SI Appendix*, Table S1. (*B*) α-synuclein of human brains (nucleus basalis of Meynert) were immunoprecipitated and subjected to liquid chromatography–tandem mass spectrometry. The figure indicates comparison of the phosphorylation sites identified in human, *N. furzeri* and medaka α-synuclein through tolerant screening. Red squares show the specific phosphorylation site of *N. furzeri* α-synuclein, blue squares show the T64 phosphorylation site of human α-synuclein, and green squares show mutation sites in human familial PD. Patients' profiles are described in *SI Appendix*, Table S3. Please also refer *SI Appendix*, Table S1. (*C*) Validation of phosphorylated *N. furzeri* α-synuclein antibodies using CIP treatment or α-synuclein KO samples. CIP+: *N. furzeri* Brain lysates with CIP treatment. CIP-: *N. furzeri* Brain lysates without CIP treatment. WT: Brain lysates of wild-type *N. furzeri*. KO: Brain lysates of α-synuclein KO *N. furzeri*. (*D*) Dot blotting analysis showed a highly specific reaction of anti-human T64-P α-synuclein antibody. Each synthetic peptide corresponding to the amino acids of human α-synuclein T33-P, T44-P, T54-P, T64 and T64-P was applied to a hydrophilized PVDF membrane. (*E*) The western blotting shows the analysis of *N. furzeri* brains (TBS-insoluble SDS-soluble fraction) at 1 and 5 mo using anti-S60-P *N. furzeri* α-synuclein. The red arrow indicated S60 phosphorylation. The graph shows quantitative analysis of S60 phosphorylation in SDS-soluble fraction using N = 4 samples/group. **P* = 0.0358. (*F*) The western blotting shows the analysis of human PD brains (amygdala, TBS-insoluble SDS-soluble fraction) using anti-T64-P human α-synuclein. The red arrow indicated T64 phosphorylation. C: control, PD: Parkinson's disease. The graph shows quantitative analysis of T64 phosphorylation in SDS-soluble fraction using N = 4 samples/group. **P* = 0.0452. Patients' profiles are described in *SI Appendix*, Table S3.

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increased in Western blotting (*SI Appendix*, Fig. S2*B*). T64-P and S-129 α-synuclein signals were often accumulated in the same cells in immunocytochemistry, while T64-P rarely colocalized with S129-P at the subcellular level (Fig. 2*A*). These T64-P signals were often colocalized with mitochondria or lipid rafts but rarely with autophagosomal or lysosomal markers (*SI Appendix*, Fig. S2 *C* and *D*). We subsequently investigated the presence of the α-synuclein T64-P modification in animal models of PD. α-Synuclein fibrils are known to cause seed-dependent aggregation in vivo (19). We thus examined C57BL/6J mice (7 mo) in which α-synuclein fibrils were injected into the striatum (PFF mouse). As was the case with cellular and zebrafish models, S129-P and T64-P were localized in similar anatomical regions with low magnification; however, they mostly showed distinct subcellular localization with high magnification (Fig. 2 *B* and *C*). GBA mutation is a risk factor for PD, and patients with GBA mutations show intraneuronal accumulation of α-synuclein referred to as Lewy bodies and Lewy neurites (20, 21). Several studies have indicated an association between GBA mutations and α-synuclein accumulation. GBA deficiency causes lysosomal dysfunction and α-synuclein accumulation; in

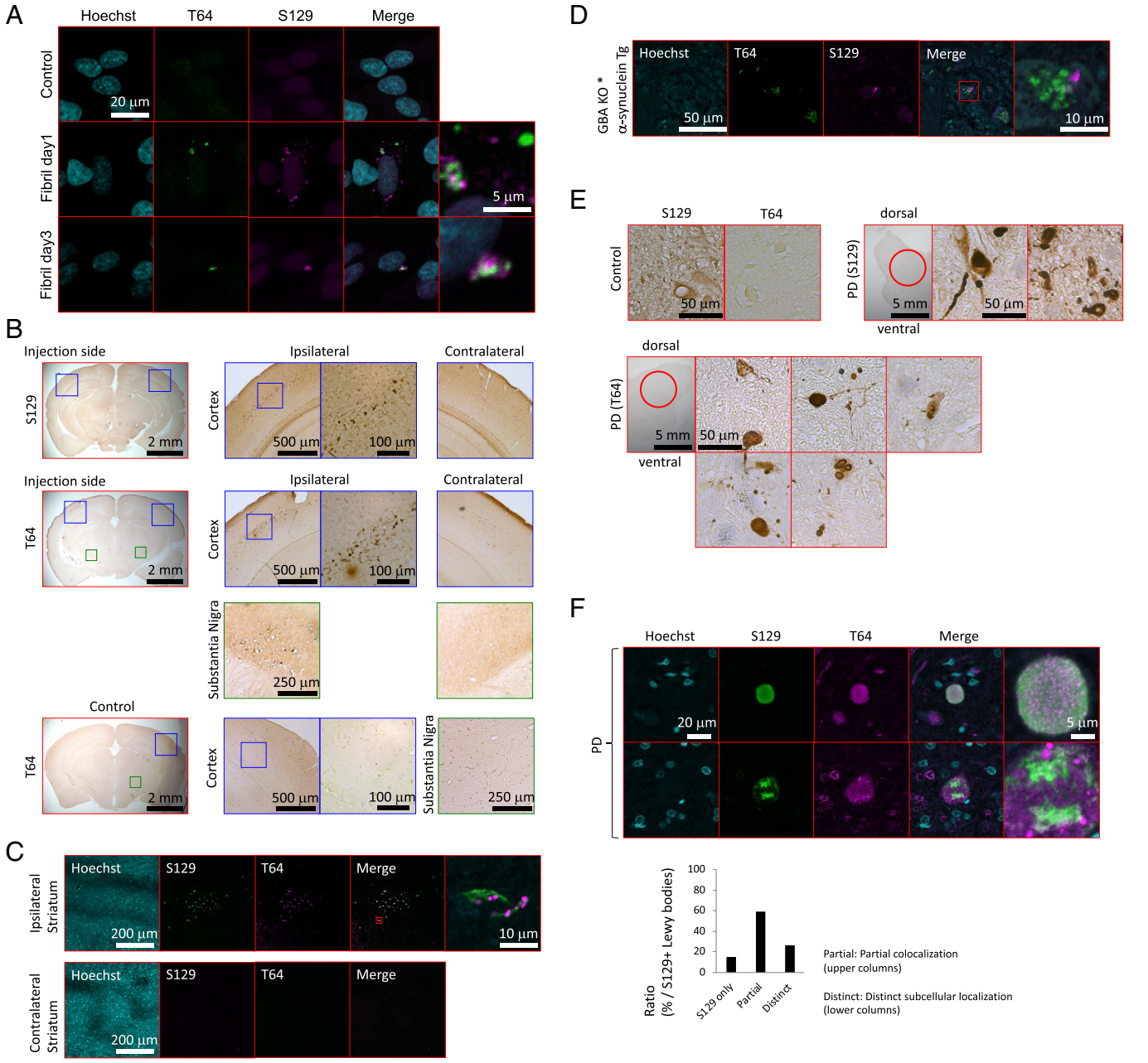

Fig. 2. Distinct pathology of T64-phosphorylated α-synuclein. (*A*) T64-P and S129-P α-synuclein immunofluorescence of the SH-SY5Y cells transfected with α-synuclein fibrils. α-Synuclein Aggregation Assay Kit was used for the transfection of α-synuclein fibrils and cells are subjected to immunofluorescence at 1 and 3 d after transfection. (*B*) Immunohistochemistry of T64-P and S129-P α-synuclein in the mouse PD models (α-synuclein PFF mouse). α-Synuclein fibrils were injected into the striatum of C57BL/6J mice at 5 mo. Two months after inoculation, the mice were sacrificed and the brains were subjected to immunohistochemistry. (*C*) Immunofluorescence of T64-P and S129-P α-synuclein in the mouse PD models (α-synuclein PFF mouse). α-Synuclein fibrils were injected into the striatum of C57BL/6J mice at 5 mo. Two months after inoculation, the mice were killed, and the brains were subjected to immunofluorescence. (*D*) Immunofluorescence of T64-P and S129-P α-synuclein in the zebrafish PD models. Zebrafish (*Tg(XenopusNBT:human α-synuclein);gba-/-*) were killed and subjected to immunofluorescence at 3 mo of age. (*E*) Immunohistochemistry of T64-P and S129-P α-synuclein in the human PD brains. The medulla oblongata sections of human postmortem brains were used for immunohistochemistry. Patient profiles are described in *SI Appendix*, Table S3. (*F*) Immunofluorescence of T64-P and S129-P α-synuclein in the human PD brains. The medulla oblongata sections of human postmortem brains were used for immunofluorescence. Patient profiles are described in *SI Appendix*, Table S3.

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turn, α-synuclein accumulation creates a vicious cycle by inhibiting the trafficking of GBA to lysosomes (22, 23). We created gba knockout (KO) zebrafish carrying human α-synuclein protein (24), and S129-P α-synuclein accumulations were observed in the gba KO fish brains. In this fish model, T64-P α-synuclein accumulations were also detected; however, the subcellular localizations of S129-P and T64-P were different in most cases (Fig. 2*D* and *SI Appendix*, Fig. S2*E*). We conducted a similar analysis for the human postmortem PD brains (medulla oblongata). S129-P α-synuclein has been used as a pathological hallmark of PD and showed robust aggregations in the PD brains (Fig. 2*E*). T64-P α-synuclein immunostaining showed similar or stronger signals in the PD brains (Fig. 2*E*). This T64-P α-synuclein accumulation was observed mostly in neurons (*SI Appendix*, Fig. S2*F*). Consistent with the findings in animal models, S129-P and T64-P were localized in similar anatomical regions with low magnification; however, they mostly showed distinct fluorescent patterns and subcellular localization with high magnification (Fig. 2*F*). Multiple system atrophy (MSA) is a neurodegenerative disease with cerebellar symptoms, parkinsonism, and autonomic failure. α-synuclein is also a major component of the glial cytoplasmic inclusions found in MSA (25). Interestingly, glial cytoplasmic inclusions of MSA were negative for T64-P α-synuclein immunostaining, suggesting that the pathogenesis of α-synuclein is different in PD and MSA (*SI Appendix*, Fig. S2*G*). No T64-P α-synuclein signal was observed in other neurodegenerative diseases, amyotrophic lateral sclerosis and Alzheimer's disease (*SI Appendix*, Fig. S2*G*). In summary, T64-P α-synuclein was elevated in cellular and animal models of PD and human PD brains, and it showed distinct subcellular localization from S129-P.

Four candidates of protein kinases (calmodulin-dependent protein kinase II, glycogen synthase kinase 3, cyclin-dependent kinase 1, and casein kinase 1) for T64 phosphorylation of α-synuclein were selected using NetPhos 3.1 Server (http://www.cbs.dtu.dk/services/NetPhos/). We overexpressed each kinase with α-synuclein in HeLa cells, and among them, casein kinase 1 could enhance T64 phosphorylation signals in Western blots (*SI Appendix*, Fig. S3*A*). Casein kinase 1 has several isoforms, and some of them enhanced T64 phosphorylation (*SI Appendix*, Fig. S3 *B* and *C*). Ultrafiltration-based size separation disclosed that T64 phosphorylation of α-synuclein existed mainly >100 kDa fraction (*SI Appendix*, Fig. S3*D*). Casein kinase 1 also phosphorylated α-synuclein at T64 in vitro, which was demonstrated using Western blots and mass spectrometry (*SI Appendix*, Fig. S3 *E–G* and Table S2). T to A substitution at T64 completely abolished the Western blot signal of the T64-P antibody, further demonstrating the specificity of this antibody (*SI Appendix*, Fig. S3*H*). T64 phosphorylation caused a change in the electrophoretic pattern of α-synuclein (*SI Appendix*, Fig. S3*I*). When casein kinase 1 delta was overexpressed in SH-SY5Y cells, it was diffusely localized in the cytosol under the control condition, whereas it formed puncta after α-synuclein fibril transfection and was colocalized with α-synuclein T64-P signals (*SI Appendix*, Fig. S3*J*). Overexpression of several isoforms of casein kinase 1 together with α-synuclein resulted in colocalization with T64-P signals (*SI Appendix*, Fig. S3*K*). We next investigated PFF mouse and human brains using immunohistochemistry, and casein kinase 1 delta, epsilon, or gamma showed colocalization with α-synuclein T64-P signals in these brains (*SI Appendix*, Fig. S4 *A* and *B*). Thus, multiple isoforms of casein kinase 1 appear to be responsible for phosphorylating α-synuclein at T64.

The accumulation of T64-P was then analyzed using blue native polyacrylamide gel electrophoresis (BN-PAGE), which revealed marked accumulation of T64-P-positive oligomers (stronger signal at 250 kDa and smear signals) in the SH-SY5Y cells transfected with α-synuclein fibrils or the PD brains (Fig. 3*A* and *SI Appendix*, Fig. S5*A*). In order to analyze the effects of phosphorylation modification of proteins, it is often done by replacing the target amino acid for phosphorylation modification with D (aspartic acid) or E (glutamic acid) to mimic the phosphorylation modification and analyze its effects (26). The α-synuclein of the T64D and T64E mutations was diffusely present in the cytoplasm and nucleus, as were alpha-synuclein of WT and T64A, and there were no differences in the fractions present (*SI Appendix*, Fig. S5 *B* and *C*). Neither the T64D nor T64E mutation showed any tendency to promote aggregation (*SI Appendix*, Fig. S5*B*). Neither the T64D nor the T64E mutation showed a tendency to spread to other cells (*SI Appendix*, Fig. S5*D*). BN-PAGE of the purified oligomers from the wild-type (WT) α-synuclein and T64D phosphomimetic mutant showed clearly different patterns of electrophoresis which suggested larger size oligomers in WT and smaller size oligomers in T64D (Fig. 3*B*). Two-dimensional electromicroscopic analysis of purified α-synuclein oligomers showed barrel-shaped oligomers in the WT and T64A mutant groups, while the T64D phosphomimetic mutant and A53T mutant [one of the causative mutations in familial PD (2)] groups showed irregular and smaller oligomers (Fig. 3*C* and *SI Appendix*, Fig. S5*E*). Real-time quaking-induced conversion (RT-QuIC) demonstrated that T64D phosphomimetic mutant tended to be less prone to aggregation labeled by Thioflavin T (Fig. 3*D*). Thus, α-synuclein T64D and possibly α-synuclein T64-P are less likely to aggregate, but can form oligomers with unique morphologies.

In summary, T64-P α-synuclein was increased in cellular and animal models of PD and human PD brains, and it showed distinct subcellular localization and oligomer formation.

Toxicity and Neurodegeneration Caused by α-Synuclein Phosphorylation at T64. The overexpression of phosphomimetic T64D mutant α-synuclein in SH-SY5Y cells induced various changes at the transcriptome level (Fig. 4*A* and Dataset S1), damaged the mitochondrial membrane potential (Fig. 4*B*), and disturbed the acidic condition of the lysosomes with abnormal large lysosomal structures (Fig. 4*C* and *SI Appendix*, Fig. S6*A*). HEK293T cell has very few endogenous α-synuclein protein, and it made easy to observe the toxicity of the transfected α-synuclein. To determine whether T64-P α-synuclein induces cytotoxicity, we assessed cell survival and death using WST-8 and LDH assays after transfection of HEK293T cells with the α-synuclein phosphomimetic T64D mutant. T64D α-synuclein transfection induced elevated LDH and decreased cell viability (Fig. 4*D*). T64D α-synuclein also induced the activation of Caspase-3 in both HEK293T and SH-SY5Y cells (Fig. 4 *E* and *F* and *SI Appendix*, Fig. S6*B*).

Finally, we investigated whether T64-P α-synuclein causes neurodegeneration in vivo. We generated zebrafish that overexpressed WT, T64D, and T64A human α-synuclein under the *Xenopus* neural-specific beta-tubulin (NBT) promoter control [zebrafish: Tg(NBT:human α-synuclein IRES GFP)]. The expression levels of the transgene were comparable among the WT, T64D, and T64A Tg fish (Fig. 4 *G* and *H*). The RNA sequences of SH-SY5Y cells and zebrafish indicated that endoplasmic reticulum (ER)—Golgi pathway, intracellular vesicles, and extracellular secretion were disrupted by α-synuclein phosphomimetic T64D/E mutant (Fig. 4*A*, *SI Appendix*, Fig. S6*C*, and Datasets S1 and S2). Electron microscopic analysis of T64D Tg fish showed many abnormal membranous lysosomes (*SI Appendix*, Fig. S6*D*). Western blotting of Caspase-3 indicated elevated cell death in the T64D Tg fish (Fig. 4*H* and *SI Appendix*, Fig. S6*B*). We assessed the dopaminergic neurons in these zebrafish using tyrosine hydroxylase (TH, a marker of dopaminergic neurons) immunoblotting and immunohistochemistry. These experiments confirmed the loss of dopaminergic neurons in

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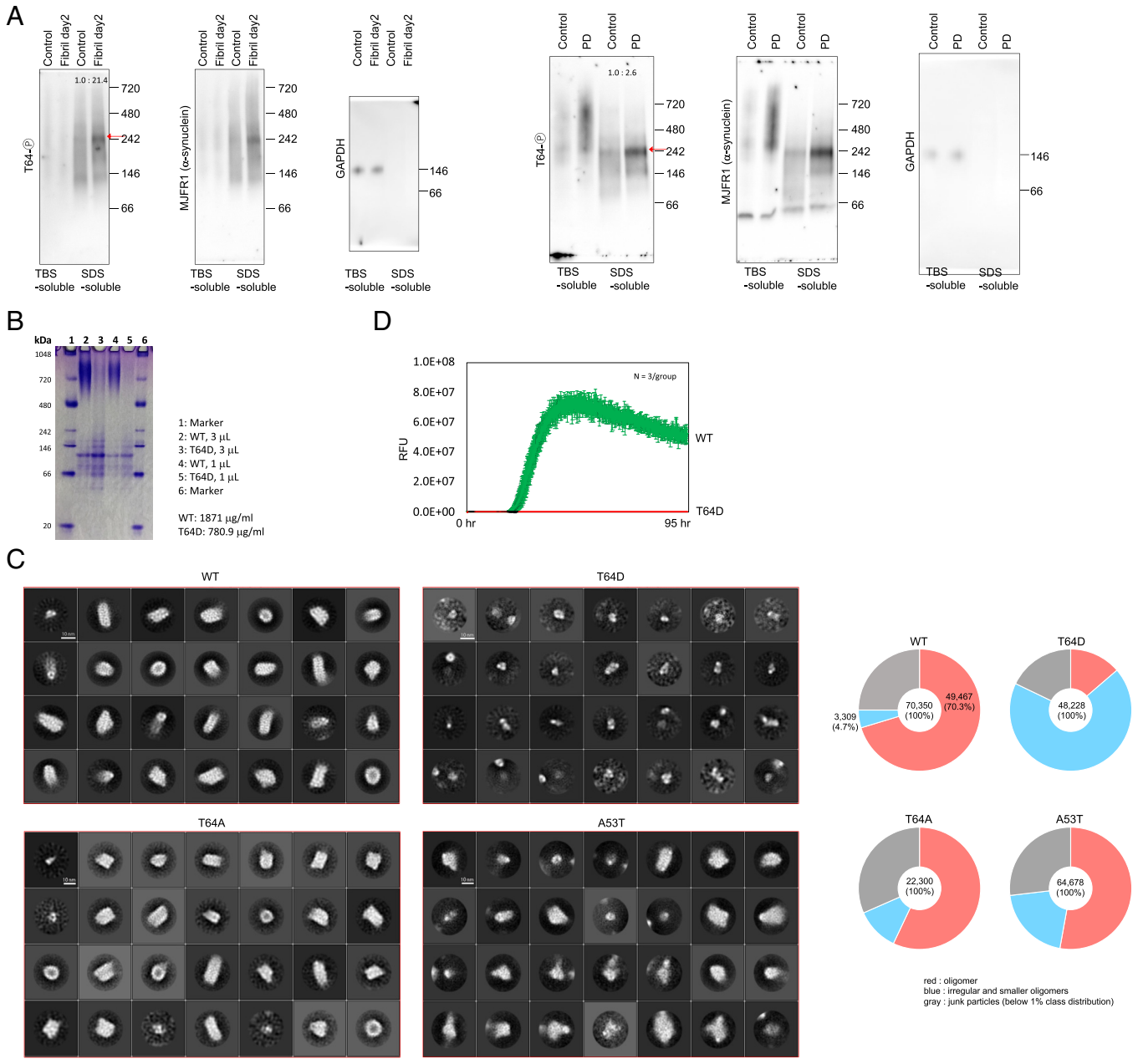

Fig. 3. Oligomer structure of T64-phosphorylated α-synuclein. (*A*) BN-PAGE and Western blotting of T64-P α-synuclein in the SH-SY5Y cells transfected with α-synuclein fibrils or the human PD brains. α-Synuclein Aggregation Assay Kit was used for the transfection of α-synuclein fibrils and cells are used at 2 d after transfection. TBS-soluble or TBS-insoluble SDS-soluble fractions of the SH-SY5Y cells and human brains were used for BN-PAGE and Western blotting. (*B*) BN-PAGE and CBB staining analysis of purified α-synuclein oligomers in the WT and T64D phosphomimetic mutant. Each purified α-synuclein (WT or T64D) was incubated for 8 h at 4 °C without agitation and then subjected to ultracentrifugation. The supernatants were subjected to multiple filtration steps to purify α-synuclein oligomers. (*C*) Two-dimensional electromicroscopic analysis of purified α-synuclein oligomers in the WT, T64A mutant, T64D phosphomimetic mutant and A53T mutant. All particles used for 2D classification were divided into oligomers, particles of lengths of 60 Å or less, and particles with a class distribution of 1% or less, and the number of particles and percentages are plotted in the pie chart. Original electromicroscopic images are shown in *SI Appendix*, Fig. S5*E*. (*D*) Realtime quaking-induced conversion (RT-QuIC) of WT (green) and T64D (red) α-synuclein. RFU: Relative Fluorescence Unit. N = 3 samples/group.

the T64D Tg fish (Fig. 4 *H* and *I*). Acridine orange staining further indicated substantial cell death in the T64D Tg fish (Fig. 4*J*). The T64D α-synuclein Tg zebrafish showed fewer spontaneous swimming movements than the control fish (Fig. 4*K* and Movie S1).

In conclusion, α-synuclein phosphorylation at T64, which is elevated in PD models and PD patients, is highly toxic in cultured cells and in vivo.

Discussion

Various post-translational modifications of human α-synuclein, including phosphorylation, ubiquitination, nitration, truncation, and acetylation, have been reported (11, 13). Post-translational modifications have emerged as important determinants of α-synuclein's physiological and pathological functions (11, 13). Some modifications may occur as a consequence of inflammation or cell death, while other modifications can be neuroprotective. Among these modifications, the phosphorylation of α-synuclein at S129 is one of the best known, and it has been utilized to clearly illustrate Lewy bodies (8, 9). S129 phosphorylation is not commonly observed in the physiological condition, but it is significantly elevated in the brains of individuals with PD. This suggests that it may be associated with the development or progression of PD (8–10). Many studies have explored the pathological roles of S129

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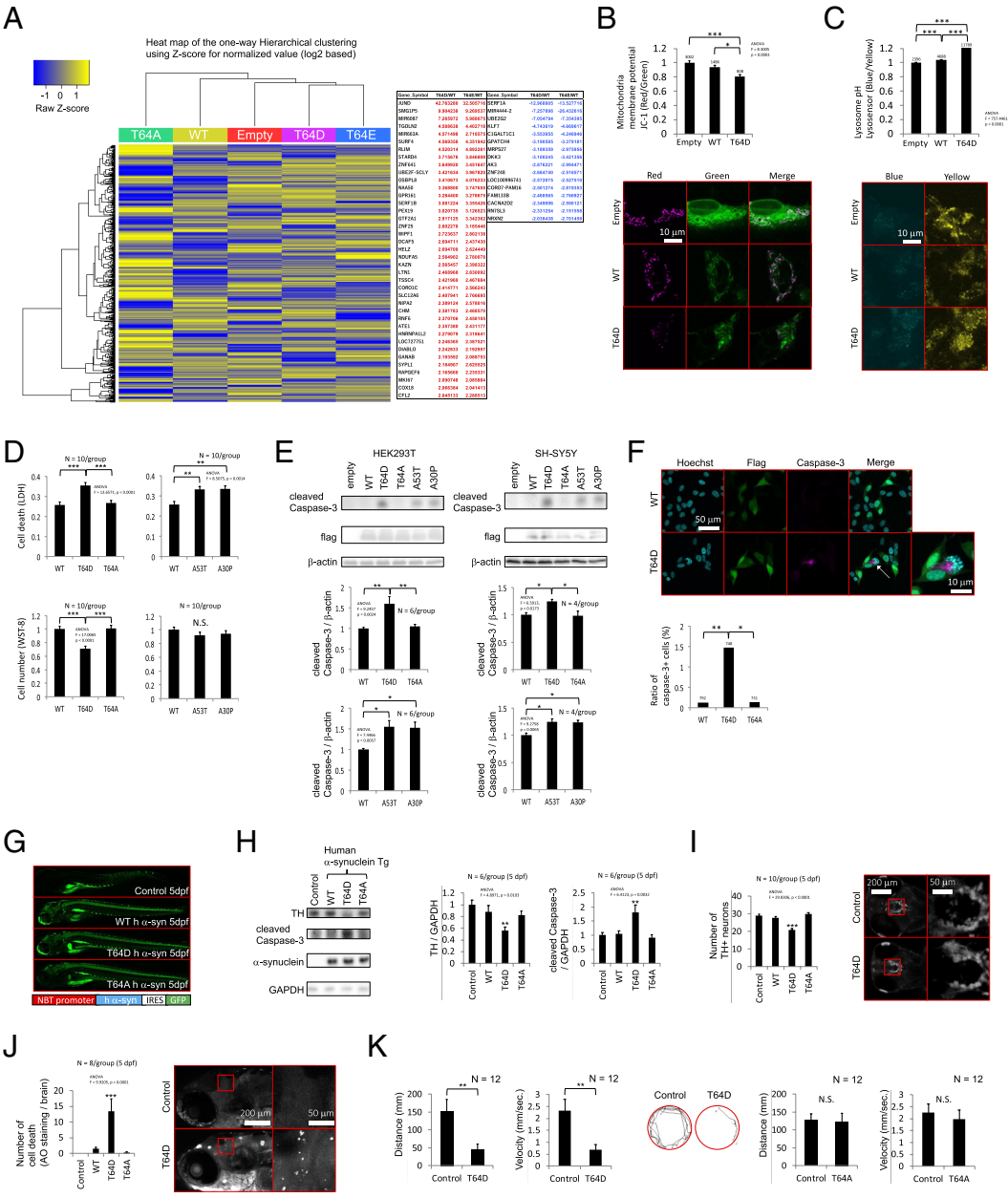

Fig. 4. Cytotoxicity and death of dopaminergic neurons induced by α-synuclein phosphorylation at T64. (*A*) RNA sequence analysis of the SH-SY5Y cells transfected with WT, T64A, T64D, or T64E α-synuclein. RNA was isolated 7 d after electroporation. The mRNAs with a more than twofold increase (red) or decrease (blue) in both the T64D and T64E samples are listed. (*B*) Mitochondrial membrane potential in the SH-SY5Y cells transfected with WT or T64D α-synuclein. Mitochondrial membrane potential was measured 5 d after electroporation using a JC-1 MitoMP Detection Kit. The numbers on the bar graph indicate the total cell number analyzed. **P* = 0.0150, ****P* < 0.0001. (*C*) Lysosomal pH in the SH-SY5Y cells transfected with WT or T64D α-synuclein. Lysosomal pH was evaluated 5 d after electroporation using LysoSensor Yellow/Blue DND-160 (PDMPO). The numbers on the bar graph indicate the total cell number analyzed. ****P* < 0.0001. (*D*) Cell death or viability in the HEK293T cells transfected with WT, T64D, T64A, A53T, or A30P α-synuclein. Cell death was measured by LDH Cytotoxicity Detection Kit 4 d after plasmid transfection using Lipofectamine 3000 reagent. Cell viability was measured by Cell Counting Kit-8 4 d after plasmid transfection using Lipofectamine 3000 reagent. N = 10 samples/group. Cell death: WT vs. T64D: *P* < 0.0001, T64A vs. T64D: *P* = 0.0002, WT vs. A30P: *P* = 0.0012, WT vs. A53T: *P* = 0.0016. Cell viability: WT vs. T64D: *P* < 0.0001, T64A vs. T64D: *P* < 0.0001. N.S.: not significant. (*E*) Western blotting of cleaved Caspase-3 in the HEK293T or SH-SY5Y cells transfected with WT, T64D, T64A, A53T or A30P α-synuclein. HEK293T cells were collected 4 d after plasmid transfection using Lipofectamine 3000 and SH-SY5Y cells were collected 5 d after electroporation. The graphs show quantitative analysis of cleaved Caspase-3 using N = 6 (HEK293T) or 4 (SH-SY5Y) samples/group. HEK293T: WT vs. T64D: *P* = 0.0015, T64A vs. T64D: *P* = 0.003, WT vs. A30P: *P* = 0.0052, WT vs. A53T: *P* = 0.0038. For the ANOVA comparison among T64D, A53T and A30P, the F value = 0.0508 and *P* = 0.9506 (N.S.). SH-SY5Y: WT vs. T64D: *P* = 0.0145, T64A vs. T64D: *P* = 0.0099, WT vs. A30P: *P* = 0.0057, WT vs. A53T: *P* = 0.0039. For the ANOVA comparison among T64D, A53T and A30P, the F value = 0.0364 and P = 0.9644 (N.S.). N.S.: not significant. (*F*) Caspase-3-positive SH-SY5Y cells (white arrow) transfected with T64D α-synuclein. SH-SY5Y cells were subjected to immunofluorescence 5 d after electroporation. The graph shows the percentage of caspase-3-positive SH-SY5Y cells transfected with WT, T64A or T64D α-synuclein. The numbers on the bar graph indicate the total cell number counted. **P* = 0.0043, ***P* = 0.0027. (*G*) Images of Zebrafish: Control fish, *Tg(NBT:human α-synuclein WT IRES GFP)Tg(NBT:human α-synuclein T64D IRES GFP)* and *Tg(NBT:human α-synuclein T64A IRES GFP)* at 5 dpf. (*H*) Western blotting of TH, Caspase-3 and α-synuclein in zebrafish: Control fish, *Tg(NBT:human α-synuclein WT IRES GFP)Tg(NBT:human α-synuclein T64D IRES GFP)* and *Tg(NBT:human α-synuclein T64A IRES GFP)* at 5 dpf. The graphs show quantitative analysis of TH and Caspase-3 using N = 6 fishes/group. Dunnett's test was applied (Control = Control fish). ***P* = 0.0038 (TH), ***P* = 0.0067 (cleaved Caspase-3). (*I*) Dopaminergic neurons in zebrafish: Control fish, *Tg(NBT:human α-synuclein WT IRES GFP)Tg(NBT:human α-synuclein T64D IRES GFP)* and *Tg(NBT:human α-synuclein T64A IRES GFP)* at 5 dpf. The graph shows the number of DC2 and DC4 dopaminergic neurons of the posterior tuberculum. N = 10 fishes/group. Dunnett's test was applied (Control = Control fish). ****P* < 0.0001. (*J*) Cell death in zebrafish brain: Control fish, *Tg(NBT:human α-synuclein WT IRES GFP)Tg(NBT:human α-synuclein T64D IRES GFP)* and *Tg(NBT:human α-synuclein T64A IRES GFP)* at 5 dpf. Cell death was analysed using Acridine Orange Staining. N = 8 fishes/group. Dunnett's test was applied (Control = Control fish). ****P* = 0.0002. (*K*) Spontaneous swimming movement in zebrafish. Control fish and *Tg(NBT:human α-synuclein T64D IRES GFP)* at 5 dpf. N = 12 fishes/group. ***P* = 0.0071 (Distance), *P* = 0.0070 (Velocity). Control fish and *Tg(NBT:human α-synuclein T64A IRES GFP)* at 5 dpf. N = 12 fishes/group. N.S.: not significant. Tracking shows a representative trace of the zebrafish movements for 1 min.

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phosphorylation in PD (11–13), and one recent study indicated that it may have a protective effect against the formation of toxic α-synuclein fibrils (14).

In this paper, we describe a novel phosphorylation site of α-synuclein at T64 and the detailed characteristics of this post-translational modification. We conducted various validations and utilized both western blotting and mass spec to show the presence of phosphorylation at T64. According to these validations and experiments, we concluded that phosphorylation at T64 was elevated in various PD models and human PD brains. However, the quantitativeness of phosphorylation at T64 was partially lost due to the multistep nature of the experimental procedures and the fact that T64 phosphorylation spectra were not separated in crude brain samples. We ventured a rough approximation by enumerating the number of tandem mass spectra. In this instance, the proportion was approximately 2.0% for the control case and 3.3% for the PD case. Nevertheless, the number of tandem mass spectra for T64 phosphorylation in the control case was only one, and thus, it was not particularly quantitative. In light of the results from the western blotting and other experiments, we can infer that T64 phosphorylation was augmented in PD cases; however, our conjecture as to the extent of T64 phosphorylation of α-synuclein was that it was relatively low. This constitutes one of the limitations of our study. A further limitation of this study was the inability to isolate a single kinase responsible for T64 phosphorylation of α-synuclein. There exist multiple isoforms of casein kinase 1, several of which have been shown to augment T64 phosphorylation. Our endeavor to isolate a specific kinase was impeded, leading us to tentatively conclude that multiple kinases may phosphorylate the T64 site of human α-synuclein.

Phosphomimetic mutation at T64 resulted in the formation of irregular and smaller oligomers, was highly toxic and induced neurodegeneration in vivo. Surprisingly, the structure of the oligomer induced by T64 phosphorylation was similar to that of α-synuclein oligomer with A53T mutation which is one of the causative mutations in familial PD (2). T64 phosphorylation of α-synuclein does not appear to elicit the formation of aggregates that can be identified through RT-QUIC, instead exhibiting an inhibitory effect. The overexpression of T64D or T64E in cultured cells also failed to induce the propagation of the α-synuclein. It is our belief that there may exist more than two types of α-synuclein that contribute to PD, one that drives propagation—if the propagation hypothesis holds true—and the other that exerts cytotoxic effects. The former would encompass modifications or mutants that induce aggregate formation through RT-QUIC, whereas the latter would encompass T64 phosphorylation, as found in this study, or other molecules that facilitate oligomer formation. Phosphorylation at the T64 residue was also observed in the mass spectrometry analysis of individuals with MSA, albeit the proportion of α-synuclein that underwent T64 phosphorylation was relatively scant (27). Indeed, Lewy bodies in PD were stained with immunohistochemistry of T64-P α-synuclein in this study, whereas glial cytoplasmic inclusions of the MSA were not stained with the same method. Phosphorylation at T64 appears to be one of the essential key players in PD pathogenesis, and could be a drug target and a diagnostic marker in future.

Phosphomimetic mutations have been commonly used to study the effect of protein phosphorylation (26). In this method, the desired phosphorylated residue is mutated to a negatively charged residue to mimic the negative charge associated with phosphorylation. However, these mutations are not reversible. Furthermore, these amino acids do not exactly reproduce the steric and charge properties of phosphorylation. This is one of the limitations in our study and we will overcome this problem by unveiling more detailed mechanisms of T64 phosphorylation in the near future.

T64 phosphorylation of α-synuclein was observed to increase with age in *N. furzeri* brains, while in human brains it was specifically observed in PD. The T64-phosphorylated α-synuclein is found in TBS-insoluble SDS-soluble fraction, specifically as oligomers. These oligomers are similar in shape to the oligomers of mutant α-synuclein found in familial PD, suggesting that T64 phosphorylation of α-synuclein and these oligomers are strongly involved in the neurodegeneration of PD. The oligomeric form imaged in the electron microscope lacks any discernible structure, suggesting a breadth in molecular weight. Given the limitations of the phosphorylation mimetic technique, where T64 phosphorylation and T64D may not exhibit identical properties, it is challenging to definitively determine the molecular weight of the oligomer formed by T64 phosphorylation. SDS destabilizes noncovalent bonds both within and between proteins, inducing their denaturation and resulting in the alteration of their native configuration and function. However, the detection of monomer-sized bands can only be achieved through several cycles of freezing and thaw or concentration via immunoprecipitation. There exist two potential explanations for this phenomenon. Firstly, the T64-P-modified species might persist as trimers, tetramers, or pentamers. Alternatively, multiple and/or complex protein modifications might alter the electrophoretic velocity of T64-P-modified species.

Our investigation has revealed that several kinases may participate in the phosphorylation of α-synuclein at T64, and it is plausible that T64-P represents one of the potential molecules that contribute to the toxic effects observed in PD. Further clarification of the mechanism of this pathological phosphorylation may lead to elucidation of the pathogenesis of PD and the development of therapeutic drugs.

Data, Materials, and Software Availability. All data are available in the main text or the supporting information. The study did not generate a unique code.

ACKNOWLEDGMENTS. We acknowledge Prof. Hiroyuki Nawa, Prof. Kenji Sakimura, Prof. Hitoshi Takahashi, Dr. Yoshiyuki Tsujihata and Dr. Tsuneaki Hida for helpful discussions and continuous support. This work was supported by the Takeda Science Foundation (Hideaki Matsui); JSPS KAKENHI Grant Numbers JP 22484842 (Hideaki Matsui), JP 18955907 (Hideaki Matsui), JP 14516799 (Hideaki Matsui), JP 16690735 (Hideaki Matsui), and JP 17925674 (Hideaki Matsui); AMED [Grant Numbers JP19gm6110028 and JP19dm0107154 (Hideaki Matsui)]; Sumitomo Foundation Grant Number 150081 (Hideaki Matsui); Tokyo Biochemical Research Foundation (Hideaki Matsui); the Uehara Memorial Foundation (Hideaki Matsui); JST [Moonshot R&D][Grant Number JPMJMS2024] (Hideaki Matsui); the Cell Science Research Foundation (Hideaki Matsui); AMED [Grant Numbers JP21wm0425019 (A. Kakita)]; Crowd Funding (Hideaki Matsui); COCKPI-T (Co-Create Knowledge for Pharma Innovation with Takeda) and Takeda Pharmaceutical Company, Limited. H. Matsui, M.T., S.-i.K. and K.H. are employees of Takeda Pharmaceutical Company, Limited.

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