# Synthetic polyubiquitinated α-Synuclein reveals important insights into the roles of the ubiquitin chain in regulating its pathophysiology

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**Ubiquitination regulates, via different modes of modifications, a variety of biological processes, and aberrations in the process have been implicated in the pathogenesis of several neurodegenerative diseases. However, our ability to dissect the pathophysiological relevance of the ubiquitination code has been hampered due to the lack of methods that allow site-specific introduction of ubiquitin (Ub) chains to a specific substrate. Here, we describe chemical and semisynthetic strategies for site-specific incorporation of K48-linked di- or tetra-Ub chains onto the side chain of Lys12 of α-Synuclein (α-Syn). These advances provided unique opportunities to elucidate the role of ubiquitination and Ub chain length in regulating α-Syn stability, aggregation, phosphorylation, and clearance. In addition, we investigated the cross-talk between phosphorylation and ubiquitination, the two most common α-Syn pathological modifications identified within Lewy bodies and Parkinson disease. Our results suggest that α-Syn functions under complex regulatory mechanisms involving cross-talk among different posttranslational modifications.**

chemical protein synthesis | proteasome | amyloid | post-translational modifications | fibrils

One of the most common posttranslational modifications in eukaryotes is the covalent attachment of ubiquitin (Ub) to proteins. This reversible modification, which regulates a variety of biological processes, such as protein degradation, trafficking, and DNA damage response (1, 2), involves the attachment of the C terminus of Ub mainly to the side chain of a Lys residue in a protein substrate via an isopeptide linkage. The process is catalyzed by three enzymes that act in concert: the Ub-activating enzyme (E1), the Ub-conjugating enzyme (E2), and the Ub ligase (E3). The reaction is repeated, and a second Ub is attached to an internal Lys in the previously conjugated ubiquitin. Several repeats result in the synthesis of a poly-Ub chain that can be of varying lengths and internal linkages. The presence of seven Lys residues as possible anchoring sites within Ub in addition to the N-terminal amine results in a highly complex landscape of diverse Ub bioconjugates, which accounts for the diversity of the Ub signaling (3).

Research in the Ub field, which aims at understanding the ubiquitination system at the molecular level, has been hampered by the difficulties of controlling ubiquitination in the cell and challenges associated with the preparation of specific Ub conjugates in vitro. These limitations have inspired the development of novel synthetic strategies to facilitate site-specific ubiquitination of proteins (4, 5). Recent advancements in the field have enabled the synthesis of relatively large amounts of highly complex Ub conjugates of defined covalent structure and provided novel insights into the structural, biochemical, and functional consequences of protein ubiquitination, along with unique opportunities to elucidate the molecular basis of Ub signaling. For example, monoubiquitinated α-Synuclein (α-Syn) and histone H2B bearing native isopeptide bonds were prepared and used to shed light on the role of monoubiquitination in regulating α-Syn aggregation and degradation by the proteasome (6, 7) and histone H2B functions within the nucleosomal context (8). Successful synthesis of all Lys-linked di-Ub chains with native isopeptide bonds were also achieved and proved to be instrumental in studies aimed at understanding the structures and dynamics of these chains, as well as their relative sensitivities towards deubiquitinases (9, 10). These advances provided access to new structural information about K33- and K6-linked di-Ub chains using NMR and X-ray crystallography, respectively (11, 12). More recently, tetra-Ub chains in a free form or anchored to a tripeptide were also synthesized chemically (13–15).

Despite these important achievements, one of the most important synthetic targets in these endeavors, the synthesis of homogeneous and well-defined polyubiquitinated proteins, has remained out of reach. Success in preparing these highly challenging targets is crucial for dissecting the effects of poly-Ub chain lengths and the role of a particular Ub chain in regulating protein structure and function in different cellular pathways. Toward achieving this goal, we sought to expand our tools and synthetic methodologies to enable the preparation of di- and tetraubiquitinated proteins in sufficient quantities for biophysical, biochemical, and cellular studies.

## Significance

**This study describes the nonenzymatic preparation of a protein conjugated to a tetraubiquitin Lys48-based chain made of native isopeptide bonds. We show that certain properties of our model protein, the Parkinson disease-associated protein α-Synuclein (α-Syn), are differentially regulated depending on the length of the conjugated ubiquitin (Ub) chain. In addition, we show that cross-talks between phosphorylation and ubiquitination may play important roles in regulating α-Syn aggregation and pathophysiology. The versatile synthetic strategy described here will enable future studies to further dissect the roles of Ub chain lengths and linkage types, conjugated in a site-specific manner, in regulating the physiological and pathological functions of α-Syn and other proteins.**

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Here, we report a semisynthetic strategy that enables site-specific conjugation of poly-Ub chains and generation of di- and tetraubiquitinated proteins of well-defined covalent structure. We focused our efforts on the conjugation of di- and tetra-Ub chains, because the effect of the chain length in protein degradation remains poorly understood and a tetra-Ub chain is proposed to be the shortest and minimal signal required for recognition and targeting of proteins for proteasomal degradation (16).

As a model system, we chose α-Syn, a 140-residue intrinsically disordered presynaptic protein that plays a central role in the pathogenesis of Parkinson disease (PD) and several other neurodegenerative diseases (collectively known as synucleinopathies). In diseased brains, α-Syn self-associates to form fibrillar aggregates, which are the primary constituents of Lewy bodies (LBs), the pathological lesions that define PD. LBs are immunoreactive for Ub (17, 18), and biochemical analyses of α-Syn purified or immunoprecipitated from LBs or insoluble α-Syn preparations (19) from a patient who had dementia with LBs demonstrated evidence for mono-, di-, tri-, and polyubiquitinated forms of the protein (17, 20). Similar ubiquitinated α-Syn species were also reported in cell culture models of synucleinopathies (17) and in in vitro ubiquitination assays (21, 22). Recent studies from our laboratories (6), which were independently confirmed by Meier and colleagues (23), demonstrated that monoubiquitination inhibits rather than promotes α-Syn fibrillization. These findings suggested that ubiquitination may play an important role in α-Syn aggregation and LB formation. However, very little is known about the effect of poly-Ub chain length on α-Syn aggregation and degradation or on how the pattern of ubiquitination [i.e., linkage type, anchoring site(s)] may influence the functional and pathogenic properties of α-Syn.

In this study, we describe chemical and semisynthetic strategies that enabled site-specific incorporation of K48-linked di- or tetraubiquitin chains onto the side chain of Lys12 of α-Syn. These advances provided unique opportunities to elucidate the role of ubiquitination in regulating α-Syn stability, aggregation, and clearance, and to investigate cross-talk between the two most common α-Syn pathological modifications found within LBs, phosphorylation and ubiquitination. Our results demonstrate that the length of the Ub chain plays an important role in regulating α-Syn fibril formation, phosphorylation, and clearance, and suggest that α-Syn structure, function, and pathophysiology could be under complex regulatory mechanisms involving cross-talk among different posttranslational modifications.

## Results

**Chemical Synthesis and Purification of Di- and Tetraubiquitinated α-Syn.** Although the syntheses of monoubiquitinated proteins and free di- and tetra-Ub chains have been accomplished (6, 10, 15), the synthesis of di- or tetraubiquitinated proteins has remained out of reach. Such synthesis requires site-specific and efficient coupling of the Ub chain to the desired residue in the protein of interest. In this study, we sought to determine the effect of K48-linked di- and tetra-Ub chains into the side chain of Lys12 on the biochemical properties of α-Syn. We chose to incorporate these Ub chains at position K12 in α-Syn because this residue was shown to be ubiquitinated in α-Syn isolated from LBs (20). However, the approach described here can be easily applied to conjugate poly-Ub chains at other putative ubiquitination sites in the N-terminal region of the protein.

We envisioned the development of semisynthetic approaches that allow the generation of di- and tetraubiquitinated forms of α-Syn via the incorporation of our δ-mercaptolysine at the desired lysine residue (24). We first synthesized α-Syn bearing a δ-mercaptolysine at K12 (K*12), using a chemically synthesized α-Syn(1–29)–thioester peptide and recombinant α-Syn(30–140) fragment harboring N-terminal Cys (Fig. 1) that was expressed in *Escherichia coli* and purified as described in *SI Materials and Methods*. This led to a more efficient synthesis of α-Syn[(1–140)-(K12K*)], compared with the previously described methods for the synthesis of α-Syn[(1–140)-(K6K*)] (6). This was due to our ability to prepare the thioester peptide [i.e., α-Syn(1–29)-thioester] using Fmoc-solid phase peptide synthesis, which allowed the use of the thiazolidine (Thz)-protected form of δ-mercaptolysine, hence enabling in situ deprotection of the δ-mercaptolysine subsequent to the native chemical ligation step (25). Previously, we used the acetamidomethyl (Acm)-protected version of δ-mercaptolysine (6), which required the isolation of the ligation product to remove the Acm protecting group.

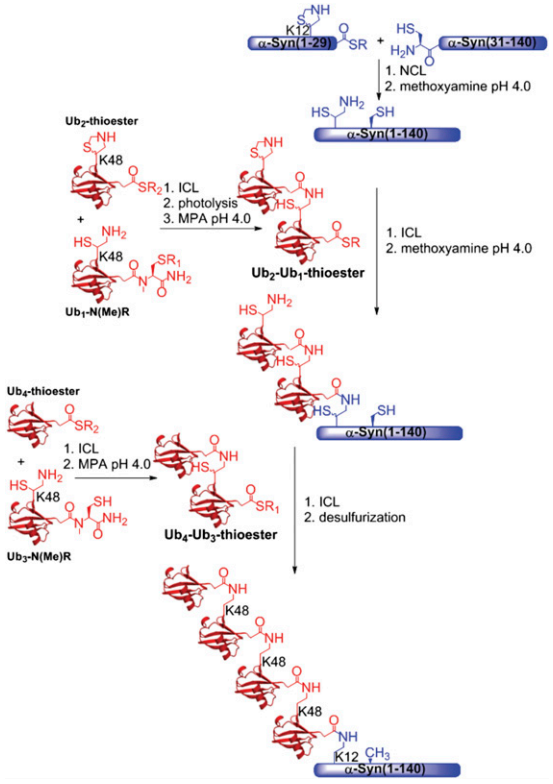


**Fig. 1.** Semisynthesis strategy for the preparation of di- and tetraubiquitinated α-Syn. The scheme shows how tetra-Ub–α-Syn was assembled from the recombinant fragment α-Syn[30–140(A30C)], the synthetic fragment α-Syn(1–29) K12K* thioester, and two synthetic diubiquitin thioester building blocks.

Next, we turned our attention to the most challenging aspect of the preparation of tetraubiquitinated α-Syn, namely the attachment of the K48-linked tetra-Ub chain, composed of 304 residues, to K*12 of α-Syn. This could, in principle, be achieved by preparing tetra-Ub–thioester for isopeptide chemical ligation (ICL) with α-Syn–bearing δ-mercaptolysine. However, several attempts to prepare the tetra-Ub–thioester using *N*-methylcysteine–mediated latent thioester formation (26) were unsuccessful. Despite being able to prepare the tetra-Ub chain with the proximal Ub having *N*-methylcysteine, we failed to identify conditions that allow efficient thioester formation. As an alternative approach, we envisioned the preparation of two different fragments of di-Ub–thioester, followed by two sequential ligation steps with α-Syn (Fig. 1). Here, the *N*-methylcysteine–mediated latent thioester formation was successful and enabled the preparation of $Ub_2$-$Ub_1$-thioester and $Ub_4$-$Ub_3$-thioester in high yields, sufficient for the ligation with α-Syn[(1–140)-(K12K*)]. Hence, α-Syn[(1–140)-(K12K*)] was ligated with $Ub_2$-$Ub_1$-thioester to give diubiquitinated α-Syn(1–140), $Ub_{1,2}$-Syn(1–140), in good yield, which was then subjected to methoxylamine treatment at pH 4 to expose the δ-mercaptolysine for the subsequent

ICL with $Ub_4$-$Ub_3$-thioester. The latter ligation also proceeded efficiently, as indicated by the disappearance of the $Ub_4$-$Ub_3$-thioester and the formation of tetraubiquitinated Syn(1–140), $Ub_{1,2,3,4}$-Syn(1–140), as a major product, as evidenced by HPLC-MS and SDS/PAGE analyses. The ligation mixture was then subjected to desulfurization using radical-based conditions [Tris (2-carboxyethyl)phosphine (TCEP), 2,2′-Azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044), and 2-methylpropane-2-thiol] (27), which was completed after 12 h (Fig. 2*D* and Fig. S1*B*).

To be able to study the effect of α-Syn di- or tetraubiquitination at K12, we attempted first to separate the two species from the unreacted materials [more specifically, $Ub_{1,2}$-Syn(1–140) and the unconjugated α-Syn]. After exploring several techniques, we found that only the use of gel-eluted liquid fraction entrapment electrophoresis (28), a molecular-weight–based purification technique akin to preparative SDS/PAGE, allowed the separation and isolation of highly pure (>95%) di- and tetraubiquitinated α-Syn (Fig. 2*A*). Western blot analysis confirmed that the purified proteins could be recognized by antibodies specific for anti–α-Syn and anti-Ub (Fig. 2*B*). We then verified the folding state of di- and tetraubiquitinated α-Syn by CD spectroscopy. The CD spectra shown in Fig. 2*C* are consistent with those of the correctly folded Ub and Ub chains as determined by comparison with previously published CD spectra of unanchored di- and tetra-Ub (10, 15), and they suggest that the α-Syn moiety remains unstructured, as previously observed with monoubiquitinated α-Syn at K6 (6).

**Tetraubiquitinated α-Syn Forms Soluble but SDS-Resistant High-Molecular Weight Aggregates.** Although polyubiquitinated α-Syn species have been detected in LBs and insoluble α-Syn–rich fractions from human brains, the role of poly-Ub in α-Syn aggregation and LB formation remains unknown. To examine the effect of Ub chain length on α-Syn fibril formation, we compared the aggregation propensities of WT and K12 tetraubiquitinated α-Syn. The in vitro fibrillization studies were carried out at a low protein concentration (5 μM), to mimic to the best possible extent the estimated physiological concentration of α-Syn in neurons (~1 μM) (29). At lower concentrations, α-Syn does not form fibrils readily in vitro. Using a Western blot assay, we monitored the amount of soluble protein during incubation at 37 °C with shaking. Under these conditions, WT α-Syn remains completely soluble (Fig. 3*A*) while tetraubiquitinated α-Syn rapidly forms soluble but SDS-resistant high-molecular weight species (Fig. 3*A*). Further analyses using transmission electron microscopy (TEM) confirmed that at 5 μM, WT α-Syn only forms mature amyloid fibrils after a long incubation time (8 d), whereas tetraubiquitinated α-Syn forms electron-dense aggregates that do not possess an amyloid-like structure or morphology (Fig. 3*B*). These amorphous tetra-Ub–α-Syn aggregates are already present at 84 h but do not proceed to form amyloid fibrils at longer incubation times (Fig. 3*B*). These results suggest that the addition of a tetra-Ub chain at K12 results in the formation of nonfibrillar aggregates but blocks α-Syn fibrillization in vitro.

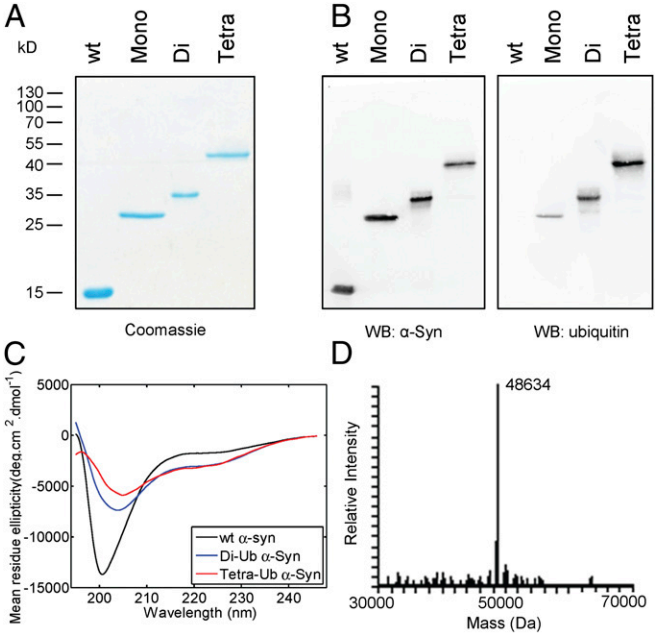


**Fig. 2.** Purity and folding analyses of ubiquitinated α-Syn. (*A*) SDS/PAGE/Coomassie staining of purified unconjugated (wt), mono-, di-, and tetraubiquitinated α-Syn. (*B*) SDS/PAGE/Western blot (WB) analysis of the same proteins as in *A*. The primary antibodies are indicated below each blot. Ub-conjugated α-Syn variants were purified using a 12% Tris·acetate GELFREE cartridge (Expedeon). (*C*) Characterization of di- and tetra-Ub–α-Syn by CD, in which wt (14.6 μM, black), di-Ub–α-Syn (6.71 μM, blue), and tetra-Ub–α-Syn (4.85 μM, red) are shown. (*D*) Electrospray ionization MS of tetraubiquitinated α-Syn was done to confirm the identity of the protein (also Fig. S1*B*). The observed mass of 48,634 Da is consistent with oxidation of the four methionine residues within α-Syn to the sulfoxide form (calculated: 48,639.2 Da).

**Cross-Talk Between Ubiquitination and C-Terminal Phosphorylation.** Both immunohistochemical (17, 22) and proteomic studies (20) have shown that α-Syn within LBs and other different inclusions can be ubiquitinated and phosphorylated at S129 and/or at Y125 (17, 22, 30, 31). Interestingly, exogenously prepared α-Syn fibrils are rapidly ubiquitinated and phosphorylated at S129 upon internalization in mammalian cell lines and following stereotaxic injection and uptake by neurons in mouse brain (32, 33). These findings suggest potential cross-talk between α-Syn phosphorylation at S129 and Y125 and ubiquitination. Nonaka et al. (34) suggested that S129 phosphorylation of α-Syn may not affect its ubiquitination, using an in vitro assay based on E3 ligases present in rabbit reticulocyte lysate. Recently, we showed that monoubiquitination at K6 has no major impact on α-Syn phosphorylation at S87 by casein kinase I and at S129 by casein kinase I, G protein-coupled receptor kinase 5, and polo-like kinase (PLK) 3 (6). To the best of our knowledge, the effects of polyubiquitination on both serine and tyrosine phosphorylation of α-Syn have not been investigated.

To determine whether the addition of Ub chains on N-terminal region lysine residues could affect C-terminal phosphorylation of α-Syn, we first investigated whether our semisynthetic proteins could be phosphorylated by PLK3, which efficiently phosphorylates α-Syn at S129 (35). Fig. 4*A* shows that no phosphorylation of S129 could be detected on K12 di- or tetraubiquitinated α-Syn, whereas monomeric unconjugated α-Syn used as an internal control was efficiently phosphorylated at S129. This suggests that the addition of a poly-Ub chain at K12 may disrupt the interaction between α-Syn and PLKs, consistent with previous studies from our group and others demonstrating that the N-terminal domain of α-Syn is important for α-Syn–PLK2/3 interaction and PLK-mediated α-Syn phosphorylation (35). For example, Wang et al. (36) showed that deletion of α-Syn's first nine residues completely abolished its interaction and phosphorylation by PLK2.

To determine whether there could be cross-talks between di- and tetraubiquitination at K12 and C-terminal tyrosine phosphorylation, we examined whether tetraubiquitinated α-Syn could be phosphorylated by the nonreceptor spleen tyrosine kinase (Syk), which is known to phosphorylate α-Syn at Y125, Y133, and, to a lesser extent, Y136 (37). Fig. 4*B* shows that diubiquitinated and tetraubiquitinated α-Syn are both phosphorylated at Y125 by Syk. However, both polyubiquitinated α-Syn species are destabilized upon phosphorylation and exhibited a tendency to precipitate, unlike WT α-Syn, which was used as an

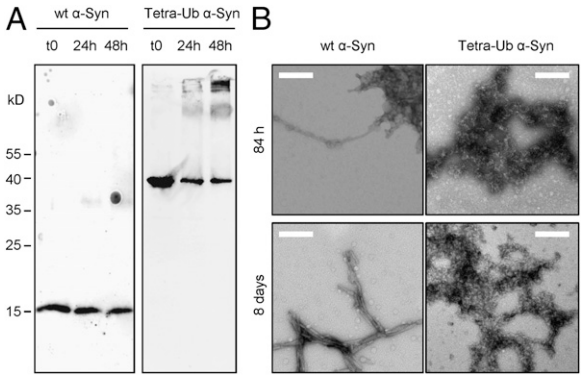


**Fig. 3.** Aggregation of tetraubiquitinated α-Syn. (*A*) wt and tetra-Ub–α-Syn (50 μL) were incubated at 5 μM in 50 mM Tris and 150 mM NaCl (TBS) (pH 7.5) with orbital shaking at 1,000 rpm. Aliquots (5 μL) were withdrawn at the indicated time points, diluted to 10 μL with TBS, and centrifuged (20,000 × *g*, 10 min, 4 °C). The supernatant (7 μL) was loaded on SDS/PAGE gels for WB analysis. (*B*) TEM analyses of uranyl acetate-stained samples analyzed after 84 h (*Upper*) and 8 d (*Lower*) of incubation. (Scale bars: 200 nm.)

internal control and remained soluble after Syk phosphorylation. Moreover, phosphorylated tetraubiquitinated α-Syn is significantly more sensitive to destabilization than its diubiquitinated counterpart, suggesting that the length of the K12-linked poly-Ub chain may affect the stability and solubility of C-terminally tyrosine-phosphorylated α-Syn (Fig. 4*B*, *Lower*). In the absence of Y125 phosphorylation and agitation, tetraubiquitinated α-Syn is stable and does not aggregate (Fig. 4*A*). It is noteworthy that phosphorylation at Y125 on its own does not influence the conformational or aggregation properties of α-Syn significantly (38). Together, our results suggest that the combination of phosphorylation at Y125 and tetraubiquitination renders α-Syn more susceptible to aggregation and supports our hypothesis for cross-talk between these two types of posttranslational modifications.

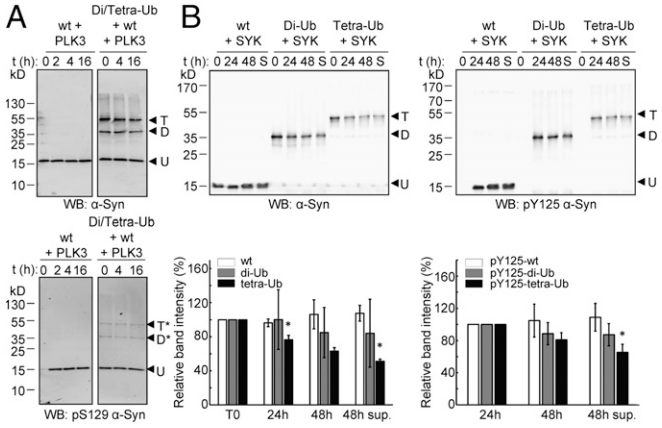


**Fig. 4.** Interplay between di- and tetraubiquitination of α-Syn at K12 and C-terminal phosphorylation. (*A*) K12 diubiquitination and K12 tetraubiquitination abolish α-Syn phosphorylation at S129 by PLK3. wt α-Syn alone (4 μM) or added to 4 μM diubiquitinated/tetraubiquitinated α-Syn mixture as an internal control was incubated with 0.42 μg of PLK3 and analyzed at the indicated time points by SDS/PAGE/Western blot (WB). (*Lower*) Bands indicated with an asterisk indicate bands not specific for pS129, because they are detected at $t = 0$ before adding the kinase. (*B*) Phosphorylation of the C-terminal tyrosines of α-Syn by SYK destabilizes tetraubiquitinated α-Syn, whereas monomeric, unconjugated wt α-Syn remains stable. Purified wt, diubiquitinated, or tetraubiquitinated α-Syn (3 μM) were incubated with 0.23 μg of SYK for the indicated time points and analyzed by SDS/PAGE/WB. (*Upper*) Representative WBs from three independent experiments. Lanes labeled "S" correspond to soluble fractions (supernatant after high-speed centrifugation) at $t = 48$ h. (*Lower*) Quantification of the band intensities in three independent experiments. Asterisks indicate significant differences between the tetra-Ub–α-Syn levels compared with wt α-Syn ($P < 0.05$, paired *t* tests). D, di-Ub–α-Syn; T, tetra-Ub–α-Syn; U, unconjugated wt α-Syn monomer.

**Effect of Mono-, Di-, and Tetraubiquitination on α-Syn Degradation by the Proteasome.** Several studies have shown that ubiquitination plays an important role in regulating α-Syn degradation by the proteasome (39–44). Interestingly, Tofaris et al. (45) showed that ubiquitination of α-Syn by Nedd4 targets it for degradation via the endosomal/lysosomal pathway. However, the nature of the ubiquitination signal required for targeting α-Syn for proteasomal or lysosomal degradation remained unexplored. Previous studies have also suggested that the affinity of protein-Ub adducts for the proteasome increases with increasing length of the poly-Ub chain, although a direct comparison using homogeneously modified substrates has not been possible (16). Herein, we sought to determine the effect of mono-, di-, and tetraubiquitination on the stability of α-Syn and its susceptibility to degradation by the proteasome. Purified mono-, di-, and tetraubiquitinated α-Syn were incubated with fraction II, which contains both 26S proteasomes and the entire cohort of deubiquitinases, and the extent of α-Syn deubiquitination and/or degradation was assessed by monitoring the levels of these proteins using Western blot analysis in the absence and presence of the proteasome inhibitor MG132.

As shown in Fig. 5 *A* and *B*, di- and tetraubiquitinated α-Syn are degraded efficiently by the proteasome in fraction II. In contrast, in the case of monoubiquitinated α-Syn, the single Ub moiety is efficiently cleaved well before the molecule is degraded (Fig. 5*C*). These findings strongly suggest that a possible important role of the poly-Ub chain is to protect the adduct from deubiquitinases activity and to enable a long enough residence time of the conjugated substrate on the proteasome to allow its efficient degradation. We have previously shown that monoubiquitinated α-Syn can be efficiently degraded by purified 26S proteasome (7). The current experiment was carried out in crude cell extract that contains both 26S proteasomes and the entire cohort of deubiquitinases, which represents the intracellular environment more faithfully. In the experiment carried out with purified proteasome, soluble deubiquitinases were not present and the proteasomal deubiquitinases were inhibited by *o*-phenanthrolene as a metal chelator. Under these conditions, the monoubiquitinated substrate was protected from all deubiquitinases activities. It is still not clear whether the poly-Ub chain provides true protection or whether its gradual cleavage allows the substrate the time necessary for its capture and degradation by the proteasome. It should be noted that the hypothesis that one role of the poly-Ub chain is to provide protection against the activity of deubiquitinases was previously raised by Schaefer and Morgan (46). However, they demonstrated it on conjugates containing chains with different lengths but not under conditions where the deubiquitinases and the proteasomes are present in the system in their physiological concentrations, allowing the two to "compete" in displaying the effect of the chain length on the fate of the protein: deubiquitination or degradation.

## Discussion

Our ability to synthesize and conjugate site-specifically mono-, di-, and tetra-Ub chains of defined covalent structure has provided unique opportunities to elucidate the role of ubiquitination in regulating α-Syn stability, aggregation, phosphorylation, and clearance. Our observation that diubiquitinated α-Syn and tetraubiquitinated α-Syn, but not monoubiquitinated α-Syn, are more resistant to deubiquitinases and are degraded by the

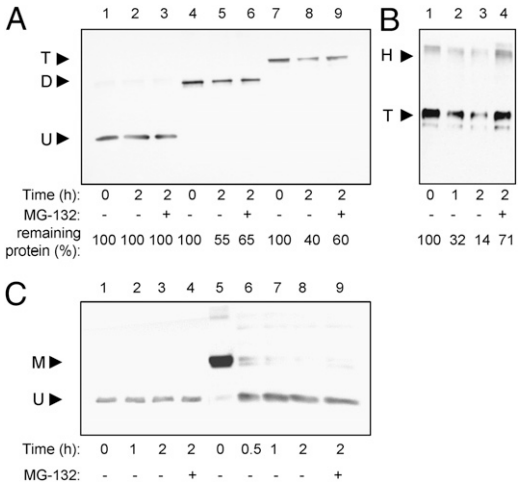


**Fig. 5.** Proteasomal degradation of ubiquitinated α-Syn in a cell-free reconstituted system. (*A*) Degradation of unconjugated (lanes 1–3), diubiquitinated (lanes 4–6), and tetraubiquitinated (lanes 7–9) α-Syn in crude fraction II. The corresponding proteins were incubated in the presence of crude fraction II for the indicated times in the absence or presence of the proteasome inhibitor MG132 as specified. Following incubation, samples were analyzed by SDS/PAGE/Western blot. (*B*) Repeat of the experiment depicting the degradation of tetraubiquitinated α-Syn. In both experiments, the percentage of remaining protein is shown. (*C*) Susceptibility of monoubiquitinated α-Syn to the activity of deubiquitinases. α-Syn (lanes 1–4) and monoubiquitinated α-Syn (lanes 5–9) were incubated for the indicated times in the presence of crude fraction II in the presence and absence of the proteasome inhibitor MG132. D, di-Ub–α-Syn; H, high-molecular weight species; M, mono-Ub–α-Syn; T, tetra-Ub–α-Syn; U, unconjugated α-Syn monomer.

proteasome suggests that polyubiquitination serves as a signal for α-Syn degradation by the proteasome.

Our findings are consistent with the emerging view that ubiquitination is not required for α-Syn fibrillization and LB formation. This hypothesis is supported by previous studies showing that not all α-Syn within LBs is ubiquitinated (19), as well as our own findings that monoubiquitination stabilizes monomeric α-Syn and tetraubiquitination favors the formation of nonfibrillar aggregates. Furthermore, polyubiquitination of α-Syn by the E3 ligase carboxy-terminus of Hsp70-interacting protein (CHIP) in a cell-free system and in cells results in significant reduction of the amount of insoluble α-Syn species (43) and oligomers (21). These results suggest that mono- or polyubiquitination of α-Syn inhibits its fibrillization, and they are likely to occur after fibril formation. Interestingly, in vitro ubiquitination of preformed α-Syn fibrils results in mono- and diubiquitination patterns similar to those observed in pathological brain samples (20). Finally, recent studies showed that exogenous α-Syn fibrils become rapidly ubiquitinated upon internalization into neurons in culture as well as in mouse brain (32, 33). Together, these results suggest that ubiquitination of α-Syn may occur after α-Syn fibril formation and could represent an active cellular response to disassociate these aggregates and/or promote clearance of α-Syn by targeting it for degradation by the proteasome.

Despite several reports demonstrating that ubiquitinated α-Syn is also phosphorylated at its C terminus (S129 and Y125) (17, 22, 30, 31), the interplay across these modifications has not previously been investigated. Our results demonstrate that the effect of phosphorylation at Y125 on α-Syn aggregation is dependent on the length of the poly-Ub chain. Tetraubiquitinated α-Syn exhibited higher aggregation propensity than the diubiquitinated α-Syn, whereas monoubiquitinated α-Syn does not aggregate upon phosphorylation at Y125. The mechanism by which phosphorylation enhances the aggregation of tetraubiquitinated α-Syn is not clear, but it is plausible that the combination of tetraubiquitination and phosphorylation at Y125 induces conformational changes that lead to the formation of an aggregation-prone structure. These results suggest that α-Syn function in health and/or disease could be under complex regulatory mechanisms involving cross-talk among different posttranslational modifications.

The synthesis of tetraubiquitinated α-Syn represents an example of a previously undescribed semisynthesis of a polyubiquitinated protein of defined covalent structure and is a significant advance in the fields of chemical synthesis of proteins and Ub research. These advances, combined with recent improvements in Ub bioconjugate synthesis, provide unique opportunities for introducing various fluorescent labels and chemical probes to enable investigation of the role of the ubiquitination signal on the structure, function, and interactome of proteins with spatial and temporal resolution. Our work perfectly illustrates the power and potential of combining synthetic protein chemistry enabled by modern chemical ligation methods with recombinant DNA molecular biology to address fundamental questions in biochemistry and cell biology, especially the all-important fine details of the ubiquitination signaling pathways.

## Materials and Methods

**Generation of Tetraubiquitinated α-Syn at K12.** ***Ligation of α-Syn[1–29(K12K*)]–thioester and α-Syn[30–140(A30C)].*** The ligation was carried out as previously described for monoubiquitinated synuclein (6) starting from α-Syn[1–29 (K12K*)] peptide thioester (4.16 mg, 1.3 μmol) and α-Syn[30–140(A30C)] (10 mg, 0.87 μmol), generating the ligation product at a yield of ∼56% (7.1 mg).
***Synthesis of $Ub_{12}$–α-Syn(1–140).*** Purified peptide $Ub_2$-$Ub_1$-thioester (6.2 mg, 0.36 μmol) was dissolved in 69 μL of argon-purged 6 M guanidine·HCl and 200 mM phosphate buffer (pH ∼7.0). In a separate Eppendorf tube, the α-Syn[1–140(K12K*)] (4 mg, 0.27 μmol) was dissolved in a 69-μL solution of 4-mercaptophenylacetic acid (MPAA) and TCEP (20 eq and 25 eq, respectively) in argon-purged guanidine·HCl and phosphate buffer pH 7, and both solutions were mixed together. The mixture was left at 37 °C for overnight. Subsequently, the mixture was treated with methoxylamine·HCl (0.2 M) and TCEP (30 eq) at pH 4 for 12 h at 37 °C to unmask the δ-mercaptolysine. The reaction was followed by liquid chromatography MS using a C4 analytical column and a gradient of 5–25–60% B over 45 min. For preparative HPLC, a similar gradient was used to afford the ligation product $Ub_{12}$–α-Syn(1–140) at a yield of ∼47% (4.0 mg).
***Synthesis of $Ub_{1234}$-Syn(1–140).*** Purified peptide $Ub_3$-$Ub_4$-thioester (2.8 mg, 0.16 μmol) was dissolved in 30 μL of argon-purged 6 M guanidine·HCl and 200 mM phosphate buffer (pH ∼7.0). In a separate Eppendorf tube, $Ub_{12}$-Syn [1–140(K12K*)] (4 mg, 0.13 μmol) was dissolved in a 33-μL solution of MPAA and TCEP (20 eq and 25 eq, respectively) in argon-purged guanidine·HCl and phosphate buffer (pH 7), and both solutions were mixed together. The mixture was left at 37 °C overnight. The reaction was followed using an analytical column and a gradient of 5–25–60% B over 45 min. For preparative HPLC, a similar gradient was used to afford the ligation product $Ub_{1234}$–α-Syn(1–140) at a yield of ∼39% (2.4 mg).
***Desulfurization.*** The reaction was carried out as previously described (15) to afford desulfurized protein $Ub_{1234}$–α-Syn(1–140) at a yield of 56% (1.3 mg).

**In Vitro Phosphorylation Assays.** Phosphorylation of WT and ubiquitinated α-Syn was carried out by incubation of the proteins with 0.42 μg of PLK3 kinase (PV3812; Invitrogen) in 50 mM Hepes (pH 7.4) in the presence of 1 mM $MgCl_2$, 1 mM EGTA, 2 mM Mg-ATP, and 1 mM DTT for 16 h at 30 °C in a total volume of 50 μL. For the phosphorylation with PLK3, the substrate protein concentration was 4 μM. Phosphorylation of WT and ubiquitinated α-Syn at Y125 was carried out by incubation with 0.23 μg of Syk kinase (PV3857; Invitrogen) in 50 mM Tris (pH 7.5) in the presence of 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 2 mM Mg-ATP, and 1 mM $Na_3VO_4$ for up to 48 h at 30 °C in a total volume of 50 μL. The final substrate protein concentration was 3 μM.

**Degradation Assays.** Purified α-Syn (∼0.5 μg), monoubiquitinated α-Syn (∼0.75 μg), diubiquitinated α-Syn (∼1.0 μg), and tetraubiquitinated α-Syn (∼1.5 μg) were incubated at 37 °C for the indicated times in a reaction mixture containing in a final volume of 12.5 μL 25 μg of fraction II (Ub-depleted HeLa cell extract), supplemented with ATP and the ATP-regenerating system as described previously (47). When indicated, the proteasome inhibitor MG132 was added at a concentration of 100 μM. Reactions were terminated by the addition of threefold concentrated sample buffer. Boiled samples were resolved via

SDS/PAGE (15%), and proteins were visualized after blotting to a nitrocellulose membrane using anti–α-Syn antibody and chemiluminescence. Band intensities were quantified using the Total Labs TL100 1D gel analysis software.

**Aggregation Assays.** Purified proteins [5 μM in Tris-buffered saline (TBS) buffer (50 mM Tris, 150 mM NaCl [pH 7.5]) at a final volume of 50 μL] were incubated at 37 °C with orbital shaking at 1,000 rpm. For Western blot analyses, 5-μL aliquots were diluted to 10 μL in TBS and centrifuged at 20,000 × *g* for 10 min at 4 °C. The supernatant (7 μL) was mixed with 2× Laemmli sample buffer and loaded on 15% SDS/PAGE gels. Western blot processing was done as described above. For TEM analyses, 5-μL aliquots were mixed with 5 μL of TBS, and 5 μL was deposited on Formvar/carbon-coated 200-mesh copper grids (Electron Microscopy Sciences). Grids were then washed twice with 5 μL of ultrapure water, stained twice with 5 μL of 2% (wt/vol) uranyl acetate (Electron Microscopy Sciences), and then vacuum-dried. Grids were imaged using a Tecnai Spirit BioTWIN electron microscope equipped with a $LaB_6$ source operated at 80 kV. Digital micrographs were recorded with a 4096 × 4096 pixels FEI Eagle CCD camera (FEI).

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