



## Proteasome inhibition induces $\alpha$ -synuclein SUMOylation and aggregate formation

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### ABSTRACT

Parkinson's disease (PD) and Dementia with Lewy Bodies (DLB) are characterized pathologically by intraneuronal inclusions called Lewy bodies (LBs) and Lewy neurites. A major component of these inclusions is the protein  $\alpha$ -synuclein, which is natively unfolded but forms oligomers and insoluble fibrillar aggregates under pathological conditions. Although  $\alpha$ -synuclein is known to undergo several posttranslational modifications, the contribution of SUMOylation to  $\alpha$ -synuclein aggregation and the pathogenesis of  $\alpha$ -synucleinopathies have not been elucidated. Here, we provide evidence that aggregates and inclusions formed as a result of impaired proteasome activity contain SUMOylated  $\alpha$ -synuclein. Additionally, SUMO1 is present in the halo of LBs colocalizing with  $\alpha$ -synuclein in the brains of PD and DLB patients. Interestingly, SUMOylation does not affect the ubiquitination of  $\alpha$ -synuclein. These findings suggest that proteasomal dysfunction results in the accumulation of SUMOylated  $\alpha$ -synuclein and subsequently its aggregation, pointing to the contribution of this posttranslational modification to the pathogenesis of inclusion formation in  $\alpha$ -synucleinopathies.

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### 1. Introduction

Parkinson's disease (PD) and Dementia with Lewy Bodies (DLB) are progressive neurodegenerative disorders characterized by the presence of cytoplasmic inclusions in surviving neurons called Lewy bodies (LBs) and Lewy neurites [1]. A major component of these inclusions is the protein  $\alpha$ -synuclein which accumulates as insoluble fibrillar aggregates. Multiple factors promote the aggregation of this natively unfolded protein [2–5] including post-translational modifications. These include phosphorylation [6,7], nitration [8,9] and ubiquitination [10,11]. These modifications affect the ability of  $\alpha$ -synuclein to form aggregates and its sub-cellular localization.

The intracellular 26S proteasome is the major proteolytic complex responsible for ubiquitin-mediated substrate degradation in eukaryotic cells. Impaired proteasome activity is implicated in the patho-

genesis of several neurodegenerative disorders including PD [12]. Postmortem analyses of brains from sporadic PD patients have demonstrated relatively low proteasomal activity in the substantia nigra, which bears the brunt of pathology in this disease [13–15]. The origin of proteasomal dysfunction in these neurodegenerative disorders remains largely unknown.

Small ubiquitin-like modifier (SUMO) belongs to a family of ubiquitin-related proteins and is covalently conjugated to lysine residues in its substrates. SUMOylation occurs through a series of enzymatic reactions analogous to that of the ubiquitination pathway, and this process leads to the modification of the biochemical and functional properties of substrates [16]. To date, four mammalian SUMO isoforms, a single heterodimeric SUMO-activating E1 enzyme SAE1/SAE2, a single SUMO-conjugating E2 enzyme UBE2I (also known as Ubc9 in yeast), and several families of SUMO E3 ligases have been identified [16].

The implications of SUMOylation have been studied in relation to the pathogenesis of several neurodegenerative disorders [17–23]. Although  $\alpha$ -synuclein has been reported to be SUMOylated in cultured cells [24], the contribution of this posttranslational modification to the pathological picture of  $\alpha$ -synucleinopathies was not established. Here, we provide evidence that impaired proteasomal function significantly promotes the formation of SUMOylated  $\alpha$ -synuclein containing aggregates in aggresome-like structures in cultured cells as well as in the LBs of PD and DLB affected brains.

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## 2. Methods

### 2.1. Chemicals and antibodies

MG-132 was purchased from EMD Chemicals (Gibbstown, NJ, USA). The mouse monoclonal anti- $\alpha$ -synuclein (Syn-1) antibody was purchased from BD Biosciences (San Jose, CA, USA), and mouse monoclonal anti-SUMO1 (GMP-1) from Zymed Laboratories (San Francisco, CA, USA). Rabbit polyclonal anti-SUMO1, anti-ubiquitin, and anti-HA antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA, USA). FuGene 6 transfection reagent was purchased from Roche Applied Science (Indianapolis, IN, USA). Protein A-Sepharose beads were from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Unless otherwise specified, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

### 2.2. Plasmid constructs

The mammalian plasmid vector expressing human  $\alpha$ -synuclein was previously described [25]. The mature form of human SUMO1 cDNA was isolated by PCR using a human brain cDNA library and subcloned into pHM-6 (Roche Applied Science) to generate HA-tagged wild-type SUMO1 (HA-SUMO1-GG). The conjugation-defective SUMO1 mutant (HA-SUMO1- $\Delta$ GG), which lacks the C-terminal Gly-Gly motif required for conjugation of SUMO-1 to substrates [16,26,27], was generated using PCR.

### 2.3. Cell culture and DNA transfection

COS-7 cells were maintained in DMEM supplemented with 10% FBS and 100 units/mL penicillin–streptomycin (Invitrogen). Cells were transfected using FuGene 6 (Roche), according to the supplier's instructions.

### 2.4. Immunocytochemistry

Cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS, and permeabilized with 0.2% Triton X-100 in PBS. Cells were then incubated with the indicated primary antibodies, which were diluted in PBS containing 1% bovine serum albumin (BSA), for 24 h at 4 °C. After washing with PBS three times, cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 594-conjugated goat anti-rabbit antibodies (Molecular Probes, Eugene, OR, USA), which were diluted in PBS containing 1% BSA for 2 h. For nuclear staining, cells were incubated with 1  $\mu$ g/mL 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) in PBS for 5 min. After washing with PBS three times, the cells were analyzed under a fluorescence microscope (IX71, Olympus).

### 2.5. Immunohistochemistry

Postmortem brain tissue specimens from patients with PD and DLB were fixed in paraformaldehyde for 2 weeks and embedded in paraffin. Six-micrometer sections from the substantia nigra were immunostained with mouse monoclonal anti-SUMO1 antibody or rabbit polyclonal anti-SUMO1 antibody using the avidin–biotin–peroxidase method (EnVision Plus kit, DAKO, Glostrup, Denmark), described previously [25].

### 2.6. Western blot analysis

After rinsing with ice-cold PBS twice, cells were lysed with ice-cold lysis buffer consisting of 10 mM Tris (pH 7.4), 1% Triton X-100, 150 mM NaCl, 10% glycerol, 20 mM N-ethylmaleimide, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1  $\mu$ g/mL leupeptin, 1  $\mu$ g/mL aprotinin, 10 mM NaF, and 0.2 mM

phenylmethylsulfonyl fluoride for 1 h. Lysates were centrifuged at 15,000 $\times$ g for 20 min at 4 °C. The supernatant was recovered as the soluble fraction. The pellet was washed twice with the lysis buffer, dissolved in lysis buffer supplemented with 4% SDS and boiled for 30 min, yielding the insoluble fraction. The concentration of proteins in each fraction was determined using the BCA Protein Assay Reagent Kit (Roche). Western blotting was performed as described previously [25]. To perform immunoprecipitation of  $\alpha$ -synuclein from the Triton X-100-insoluble fraction, detergent-insoluble fraction (~100  $\mu$ g) was diluted 20-fold with PBS, and pre-cleared using Protein A-Sepharose beads (Amersham). Pre-cleared lysates were incubated with anti- $\alpha$ -synuclein antibody (SYN-1) overnight at 4 °C and then incubated with Protein A-Sepharose beads for 2 h at 4 °C with gentle rotation. After washing with the lysis buffer four times, immunoprecipitated proteins were analyzed by Western blotting.

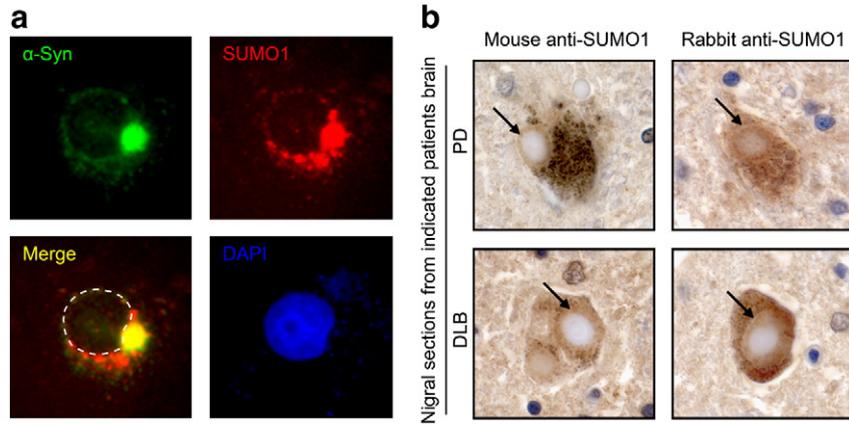
## 3. Results

### 3.1. $\alpha$ -Synuclein co-localizes with SUMO1 in aggresome-like structures and in LBs

To gain insight into the physiological relevance of  $\alpha$ -synuclein SUMOylation, we investigated whether SUMO modification is closely associated with the formation of  $\alpha$ -synuclein aggregates. After co-transfection with plasmids encoding  $\alpha$ -synuclein and HA-SUMO1-GG, COS-7 cells were treated with the non-specific proteasome inhibitor MG-132. Immunofluorescence staining with antibodies specific to  $\alpha$ -synuclein and HA revealed that MG-132 treatment promoted the formation of  $\alpha$ -synuclein- and SUMO1-containing aggresome-like structures in the juxtannuclear area (Fig. 1a), similar to inclusions reported by Tanaka et al. [28]. To investigate whether SUMOylated proteins are also present in the brains of patients with  $\alpha$ -synucleinopathies, we performed immunohistochemistry for SUMO1 in mid-brain sections of brains through the substantia nigra pars compacta from patients with PD and DLB. As shown in Fig. 1b, immunoreactivity to SUMO1 is detected in the halo of LBs in both PD and DLB brains (Fig. 1b), which is similar to the staining pattern for  $\alpha$ -synuclein [25]. Omission of primary SUMO1 antibody did not reveal any significant signal (data not shown). These results suggest that SUMO and  $\alpha$ -synuclein co-localize in the LBs of PD and DLB brains.

### 3.2. Proteasome inhibition induces the SUMOylation of $\alpha$ -synuclein

The effects of proteasomal impairment on soluble  $\alpha$ -synuclein levels and insoluble  $\alpha$ -synuclein aggregates were analyzed next. After COS-7 cells were transfected with  $\alpha$ -synuclein and treated with MG-132 for 18 h, lysates were sequentially extracted with 1% Triton X-100 (soluble) and 4% SDS (insoluble), and subjected to Western blotting with an anti- $\alpha$ -synuclein antibody. As shown in Fig. 2, MG-132 treatment increased soluble monomeric  $\alpha$ -synuclein levels, consistent with a reduction in  $\alpha$ -synuclein clearance by the proteasome [10]. Furthermore, proteasomal inhibition significantly increased the accumulation of high molecular weight (HMW)  $\alpha$ -synuclein aggregates in the detergent-insoluble fraction. To further examine whether the HMW  $\alpha$ -synuclein included the SUMOylated form following MG-132 exposure, the insoluble fraction was immunoprecipitated with anti- $\alpha$ -synuclein antibody followed by Western blotting with either anti-SUMO1 or anti-ubiquitin antibody. As expected, complexes immunoprecipitated with anti- $\alpha$ -synuclein antibody consisted largely of ubiquitinated  $\alpha$ -synuclein following MG-132 treatment (Fig. 2b). Notably, anti- $\alpha$ -synuclein immunocomplexes following MG-132 treatment were also SUMOylated (Fig. 2b). In addition, MG-132 treatment significantly increased the levels of SUMOylated and ubiquitinated proteins in the detergent-insoluble fraction (Fig. 2a). These results suggest that impaired proteasomal function significantly

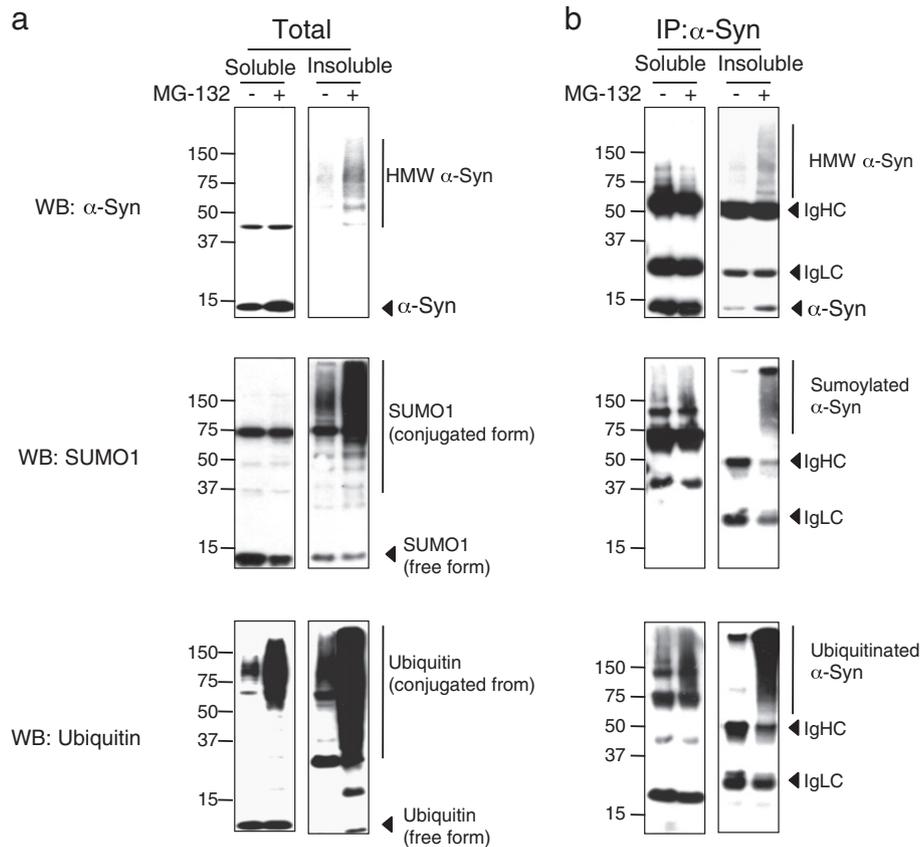


**Fig. 1.**  $\alpha$ -Synuclein and SUMO1 co-localize to aggresome-like structures in COS-7 cells and in Lewy bodies in PD and DLB patients' brains. (a) COS-7 cells were co-transfected with plasmids encoding  $\alpha$ -synuclein ( $\alpha$ -Syn) and HA-SUMO1-GG and then treated with MG-132 (5  $\mu$ M) for 18 h. Immunocytochemical staining was done with anti- $\alpha$ -Syn and anti-HA antibodies. Expression of  $\alpha$ -Syn (green) and HA-SUMO-GG (red) and DAPI-stained nuclei (blue) were observed using fluorescence microscopy. The merged image (yellow) indicates the overlay of the two proteins, and the dotted line indicates the nuclear outline. (b) Nigral sections obtained from brain specimens from patients with PD and DLB were analyzed by immunohistochemistry using mouse or rabbit-derived anti-SUMO1 antibodies, as indicated. Black arrows point to the halo of the Lewy bodies.

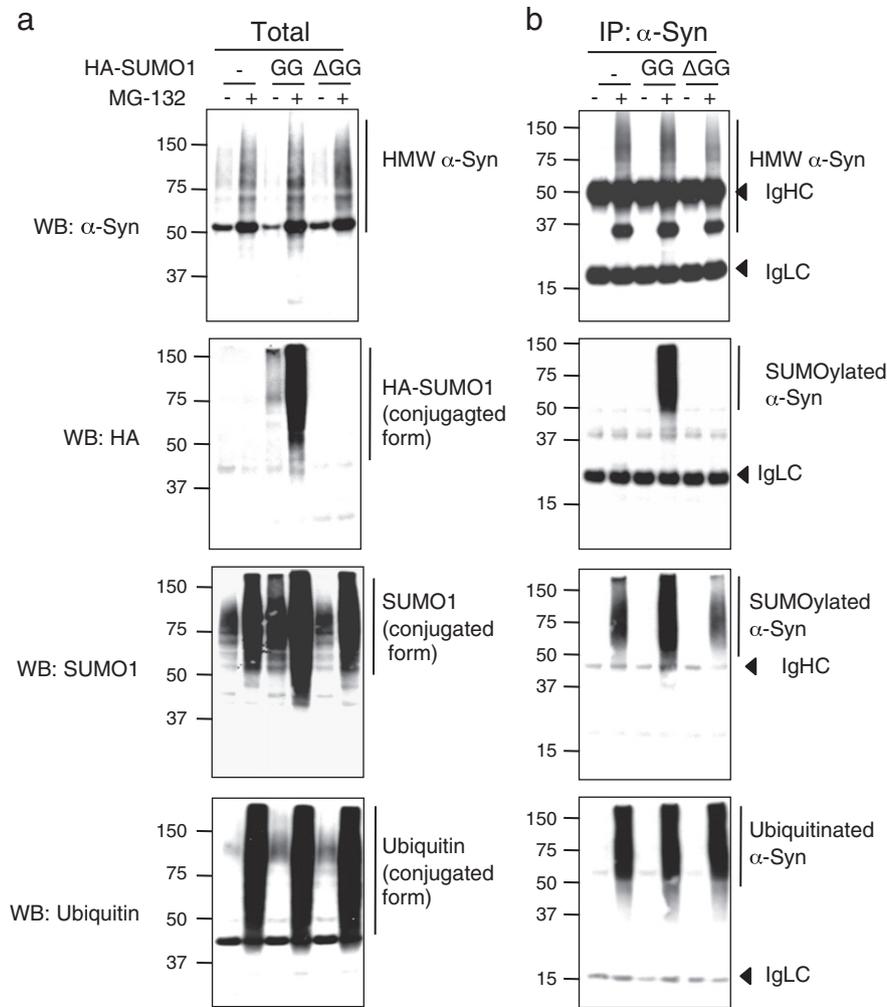
promotes the accumulation of both SUMOylated and ubiquitinated HMW  $\alpha$ -synuclein in the detergent-insoluble fraction.

Since both ubiquitination and SUMOylation target lysine residues, we addressed the question whether SUMOylation could influence the ubiquitination of  $\alpha$ -synuclein. COS-7 cells were transfected with  $\alpha$ -synuclein alone or together with either HA-SUMO1-GG or HA-SUMO1- $\Delta$ GG. The basis of this approach is because SUMO is synthesized as a precursor protein and processed by specific proteases to its mature

form to expose the Gly-Gly motif at the C-terminus, which is required for conjugation to Lys residues of substrates [16,26,27]. Therefore, SUMO lacking the Gly-Gly motif is unable to be conjugated to substrates. After treating the cells with MG-132 for 18 h, the amount of  $\alpha$ -synuclein aggregation was measured in the detergent-insoluble fraction. As shown in Fig. 3a, the formation of HMW  $\alpha$ -synuclein increased substantially in the detergent-insoluble fraction following MG-132 treatment. Furthermore, the immunoprecipitation of this



**Fig. 2.** SUMOylation and ubiquitination of  $\alpha$ -synuclein are promoted by MG-132. COS-7 cells were transfected with a plasmid expressing  $\alpha$ -synuclein ( $\alpha$ -Syn) and then treated with MG-132 (5  $\mu$ M) for 18 h. Lysates were fractionated into 1% Triton X-100-soluble and -insoluble fractions. (a) Both fractions were analyzed by Western blotting using the indicated antibodies. (b) Soluble and insoluble fractions were immunoprecipitated with an anti- $\alpha$ -Syn antibody followed by Western blotting using the indicated antibodies. (IgHC, immunoglobulin heavy chain; IgLC, immunoglobulin light chain).



**Fig. 3.**  $\alpha$ -Synuclein ubiquitination does not depend on SUMOylation. COS-7 cells were transiently transfected with  $\alpha$ -synuclein ( $\alpha$ -Syn) alone or together with either HA-SUMO1-GG or HA-SUMO1- $\Delta$ GG. Cells were then treated with MG-132 (5  $\mu$ M) for 18 h, and lysates were fractionated into 1% Triton X-100-soluble and -insoluble fractions. (a) The insoluble fraction was analyzed by Western blotting using the indicated antibodies. (b) Immunoprecipitation of the insoluble fraction with an anti- $\alpha$ -Syn antibody followed by Western blotting using the indicated antibodies was performed. (IgHC, immunoglobulin heavy chain; IgLC, immunoglobulin light chain).

detergent-insoluble fraction with anti- $\alpha$ -synuclein antibody followed by Western blotting with anti-HA antibody revealed that the HMW  $\alpha$ -synuclein aggregates are heavily SUMOylated in cells expressing both  $\alpha$ -synuclein and HA-SUMO1-GG (Fig. 3b). On the other hand, no HA-positive HMW bands were detected in cells transfected with  $\alpha$ -synuclein alone or in cells co-transfected with  $\alpha$ -synuclein and the conjugation-defective mutant HA-SUMO1- $\Delta$ GG (Fig. 3b). These findings indicate that proteasomal inhibition induces covalent modification of  $\alpha$ -synuclein by the process of SUMOylation, rather than non-covalent interaction of  $\alpha$ -synuclein and SUMO1.

Notably, the SUMOylation of  $\alpha$ -synuclein increased significantly in cells expressing  $\alpha$ -synuclein with HA-SUMO1-GG compared to cells with  $\alpha$ -synuclein alone, whereas the levels of the ubiquitinated  $\alpha$ -synuclein was not affected by the presence or absence of HA-SUMO1-GG (Fig. 3b). This result suggests that  $\alpha$ -synuclein ubiquitination and SUMOylation events are independent processes, and likely occur at different lysine residue(s) in  $\alpha$ -synuclein.

#### 4. Discussion

Accumulating evidence suggests that posttranslational modifications of  $\alpha$ -synuclein are important regulators of its pathogenic role in neurodegenerative diseases. For example, proteasomal impairment leads to the accumulation of ubiquitinated  $\alpha$ -synuclein [11], and

phosphorylation promotes its aggregation [7,29]. Here we demonstrate that insoluble  $\alpha$ -synuclein aggregates are also SUMOylated both in cultured cells subjected to proteasomal impairment in the form of perinuclear aggresomes as well as in the halo of LBs in PD and DLB affected brains. Similar immunoreactivity to SUMO-1 has been detected in  $\alpha$ -synuclein-positive glial cytoplasmic inclusions in Multiple System Atrophy, and in perinuclear aggregates in C6 glioma cells challenged with proteasome inhibitor [20]. Whether SUMOylation per se leads to  $\alpha$ -synuclein aggregation remains to be determined.

Similar to ubiquitination, the SUMO E2 enzyme UBE2I conjugates SUMO onto its substrates by a covalent bond. This conjugation occurs through direct binding of UBE2I to the consensus SUMOylation motif  $\Psi$ KXD/E (where  $\Psi$  is a large hydrophobic residue; K is a modified lysine residue; X is any residue; and D/E is an aspartic acid or a glutamic acid) in the substrate protein [30,31]. Sequence analysis of  $\alpha$ -synuclein revealed 15 lysine residues. Mutagenesis of three of these residues (K12, K96 and K102) having high predicted scores for SUMO attachment did not affect MG-132-induced  $\alpha$ -synuclein SUMOylation (data not shown), suggesting that other sites within  $\alpha$ -synuclein could be target(s) for SUMOylation, particularly since about 26% of SUMOylation occurs at lysine residues outside the consensus motif [32]. Further studies focused on identifying the SUMOylation sites in this protein are warranted.

Ubiquitination and SUMOylation pathways often compete with each other because they target the same lysine residues in their substrates. For

example, SUMOylation of the pathogenic huntingtin fragment prevents ubiquitination at the same lysine residue resulting in abrogation of proteasome-mediated degradation [19].  $\alpha$ -Synuclein is degraded by the proteasome in both ubiquitin-dependent [10] and -independent manners [33]. Nonetheless, our findings indicate that MG-132-induced ubiquitination of  $\alpha$ -synuclein is not reduced by SUMO1 overexpression, even though MG-132 also triggers the SUMOylation of  $\alpha$ -synuclein. Thus, the ubiquitination and SUMOylation of  $\alpha$ -synuclein appear to not involve the same lysine residue. On the other hand, the relationship between SUMOylation and ubiquitination is quite complex, and in many cases including those where the same lysine residue is targeted, the two modifications do not simply antagonize or compete with each other [reviewed in [16]].

In addition to altering the sub-cellular localization and function of target proteins, SUMOylation affects their solubility and subsequent aggregation. Analogous to our present finding about the role of SUMOylation in promoting  $\alpha$ -synuclein aggregation, ataxin-1 also undergoes a similar increased aggregation, a process that is further enhanced by oxidative stress [22]. However, other pathogenic proteins association with other neurodegenerative disorders may behave differently as a result of SUMOylation. For example, SUMOylation of amyloid precursor protein (APP) at lysine residues immediately adjacent to the  $\beta$ -secretase cleavage site results in decreased levels of A $\beta$  aggregates [21]. The latter finding has even raised the suggestion that up-regulating the activity of the cellular SUMOylation machinery as a potential therapeutic approach for Alzheimer's disease. In relation to Huntington's disease, SUMOylation can increase huntingtin accumulation, decrease aggregate formation, possibly increasing the amount of toxic oligomers [19]. Thus, the contribution of SUMOylation to target protein aggregation appears quite variable depending on the substrate protein and experimental paradigm. In the case of  $\alpha$ -synuclein, we find evidence that proteasome inhibition induces aggregate formation possibly through SUMOylation, which increases in the presence of wild type SUMO1 but not the conjugation-defective SUMO1.

Methodological considerations can be critical in detecting the degree and pattern of SUMOylation of particular proteins. In our hands,  $\alpha$ -synuclein SUMOylation is present at detectable levels even in the absence of over-expressed HA-tagged SUMO1. By immunoprecipitating  $\alpha$ -synuclein rather than SUMO1 followed by detecting the conjugated SUMO1 moieties by Western analysis, we were able to capture most of the SUMOylated  $\alpha$ -synuclein species. An opposite paradigm where SUMO1 is purified followed by Western blotting for  $\alpha$ -synuclein [24] does not detect  $\alpha$ -synuclein that is SUMOylated by cellular endogenous SUMO1, which is substantial according to our data. Therefore, caution should be exercised in comparing results obtained from different experimental paradigms.

In summary, our results show that impaired proteasome function significantly increases the formation of SUMOylated  $\alpha$ -synuclein-containing aggregates in COS-7 cells. Furthermore, we demonstrate that  $\alpha$ -synuclein and SUMO1 co-localize in aggresome-like structures in cells and in the LBs of brains from PD and DLB patients. Taken together, our observations suggest that impaired proteasomal function triggers the SUMOylation of  $\alpha$ -synuclein and its aggregation providing further insight into the pathogenesis of  $\alpha$ -synucleinopathies including PD and DLB.

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