Cross-talk between α-synuclein post-translational modifications in yeast as model of Parkinson's disease

Dissertation

for the award of the degree

"Doctor of Philosophy"

Division of Mathematics and Natural Sciences

of the Georg-August-Universität Göttingen

Submitted by

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From Kerman / Iran

Göttingen 2014

Declaration

Hereby I declare that the Ph.D. Thesis entitled:

“Cross-talk between α-synuclein post-translational modifications in yeast as model of Parkinson’s disease”

has been written independently with no other sources and aids than quoted.

Göttingen 2014 Hedieh Shahpasandzadeh

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Date of oral examination: 17.03.2014

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Publications:

Shahpasandzadeh H, Popova B, Juckert A, Fraser PE, Outeiro TF, Braus GH (2014) Interplay between sumoylation and phosphorylation for protection against alpha-synuclein inclusions. *J Biol Chem* M114.559237

Petroi D, Popova B, Taheri-Talesh N, Irniger S, Shahpasandzadeh H, Zweckstetter M, Outeiro TF, Braus GH (2012) Aggregate clearance of alpha-synuclein in *Saccharomyces cerevisiae* depends more on autophagosome and vacuole function than on the proteasome. *J Biol Chem* **287:** 27567-27579

Dedication

To

My parents

Zahra and Hossein, Who made all of this possible.

For their endless support, encouragement and love.

And also to my beloved sisters, Mahdieh, Homa and Mina

Who have never left my side and lightened my way with their pure love.

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Summary

Post-translational modifications modulate several characteristics of proteins. They can regulate substrate activity, localization, stability, but also feature and structure, remarkably in disease-associated proteins**.** The major hallmark of Parkinson's disease is the accumulation of proteinaceous inclusions termed Lewy bodies (LBs), which are mainly composed of α-synuclein. They lead to neuronal cell death upon different mechanisms, which are often yet unknown. α-synuclein, a presynaptic neuronal protein, plays an important role in Parkinson's disease pathogenesis. It undergoes various post-translational modifications during pathological conditions. The cytotoxicity and aggregation of α-synuclein can be mimicked in yeast. In this study, the two major post-translational modifications of α-synuclein, sumoylation and serine-129 (S129) phosphorylation, are addressed. Heterologously expressed wild type α-synuclein and A30P mutant are sumoylated in yeast at the same major α-synuclein sumoylation residues, lysine 96 (K96) and lysine 102 (K102) as in human. Lowering the cellular pool of the ubiquitin like modifier SUMO resulted in severe growth reduction in cells expressing α-synuclein, which correlated with increased numbers of cells with inclusion formation. This suggests that sumoylation protects against α-synuclein-mediated toxicity and inclusion formation in yeast. Expression of sumoylation-deficient α-synuclein caused the same growth rate, validating the protective role of α-synuclein sumoylation *in cis*. Overexpression of the human kinases GRK5 and PLK2 elevated α-synuclein phosphorylation level at S129. Interestingly, α-synuclein-mediated cytotoxicity associated with sumoylation impairment was compensated by a kinase-dependent higher phosphorylation rate at S129 of α-synuclein. Phosphorylation reduced inclusion formation and improved yeast growth. In order to get more insight into the cross-talk between α-synuclein sumoylation and S129 phosphorylation, α-synuclein aggregate clearance was monitored. Promoter shut-off studies were conducted in parallel with chemical inhibition of the cellular degradation pathways. In the absence of SUMO, α-synuclein aggregates were mainly cleared via the ubiquitin proteasome system. This suggests that sumoylation supports autophagy in α-synuclein aggregate clearance. In the presence of the human kinases GRK5 or PLK2, sumoylation-deficient α-synuclein aggregates were subjected to the ubiquitin proteasome as well as the autophagy pathway in a kinase-dependent manner, which was accompanied with altering α-synuclein-ubiquitination profile. GRK5 was able to partially rescue autophagy and

further promotes the proteasome system to clear sumoylation-deficient α-synuclein aggregates. Both degradation pathways contributed equally to α-synuclein aggregate clearance in the absence of SUMO when PLK2 is overexpressed. This cross-talk between α-synuclein phosphorylation and sumoylation opens novel avenues for therapeutic intervention in Parkinson’s disease and other synucleinopathies.

Zusammenfassung

Posttranslationale Modifikationen modulieren verschiedene Charakteristika von Proteinen. Sie können die Aktivität, Lokalisierung und Stabilität ihrer Substrate regulieren, verändern aber auch Eigenschaften und Strukturvon Proteinen, die mit Krankheiten assoziiert sind. Ein wichtiges Kennzeichen der Parkinson-Krankheit ist die Akkumulation von Proteinaggregaten (Lewy Körperchen). Dies führt zu neuronalem Zelltod durch verschiedene, bisher oft unbekannte Mechanismen. α-Synuclein, ein präsynaptisches, neuronales Protein, ist der Hauptbestandteil der Lewy-Körperchen und spielt eine wichtige Rolle in der Pathogenese der Parkinson-Krankheit. Es unterliegt verschiedenen posttranslationalen Modifikationen unter pathologischen Bedingungen. Die Zytotoxizität und Aggregation von α-Synuclein kann in Hefe imitiert werden. In dieser Studie werden zwei wichtigen posttranslationalen Modifikationen von α-Synuclein, Sumoylierung und Phosphorylierung von Serin 129 (S129), untersucht. Heterolog exprimertes Wildtyp-α-Synuclein und die A30P Mutante sind in Hefe an den gleichen Resten, Lysin 96 (K96) und Lysin 102 (K102), sumoyliert wie im Menschen. Eine Absenkung des zellulären Pools des Ubiquitin-ähnlichen Proteins SUMO führte zu einer starken Wachstumsreduktion von Zellen, welche α-Synuclein exprimieren. Dies korrelierte mit einer erhöhten Zahl an Zellen, die Einschlüsse bildeten. Dies legt nahe, dass Sumoylierung die Hefen vor α-Synuclein vermittelter Toxizität und Einschlussbildung schützt. Die Expression von sumoylierungsdefizienten α-Synuclein verursachte die gleiche Wachstumsrate, was die protektive Rolle der α-Synuclein Sumoylierung *in cis* bestätigt. Eine Überexpression der humanen Kinasen GRK5 und PLK2 erhöhten den Anteil an S129 phosphoryierten α-Synuclein. Interessanterweise wurde die α-Synuclein–vermittelte Zytotoxizität in Zusammenhang mit einer beeinträchtigten Sumoylierung durch eine höhere Kinase-abhängige S129 α-Synuclein Phosphorylierungsrate kompensiert. Phosphorylierung reduzierte die Einschlussbildung und verminderte die Wachstumshemmung. Um mehr Einblicke in eine plausible wechselseitige Beeinflussung zwischen α-Synuclein Sumoylierung und S129 Phosphorylierung zu erhalten, wurde die Beseitigung der α-Synuclein Aggregate beobachtet. Promotor „shut-off“ Studien wurden parallel mit chemischer Inhibition der zellulären Abbauwege durchgeführt. In der Abwesenheit von SUMO wurden α-Synuclein-Aggregate hauptsächlich durch das Ubiquitin-Proteasom-System abgebaut. Dies legt nahe, dass Sumoylierung den Abbau der α-Synuclein-Aggregate durch Autophagie unterstützt. In Anwesenheit der humanen Kinasen

GRK5 oder PLK2, wurden die sumoylierungsdefizienten α-Synuclein-Aggregate Kinasen abhängig sowohl dem Ubiquitin-Proteasom als auch dem Autophagie-System zugeführt. Dies ging einher mit einem veränderten Ubiquitinierungs-Profil von α-Synuclein. GRK5 war in der Lage den Abbau von sumoylierungsdefizienten α-Synuclein-Aggregaten durch Autophagie partiell zu retten und außerdem das Proteasom-System zu unterstützen. In Abwesenheit von SUMO, wenn PLK2 überexprimiert wird, trugen beide Abbauwege gleich stark zur Beseitigung der α-Synuclein-Aggregate bei. Diese wechselseitige Beeinflussung zwischen α-Synuclein Phosphorylierung und Sumoylierung könnte neue Wege für eine therapeutische Intervention in der Parkinsonkrankheit und anderen Synucleinopathien eröffnen.

1 Introduction

1.1 Parkinson's Disease

Parkinson's disease is the second most frequent neurodegenerative disorder, which belongs to the wide superfamily of pathologies known as protein misfolding diseases (de Lau & Breteler, 2006). The clinical syndrome of Parkinson's disease is associated with dopaminergic neuronal loss from part of the mid-brain called *substantia nigra pars compacta*. The specific cause of dopamine generating cell death in disease progression is still unknown. In the early stage of the disease, the most noticeable symptoms are movement related and include shaking, rigidity, walking difficulties and bradykinesia (slowness of movement), which were first described by James Parkinson in 1817 (Galvin et al, 2001a; Meissner et al, 2011). In advanced stages thinking and behavioral problems may arise. Moreover, patients with this condition show some level of cognitive dysfunction including dementia. Parkinson's disease is now known as being a more complex clinicopathological object with both a movement and cognitive dysfunction.

Although incompletely understood, the etiology of Parkinson's disease is thought to involve both genetic and environmental factors. Genetic causes of Parkinson's disease comprise of two categories, sporadic/idiopathic (≈ 95% of the cases) and familial (≈5% of the cases). Studies of familial cases of Parkinson's disease introduced 17 autosomal dominant and autosomal recessive gene mutations responsible for the disease (Houlden & Singleton, 2012) (Table 1). These include α-synuclein mutation and triplication, parkin, ubiquitin carboxyl terminal hydrolase L1 (UCH-L1), DJ-1, phosphatase and tensin homolog-inducible kinases 1 (PINK1), leucine-rich repeat kinase 2 (LRRK2), and glucocerebrosidase (GBA) (Dexter & Jenner, 2013). Beside genetic there are some environmental factors that contribute to the risk of developing the disease. Toxicity of some chemicals like commercial weed killer (Rotenone) and pesticides (paraquat) are found to destroy dopaminergic cells and links to an increased risk of the disease development (McCormack et al, 2008; McCormack et al, 2002; Tanner et al, 2011). Other environmental causes include solvent exposure (n-hexane, methanol), carbon monoxide poisoning, hydrogen sulfide intoxication and perhaps manganese. One environmental factor worth noticing is head trauma, which significantly increases the risk of Parkinson's disease in population studies. Study with ex-national football players of the United

States suggests that head trauma increases the risk of developing Parkinson's disease (Lehman et al, 2012).

Moreover, recently epigenetic mechanisms, such as DNA methylation, chromatin remodeling and miRNA, which may trigger gene expression, have started to be elucidated in Parkinson's disease (de Mena et al, 2010; Frieling et al, 2007; Song et al, 2010). Since epigenetic mechanism modulation over the lifetime depends on different parameters, lifestyle condition and environmental factors, it might help to clear the link between risk factors and genetic factors involved in Parkinson's disease.

Although some symptomatic therapies exist for Parkinson's disease, the complexity of the disease makes the discovery of more efficient therapeutics difficult. Hence, great effort needs to be made to get more insight into the molecular pathways involved in this disorder and to understand how different mechanisms might affect the development of the disease. These will further help to open new horizons for generating new therapeutic treatments.

Table 1. Common gene mutations causing familial Parkinson's disease.

Gene	Locus/disease	Mode of inheritance	Age of onset (years)
SNCA	PARK1/4	Autosomal dominant	20-85
LRRK2	PARK8	Autosomal dominant	32-79
GRN	FTDP-17	Autosomal dominant	45-83
MAPT	FTDP-17	Autosomal dominant	25-76
DCTN1	Perry syndrome	Autosomal dominant	35-61
PRKN	PARK2	Autosomal recessive	16-72
PINK1	PARK6	Autosomal recessive	20-40
DJ1	PARK7	Autosomal recessive	20-40
FBXO2	PARK15/PPS	Autosomal recessive	10-19
NR4A2/NURR1		Unknown	45-67
POLG		Unknown	20-26

FTDP-17, frontotemporal dementia with parkinsonism linked to chromosome 17; PPS, palidopyramidal syndrome (Dexter & Jenner, 2013).

1.2 The pathogenesis of Parkinson's disease

1.2.1 Synucleins

The synuclein family plays a major role in pathogenesis of Parkinson's disease. It consists of small natively unfolded proteins named α-, ß- and γ-synuclein. These proteins are characterized by a highly conserved N-terminal region with 6 imperfect repeats and a less-well conserved acidic terminus (von Bohlen Und Halbach, 2004). All three synucleins are expressed in the human and rodents brain (Galvin et al, 2001b; Giasson et al, 2001; Li et al, 2002). Whereas α- and ß-synuclein are enormously expressed in central nervous system, γ-synuclein is prominently expressed in peripheral nervous system (Mori et al, 2002; Surgucheva et al, 2006). Different studies described the link of α-synuclein to Parkinson's disease pathology, while there is limited investigation on ß- and γ-synucleins-mediated pathogenicity in Parkinson's disease (Irwin et al, 2013; Taschenberger et al, 2013).

1.2.2 α-synuclein

Parkinson's disease belongs to a family of neurodegenerative disorders known as synucleinopathies includes also dementia with Lewy bodies (DLB) and multiple system atrophy that are characterized by common pathogenic mechanism involved with the aggregation and deposition of misfolded α-synuclein (Spillantini et al, 1998). The molecular hallmark of synucleinopathies is the presence of intracellular inclusions termed Lewy bodies (LBs), which mainly consist of the synaptic protein α-synuclein (Spillantini et al, 1997) (Figure 2). Although synuclein family members share high sequence similarities, α-synuclein is unique in its possession of an amyloidogenic amino acid domain in its NAC region. α-synuclein protein is abundantly expressed in the brain as well as other tissues (Ltic et al, 2004) and localizes in nucleus and presynaptic terminals (Maroteaux et al, 1988). Although the full function of α-synuclein is still unclear, this protein is certainly involved in vesicular trafficking and release depending on its association with SNARE complex proteins (Burre et al, 2010; Nemani et al, 2010). This protein was implicated in pathogenesis of Parkinson's disease when pathogenic mutations in *SNCA* gene that encode for α-synuclein were linked to hereditary forms of this disease (Polymeropoulos et al, 1997). α-synuclein consists of 140 amino acids containing three domains: (i) the amino-terminal lipid binding domain with six α-helical repeats of 11 residues with variation of the conserved central sequence KTKEGV. This region is shown to be

important in anchoring and localizing α-synuclein (Bartels et al, 2010). (ii) a central hydrophobic non-Aβ component (NAC), which is critical for aggregation. The isolated NAC domain forms amyloid structure and small deletions within this domain can dramatically reduce the propensity of α-synuclein to aggregate (Rivers et al, 2008). (iii) a C-terminal unstructured domain, which is prolin rich and highly acidic, which seems to suppress α-synuclein aggregation (Li et al, 2005) (Figure 1). These three domains are essential for the misfolding of the protein (Jo et al, 2000). However, there is a contradictory study, which showed that α-synuclein might exist as a folded protein in a stable tetrameric formation under native condition in cell lines and mouse brain tissue as well as *in vitro* assay (Bartels et al, 2011; Wang et al, 2011). However, α-synuclein is widely considered as a natively unfolded monomer protein (Conway et al, 1998; Fauvet et al, 2012; Weinreb et al, 1996).

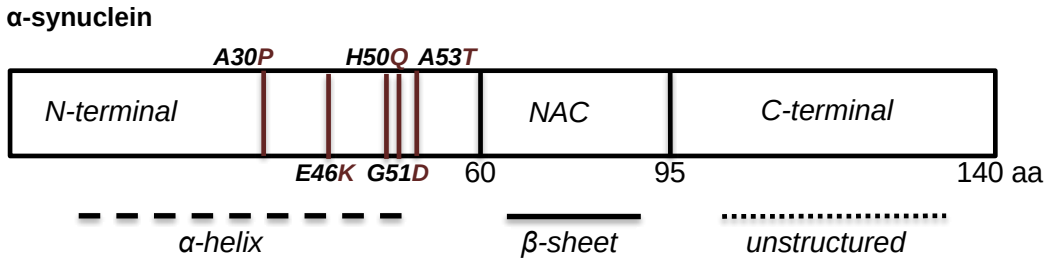

Figure 1. α-synuclein structure.

α-synuclein N-terminus adopts an α-helical structure upon binding to lipid membrane. The hydrophobic non-Aβ component (NAC) domain has the high tendency to make ß-sheet aggregates. The C-terminus is negatively charged and promotes protein solubility. Five point mutations, A30P, A53T, E46K, G51D and H50Q are Parkinson's disease-related mutations associated with an early onset of the disease (Appel-Cresswell et al, 2013; Lesage et al, 2013).

α-synuclein is not the only component of Lewy bodies in sporadic Parkinson's disease, but missense mutations A53T, A30P and E46K in the *SNCA* gene are also associated with autosomal dominant Parkinson's disease (Kruger et al, 1998; Polymeropoulos et al, 1997; Zarranz et al, 2004). Recently, a new mutation has been identified by sequencing of *SNCA* coding exons in patients with Parkinson's disease, which encodes the histidine to glutamine substitution (H50Q) (Appel-Cresswell et al, 2013). Beside that, G51D familial missense mutation of α-synuclein is also shown to be implicated in Parkinson's disease (Lesage et al, 2013).

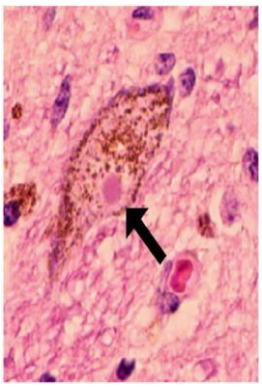

Figure 2. Lewy bodies in Parkinson's disease.

Lewy body (pointed with arrow) in dopaminergic cells of the *substantia nigra*. Lewy bodies consist of granular core that includes variety of nitrated, phosphorylated and ubiquitinated protein surrounded by filamentous halo primarily comprised of neurofilament and α-synuclein (Olanow & Brundin, 2013).

1.2.3 β-synuclein

SNCB gene is encoding human ß-synuclein protein, which is composed of 134 amino acids (Jakes et al, 1994; Spillantini et al, 1995) (Figure 3). ß-synuclein lacks 11 central hydrophobic residues compared to α-synuclein, which facilitates ß-synuclein to make random coil (Uversky et al, 2002) (Figure 3). Likewise α-synuclein, ß-synuclein is predominantly expressed in the human brain and concentrated in presynaptic nerve terminals (Jakes et al, 1994).

ß-synuclein is the closest member of the synucleins related to α-synuclein, which shows high overlapping pattern of expression in central nervous system by localizing to presynaptic nerve terminal (Clayton & George, 1998). The role of β-synuclein in Parkinson's disease pathology is poorly studied. Based on *in vitro* and *in vivo* evidences β-synuclein protects against α-synuclein toxicity by inhibiting its aggregation and fibril formation (Hashimoto et al, 2001; Park & Lansbury, 2003; Uversky et al, 2002). In addition, β-synuclein reduces α-synuclein protein level without affecting its RNA level in transgenic mouse (Fan et al, 2006). Recently, it has been shown that β-synuclein expression leads to formation of aggregates, which are proteinase resistant. This study shows the β-synuclein-mediated neurotoxicity, which leads to loss of dopaminergic neurons, suggesting β-synuclein's direct link to Parkinson's disease similar to α-synuclein (Taschenberger et al, 2013). Thus, further studies are vital to decipher the clear mechanistic role of β-synuclein in Parkinson's

disease pathology and further obtain efficient therapeutic lines in Parkinson's disease treatment.

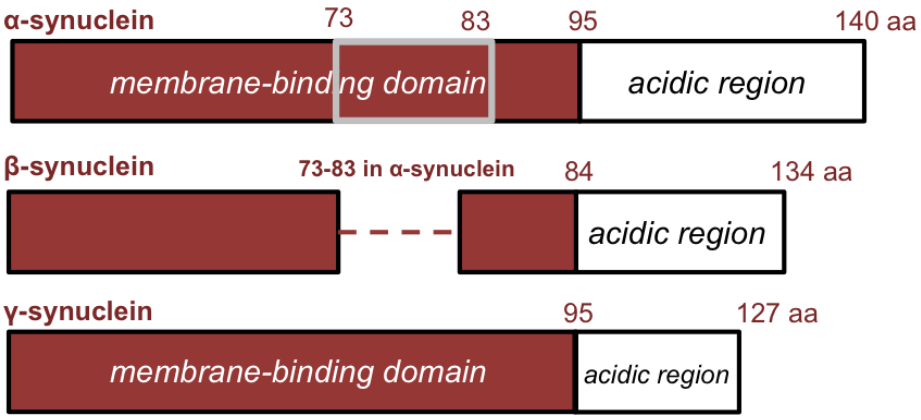

Figure 3. Schematic comparison of α-, β- and γ-synuclein proteins.

The N-terminal part is highly conserved between α-synuclein and β-synuclein (indicated in red), whereas the C-terminal part of β-synuclein is shorter and different. β-synuclein lacks 11 amino acids in non-Aβ component (NAC) (indicated in as a grey square) domain, which is responsible for amyloidogenic properties of the protein that may effect β-synuclein aggregation properties (Wales et al, 2013). γ-synuclein shares the highly conserved N-terminus with α-synuclein though it is shorter in its acidic tail.

1.2.4 γ-synuclein

The human synuclein family includes another member called γ-synuclein encoded by *SNCG* gene. As already described, synuclein family members share highly conserved N-terminal domain. The N-terminal domain of γ-synuclein, similarly to the other two members, is defined by its lipid interaction (Ueda et al, 1993). The difference of γ-synuclein exists within its C-terminal domain, which does not contain two 16-residues imperfect repeats, presents in α- and β-synuclein that makes the acidic tail shorter than the two others (Lavedan et al, 1998) (Figure 3). In contrast to α- and β-synuclein, γ-synuclein is abundant in peripheral nervous system but is also expressed in other tissues, as well as breast and ovarian cancers (Akopian & Wood, 1995; Buchman et al, 1998; Ji et al, 1997; Lavedan et al, 1998). In spite of limited information, γ-synuclein is structurally and functionally placed between α- and β-synuclein. It resembles α-synuclein in its free-state residual secondary structure, whereas in an extended-mode it resembles β-synuclein (Sung & Eliezer, 2007).

Compared to α-synuclein, γ-synuclein has lower propensity to form fibrils and aggregates *in vitro* and was shown to inhibit α-synuclein aggregation (Biere et al,

2000; Uversky et al, 2002). Unlike α-synuclein, little is known about γ-synuclein-related pathology in Parkinson's disease. Nevertheless, some evidence shows γ-synuclein deposition in Parkinson's disease, suggesting that its higher protein level leads to severe age and transgene dose-dependent neuropathology in mouse (Galvin et al, 1999; Ninkina et al, 2009). In addition, a sequencing study of Lewy bodies extracted from patients reveals that genetic variability in α- and γ-synuclein gene influences the risk of Lewy body formation, which in fact suggests the conservation between synuclein family members in Parkinson's disease pathology (Nishioka et al, 2010).

1.3 Post-translational modifications of α-synuclein

Proteins in eukaryotic cells can be edited by mechanisms known as post-translational modifications (PTMs). PTMs are critical reversible and irreversible processes, which can control the protein activity. They play an important role in regulating protein function, stability and structure. Based on biochemical reactions, PTMs are altering their target protein properties such as binding partners (protein-protein interactions), protein localization and conformation.

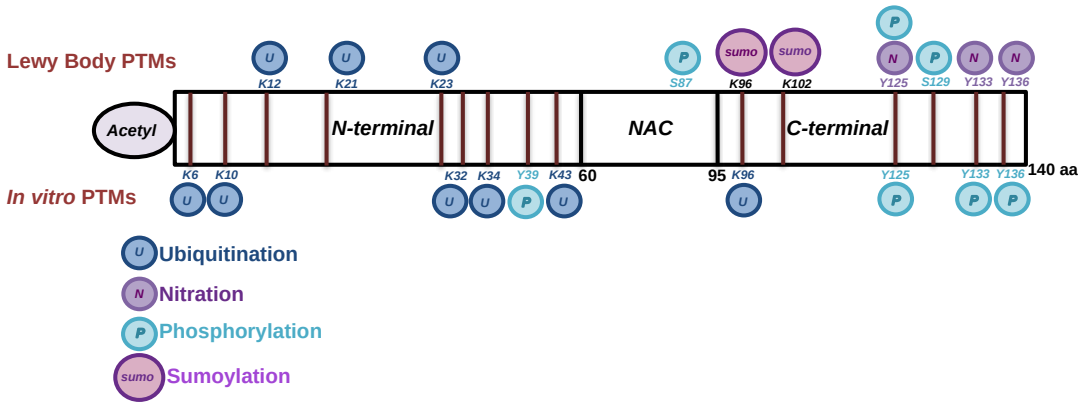

Figure 4. α-synuclein post-translational modification sites.

The position of the main α-synuclein post-translational modifications (phosphorylation, ubiquitination, nitration, acetylation and sumoylation) are shown. Disease-associated post-translational modifications in Lewy bodies are shown in the upper part of the scheme, whereas the identified PTMs from *in vitro* studies are shown below (Schmid et al, 2013).

Post-translational modifications of α-synuclein can trigger its aggregate-prone properties associated with Parkinson's disease pathology mainly linked to formation

of Lewy bodies. Inside the Lewy bodies, α-synuclein undergoes different post-translational modifications such as phosphorylation, ubiquitination, nitration, acetylation and sumoylation (Fujiwara et al, 2002; Giasson et al, 2000; Kang et al, 2012; Krumova et al, 2011; Nonaka et al, 2005) (Figure 4). However, whether these modifications enhance or inhibit α-synuclein aggregation and neurotoxicity is still debatable.

1.3.1 Sumoylation

Sumoylation is a critical post-translational modification, which controls its target protein stability, subcellular localization and activity in a dynamic and reversible manner. SUMO (small ubiquitin-like modifier protein) shares similarities with ubiquitin protein in the structure and biochemistry of its conjugation. Whereas ubiquitin often targets its substrate for proteasome-mediated degradation, sumoylation can modulate different functional consequences of its target protein. SUMO is only absent in bacteria and archea and is expressed by all eukaryotes. The importance of sumoylation in cellular processes makes it an essential system for health and even the survival of most organisms (Flotho & Melchior, 2013; Johnson, 2004; Ulrich, 2009). Sumoylation is a reversible pathway due to having the SUMO-specific proteases that can cleave and release SUMO for further cycles (Drag & Salvesen, 2008; Hickey et al, 2012). SUMO belongs to a family of protein modifiers that are covalently attached to their substrates via isopeptide bonds formed between the carboxy group of their C-terminal glycine residues and the ε-amino groups of substrates residues (Johnson, 2004). Newly synthetized SUMO protein is immature and needs to be processed in order to conjugate to its target protein. SUMO-specific proteolytic enzymes expose two glycine residues close to SUMO C-terminus by removing some carboxy residues from them (SENP proteases in mammals and ULP proteases in yeast). Mature SUMO is then activated by E1 activating enzyme in an ATP dependent manner and subsequently is transferred to the E2 conjugating enzyme (UBC9). Finally, in the last step the isopeptide bond is formed between the carboxyl group of the glycine residue at SUMO's carboxyl terminus and amino group of a lysine residue in its target protein. The last step is usually facilitated by E3 ligases, but many targets are efficiently sumoylated by E2 enzyme alone (Figure 5).

SUMO considers as one of the most soluble proteins, which regulate toxic protein properties (Marblestone et al, 2006). Sumoylation is an important candidate regulator in disease circumstances, especially in terms of neurodegenerative diseases

(Krumova & Weishaupt, 2013). SUMO protein is detected within pathological inclusions in various neurodegenerative disorders such as Huntington's, Alzheimer's and Parkinson's disease (Dorval & Fraser, 2006; Dorval & Fraser, 2007; Krumova et al, 2011; Steffan et al, 2004; Ueda et al, 2002). The sumoylation of α-synuclein has been reported *in vitro* and in mammalian cells mainly at lysine residues 96 and 102 (Dorval & Fraser, 2006; Krumova et al, 2011) (Figure 4). Sumoylation of α-synuclein can contribute to underlying Parkinson's disease molecular progression via different mechanisms. It has been shown that sumoylation negatively regulates α-synuclein aggregate formation by triggering its solubility (Krumova et al, 2011). Thus, understanding the molecular involvement of sumoylation in Parkinson's disease pathogenicity will help to achieve valuable therapeutic strategies.

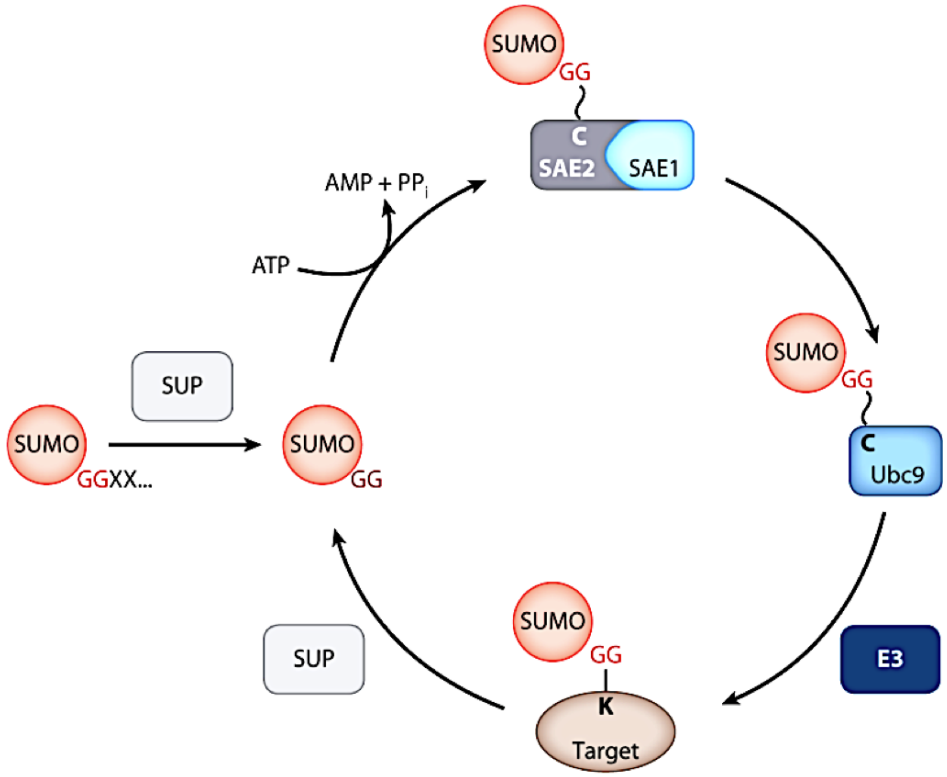

Figure 5. Reversible cycle of sumoylation.

Premature SUMO protein has to be exposed at the C-terminal glycine-glycine (GG) motif. This is catalyzed by SUMO-specific proteases (SUPs) of the Ulp/SENP family. A mature SUMO is activated by the E1 heterodimer SAE1/SAE2 (SUMO activating enzyme subunits 1 and 2) in an ATP-dependent manner, which results in thioester bond between C-terminal glycine of SUMO and the catalytic cysteine (C) of SAE2. SUMO is transferred to E2 conjugating enzyme Ubc9. Ubc9 catalyzes formation of an isopeptide bond between the C-terminal glycine of SUMO and lysine (K) residue in the substrate usually together with SUMO E3 ligase enzyme. Adapted from (Flotho & Melchior, 2013).

1.3.2 Phosphorylation

Protein phosphorylation is one of the most common and possibly the most important post-translational modification. Phosphorylation of different substrates might affect the attachment of other modifiers and consequently change the post-translational modification profile of the target. Phosphorylation is proposed to play an important role in regulating α-synuclein aggregation propensity (oligomerization and fibril formation) and neurotoxicity (Oueslati et al, 2010; Paleologou et al, 2010). α-synuclein is phosphorylated at one or multiple sites by being subjected at both serine and tyrosine residues (Fujiwara et al, 2002; Okochi et al, 2000) (Figure 4). *In vivo* studies have identified S87, S129, Y125 α-synuclein phosphorylation sites, whereas *in vitro* studies have also shown that α-synuclein is subjected to phosphorylation at Y133, Y136 and Y39 (Fujiwara et al, 2002; Negro et al, 2002; Okochi et al, 2000; Pronin et al, 2000) (Figure 4). Postmortem studies on human brains revealed that α-synuclein is predominantly phosphorylated at S129 rather than other residues (Anderson et al, 2006). In normal condition only 4% of α-synuclein is phosphorylated, whereas 90% is evident to be S129 phosphorylated in pathological cases inside Lewy bodies (Anderson et al, 2006; Fujiwara et al, 2002).

Different kinases are involved in regulation of α-synuclein S129 phosphorylation including casein kinase I (CKI), CKII, G-protein coupled receptor kinases (GRKs), LRRK2 (leucine-rich repeat kinase 2), and polo-like kinases (PLKs) (Anderson et al, 2006; Fujiwara et al, 2002; Mbefo et al, 2010; Okochi et al, 2000; Pronin et al, 2000; Waxman & Giasson, 2008). Phosphorylation of α-synuclein by GRK5 plays a crucial role in the pathogenesis of Parkinson's disease (Arawaka et al, 2006). PLK2 is the most efficient Polo-like kinase phosphorylating α-synuclein at S129 (Inglis et al, 2009; Mbefo et al, 2010; Salvi et al, 2012).

The role of α-synuclein S129 phosphorylation under physiological conditions for inclusion formation and the pathogenesis of Parkinson's disease remains controversial. It has been reported that fibrilization of α-synuclein is inhibited by S129 phosphorylation *in vitro* (Paleologou et al, 2008). Mimicking non-phosphorylated form of α-synuclein by substitution of S129 to alanine (S129A) in *Drosophila* model of Parkinson's disease resulted in inhibition of dopaminergic cell loss followed by promoting the aggregate formation (Chen & Feany, 2005). In another study, the toxic effect of α-synuclein S129 phosphorylation has been observed in transgenic mouse models of Parkinson's disease followed by increased neuronal loss (Freichel et al, 2007). Furthermore, the same observation was reported in rat model of Parkinson's

disease by comparing the effect of S129D (mimicking phosphorylated α-synuclein at S129) and S129A (non-phosphorylated α-synuclein at S129), which resulted in more toxicity by non-phosphorylated form of α-synuclein at S129 (S129A) rather than phosphorylated S129 (Azeredo da Silveira et al, 2009; Gorbatyuk et al, 2008). Study in yeast model of Parkinson's disease revealed that PLK2-mediated α-synuclein S129 increased phosphorylation level resulted in more α-synuclein cytotoxicity and intracellular inclusions (Basso et al, 2013). In contrast, another investigation reported no differences in aggregation and toxicity of α-synuclein mutants S129A and S129D in rat model of Parkinson's disease (McFarland et al, 2009).

Beside the solo importance of the effect of α-synuclein phosphorylation in Parkinson's disease pathology, the interplay between phosphorylation and other α-synuclein post-translational modifications might account as an important disease regulator. In Alzheimer's disease, increased tau phosphorylation can stimulate its sumoylation (Dorval & Fraser, 2006). There is also additional evidence indicating that the cross-talk between phosphorylation and sumoylation can affect substrates in different ways (Johnson, 2004), suggesting this might also modulate α-synuclein function and aggregation.

1.4 α-synuclein aggregation and aggregate clearance

1.4.1 Lewy body formation

Most of the efforts on understanding Parkinson's disease pathology are focused on mechanisms involved in α-synuclein aggregation and identification of the toxic species that result in disease. Although evidences support the link between the progressive accumulation of aggregated α-synuclein in patients and decrease in motor and/or cognitive function (Braak et al, 2003; Cookson, 2009; Klucken et al, 2006), the precise mechanism by which α-synuclein aggregates contribute to neuronal cell death and the events altering α-synuclein pathology are poorly understood. Recent studies show that synthetic α-synuclein pre-formed fibrils can induce Parkinson's disease-like α-synuclein pathology by initiating a cascade of pathological events in a highly lethal Lewy body-like phenotype *in vivo* (Luk et al, 2012a; Luk et al, 2012b) (Figure 6).

Although monomeric α-synuclein is a natively unfolded protein in solution, possessing a central hydrophobic region arises its affinity to oligomerize under

pathological conditions (Chandra et al, 2003). Cellular failure in degradation of natively misfolded monomeric α-synuclein under pathological conditions promotes its high self-interaction tendency and formation of unstable oligomers. The hydrophobic core of α-synuclein protein facilitates the oligomers to bind to lipid membranes, which leads to conformational change of the protein into stabilized β-sheet-rich high molecular weights (Zhu et al, 2003). Further, they aggregate into higher-order structures including protofibrils, other intermediates and amyloid fibrils. Ultimately, these higher-order structures are the building blocks for the pathological inclusions of α-synuclein termed Lewy bodies (Volpicelli-Daley et al, 2011). Interestingly, it is suggested that rather the fibrilar species of α-synuclein are cytotoxic than the aggregates in Parkinson's disease (Goldberg & Lansbury, 2000; Karpinar et al, 2009). Aggregates might play a cytoprotective role by isolating toxic forms of α-synuclein (Tanaka et al, 2004). Notwithstanding enormous investigations, the exact pathogenic species of α-synuclein (dimers, oligomers, protofibrils or fibrils) responsible for neuronal cell death and toxicity, are still unclear.

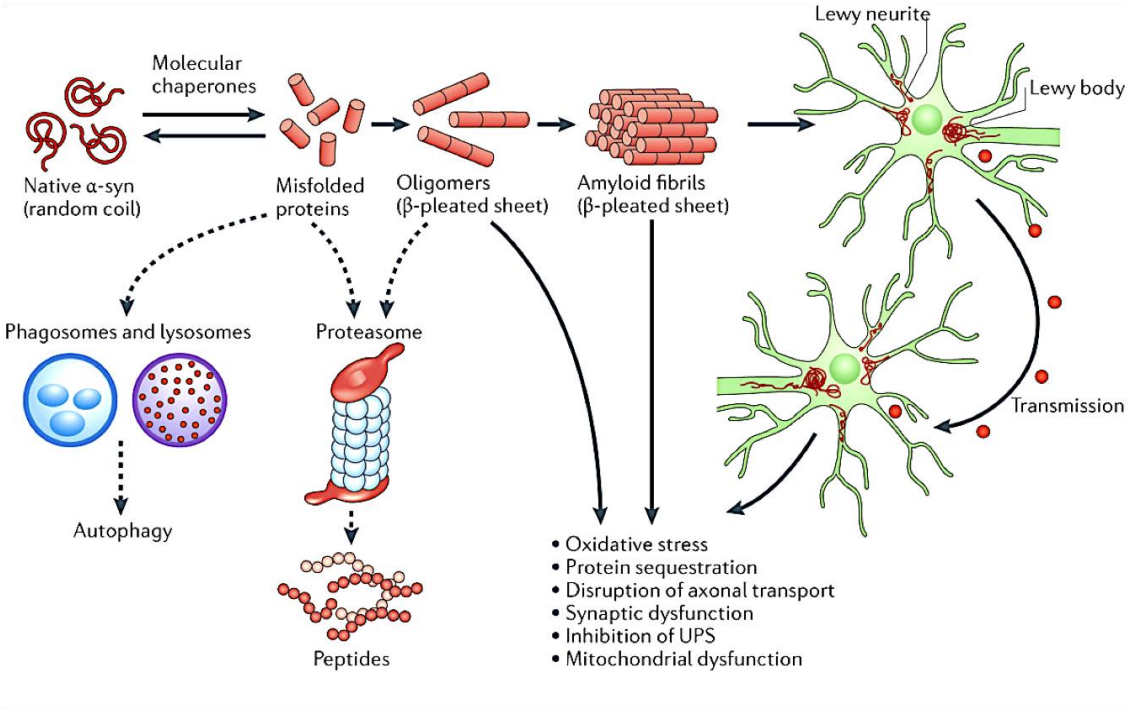

Figure 6. Hypothetical model of α-synuclein toxicity and aggregate formation.

Native α-synuclein exists as soluble random coil state. Under pathological conditions, misfolded monomeric α-synuclein homo-interact and forms unstable dimers and oligomers. Interaction of oligomers and monomers results in formation of amyloid fibrils in a stable β-sheet-rich conformation. Further accumulation of amyloid fibrils leads to Lewy body formation (Irwin et al, 2013).

1.4.2 Aggregate clearance

Accumulation of damaged or abnormally modified proteins may lead to perturbed cellular function and eventually cell death. As highlighted above, α-synuclein misfolding and aggregation is a pathological feature of Parkinson's disease that is linked to neuronal cell death. The neurons rely on particular protein quality control pathways to maintain protein intercellular homeostasis. One of the critical factors controlling the aggregation process of α-synuclein is the protein level, which is regulated by balanced equilibrium between synthesis, degradation and secretion of the protein. The two major proteolytic pathways that participate in the removal of altered proteins in neurodegenerative disorders like Parkinson's disease are the ubiquitin-proteasome system (UPS) and the autophagy-lysosomal pathway (ALP). Any dysfunction of these two important pathways contributes to accumulation of aggregated α-synuclein species and leads to disease progression. These degradation mechanisms are functionally connected and impairment of one can influence the other regulation. The systems conducting α-synuclein turnover are critical aspect of the Parkinson's disease mechanism. The exact mechanism pledged for α-synuclein aggregate clearance is still controversial depending on the system studied.

1.4.2.1 Ubiquitin-proteasome system in α-synuclein aggregate clearance

The major pathway that facilitates the degradation of short-lived intercellular soluble proteins in cell is the ubiquitin-proteasome system (UPS) (Goldberg, 2003; Wong & Cuervo, 2010). Aggregate clearance by UPS is assessed by ubiquitylation, which is a three-step cascade mechanism. The highly conserved ubiquitin protein attaches to the exposed lysine residue of the target protein by ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-ligase (E3) enzymes in an ATP-dependent manner. The substrate specificity and selectivity of the proteasome is achieved by the E3 ligases that catalyze the attachment of ubiquitin to the target protein and some number of other proteasome ancillary proteins. Ubiquitin chains act as a signal for recognition of the target protein by proteasome (Glickman & Ciechanover, 2002). Beside that, aggregated proteins may also undergo degradation in an ubiquitin-independent manner (Demartino & Gillette, 2007).

Several studies focused on the potential role of UPS in α-synuclein aggregate clearance in Parkinson's disease. According to some evidences, upon proteasome

inhibition α-synuclein aggregates are accumulated in neuronal cells in poly-ubiquitinated form, suggesting that α-synuclein is degraded via UPS system (Bennett et al, 1999; McLean et al, 2001). On the other hand, it is reported that α-synuclein does not need to be ubiquitinated to be degraded by proteasome (Tofaris et al, 2001). Rott and colleagues demonstrated that in the absence of proteolytic impairment, mono-ubiquitinated α-synuclein undergoes degradation by proteasome. They assumed that the ubiquitinase USP9X governed the α-synuclein fate of clearance (Rott et al, 2011). Recently, it has been shown that phosphorylated α-synuclein at S129 is targeted to proteasomal pathway in an ubiquitin-independent manner (Paulson et al, 2008). Furthermore, an *in vivo* study supports the involvement of UPS in α-synuclein degradation (Ebrahimi-Fakhari et al, 2011). They suggested that the degradation pathway depends on the α-synuclein protein burden inside the cell. Low expressed α-synuclein is preferentially degraded by the UPS, whereas increased expression level of α-synuclein targets the protein to the autophagosome (Ebrahimi-Fakhari et al, 2011).

1.4.2.2 Autophagy-lysosome pathway in α-synuclein aggregate clearance

The autophagy-lysosomal pathway (ALP) serves as a general degradation mechanism to degrade intercellular proteins and organelles (Wong & Cuervo, 2010). The major functional difference between UPS and ALP is the fact that ALP recruits long-lived macromolecule proteins, cytosolic components and dysfunctional organelles for degradation through the process of macroautophagy (Klionsky & Emr, 2000). Dysfunction of autophagy pathway may contribute to Parkinson's disease pathogenesis as a significant amount of α-synuclein aggregates is shown to be degraded through lysosomal pathways in neuronal cells (Vogiatzi et al, 2008). Application of pharmacological and molecular enhancement of macroautophagy showed reduced α-synuclein protein level in cell culture system study (Spencer et al, 2009). Despite UPS that mainly degrades soluble α-synuclein, the autophagy degradation pathway is shown to be predominantly responsible for clearing higher molecular weight α-synuclein species such as oligomeric intermediates and oligomers/aggregates (Alvarez-Erviti et al, 2010; Cullen et al, 2009; Lee et al, 2004; Mak et al, 2010; Tofaris et al, 2011).

In an *in vivo* study, α-synuclein turnover is addressed in living mouse brain indicating a distinct role of both degradation pathways (UPS/ALP) (Ebrahimi-Fakhari et al,

2011). They reported that both endogenous α-synuclein and pathological α-synuclein turnover is mediated by the UPS. They observed that ALP is not involved in the regular turnover of endogenous α-synuclein but turning on when the pre-existing level of α-synuclein is elevated to pathological form, suggesting the role of ALP in clearing higher molecular weight of α-synuclein (Ebrahimi-Fakhari et al, 2011; Ebrahimi-Fakhari et al, 2012). Furthermore, the clearance of α-synuclein is reported to be regulated by USP9X ubiquitinase activity. In the presence of proteolytic impairment, the de-ubiquitinated α-synuclein is cleared through ALP in cultured cells (Rott et al, 2011). Our previous study revealed the major role of ALP in α-synuclein aggregate clearance in yeast, while UPS contributes a minor role (Petroi et al, 2012).

Overall, both proteolytic pathways (UPS and ALP) are functionally connected and can take over the other responsibility in pathological conditions. Deciphering the mechanisms regulating α-synuclein turnover is a critical aspect of Parkinson's disease that helps to improve the potential therapeutic treatments.

1.5 *Humanized Saccharomyces cerevisiae*

The budding yeast *Saccharomyces cerevisiae* is the most broadly studied eukaryotic organism. *Saccharomyces cerevisiae* is a single cell organism that belongs to the group of fungi. It was the first eukaryotic organism that was fully sequenced in 1996 (Goffeau et al, 1996) and contains genes with 60% homology to human genes (Mager & Winderickx, 2005). Yeast model system possesses several advantages that make it widely used such as its ease of manipulation and amenability to genetic modification, short generation time, inexpensive growth, high transformation efficiency and easy laboratory conservation. Due to the highly conserved intercellular processes in evolution, many fundamental cellular mechanisms in eukaryotic systems are elucidated using yeast (Botstein & Fink, 2011).

The powerful genetic recourses and valuable knowledge about yeast makes it being used to demonstrate the molecular function of proteins involved in several human diseases including neurodegenerative disorders (Babcock et al, 1997; Outeiro & Lindquist, 2003; Wickner, 1994). Many disease-associated pathways and mechanisms are conserved between yeast and human (Karathia et al, 2011). If yeast cells do not harbor the human gene homolog associated with a disease, the transgene can be heterologously expressed in yeast and the obtained strain can be subjected to different functional analyses. Introduction of human genes into yeast cells often mimic the disease-relevant phenotypes. In addition, even if the gene

function is already known, we can get more insight into understanding the molecular pathways involved in the disease and the underlying basis of the disease-related pathology.

1.5.1 Yeast model of Parkinson's disease

The main aspect of studying Parkinson's disease similar to the other diseases is getting closer to improved better therapeutic strategies. In order to achieve this aim, it is essential to get a better understanding of molecular mechanisms involved in Parkinson's disease pathology. Thus, scientists are now adopting more rational approaches where different model systems are being used. Furthermore, the obtained findings can be validated in various model systems (Cooper et al, 2006; Su et al, 2010; Xiong et al, 2010).

α-synuclein does not have a yeast homolog but it can be heterologously expressed in yeast cells. Several cellular pathways involved in Parkinson's disease were either first addressed in yeast and then validated in other model systems or first identified in other Parkinson's disease model systems and then being reproduced in yeast successfully (Buttner et al, 2008; Outeiro & Lindquist, 2003; Petroi et al, 2012; Sampaio-Marques et al, 2012; Sharma et al, 2006; Su et al, 2010). The first yeast model of Parkinson's disease was introduced by Outeiro and Lindquist in 2003 (Outeiro & Lindquist, 2003). They showed that α-synuclein expression is toxic to yeast cells in gene dosage dependent manner. We reported the α-synuclein threshold for cytoxicity in yeast. Three integrated copies of wild type α-synuclein and two integrated copies of A53T mutant are the causative concentration of α-synuclein, which lead to cell death (Petroi et al, 2012).

In addition, α-synuclein forms intercellular inclusions in yeast, which are correlated with cytotoxicity. Different pathways that are involved in α-synuclein toxicity are being studied in yeast, namely oxidative stress (Flower et al, 2005; Sharma et al, 2006), proteasome impairment (Chen et al, 2005; Sharma et al, 2006), autophagy (Petroi et al, 2012), mitophagy dysfunction (Sampaio-Marques et al, 2012), mitochondrial dysfunction (Buttner et al, 2008; Su et al, 2010), vesicle trafficking defects (Soper et al, 2008) and phosphorylation (Basso et al, 2013; Sancenon et al, 2012). In addition to α-synuclein-related toxicity in Parkinson's disease, other genes such as *LRRK2* and *PARK2*, which are also involved in Parkinson's disease, are being studied in yeast model system recently (Chesi et al, 2012; Shin et al, 2008; Usenovic et al, 2012; Xiong et al, 2010; Zheng et al, 2008).

Taken together, using yeast as a model to underline molecular mechanisms involved in Parkinson's disease pathology will set up new paths toward reaching effective therapeutic approaches.

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1.6 Aim of this study

α-synuclein post-translational modifications play an important role in triggering aggregation and cytotoxicity properties. α-synuclein sumoylation and S129 phosphorylation were shown to be two key modifications involved in Parkinson's disease pathogenicity (Anderson et al, 2006; Dorval & Fraser, 2006; Fujiwara et al, 2002; Krumova et al, 2011). The mechanisms by which these modifiers might interplay and consequently effect α-synuclein-mediated toxicity is yet unclear. α-synuclein phosphorylation is well studied, while there is lack of evidences regarding sumoylation potential regulatory role in α-synuclein-mediated pathology in Parkinson's disease process. Thus, deciphering molecular mechanisms involved in α-synuclein-mediated pathology associated with Parkinson's disease needs to be studied in more details.

The main aim of this study was to use budding yeast as a model system in order to elucidate α-synuclein sumoylation and S129 phosphorylation-mediated effect on α-synuclein-related cytotoxicity, aggregation and aggregate clearance.

Three major questions were addressed in the current study:

1- Is sumoylation a conserved phenomenon in α-synuclein-related pathology from yeast to human?

In order to address this question, yeast *ulp1*^{ts} strain and *smt3*^{ts} strain expressing α-synuclein were generated and characterized to be further subjected to different molecular analyses. In addition, sumoylation-deficient α-synuclein mutants were constructed to validate the findings *in cis*.

2- Is there a cross-talk between α-synuclein S129 phosphorylation and α-synuclein sumoylation?

Due to the fact that GRK5 and PLK2 are the major kinases in phosphorylating α-synuclein at S129 (Pronin et al, 2000; Salvi et al, 2012), we further analyzed the effect of the corresponding kinases on α-synuclein S129 phosphorylation. In addition,

the probable interplay between α-synuclein sumoylation and α-synuclein S129 phosphorylation were examined in different contexts.

3- What are the mechanisms involved in α-synuclein aggregate clearance?

Since ALP has a major role in α-synuclein aggregate clearance in yeast model of Parkinson's disease, we were interested to investigate the regulatory effect of sumoylation and S129 phosphorylation on α-synuclein aggregate clearance. The involvement of ALP and UPS were analyzed through chemical approaches. Promoter shut-off studies were performed in combination with UPS and ALP impairment by chemical treatments with MG132, a proteasome inhibitor, and phenylmethanesulfonyl fluoride (PMSF), a vacuolar protease inhibitor, respectively.

Overall, our findings in yeast cell-based model of Parkinson's disease provide mechanistic insight into pathological role of α-synuclein post-translational modifications in Parkinson's disease.

2 Material and Methods

2.1 Materials

2.1.1 Chemicals, growth media and conditions

Chemicals for buffers, media and solutions preparation were achieved from AppliChem GmbH (Darmstadt, Germany), BD Becton GmbH (Heidelberg, Germany), Carl Roth GmbH und Co. KG (Karlsruhe, Germany), SERVA Electrophoresis GmbH (Heidelberg, Germany), Roche Diagnostics GmbH (Manheim, Germany) and Sigma-Aldrich Chemie GmbH (Steinheim, Germany). DNA polymerase and dNTPs were obtained from Thermo Fisher, Scientific GmbH (Dreich, Germany). Primers were received from Invitrogen GmbH (Darmstadt, Germany) or Sigma-Aldrich Chemie GmbH (Steinheim, Germany). DNA and protein standard GeneRuler (1kb DNA ladder and PageRuler Prestained Protein Ladder) were obtained from Thermo Fisher, Scientific GmbH (Dreich, Germany). DNAs were isolated with QIAprep Spin Miniprep Kit and the QIAprep Gel Extraction Kit from Qiagen (Hilden, Germany). PCRs were performed with T Professional Thermocycler, Biometra GmbH (Göttingen, Germany). DNA concentrations were determined with Nanodrop ND-1000, Peqlab Biotechnologie GmbH (Erlangen, Germany). Protein concentrations were measured with Tecan reader (Männedorf, Switzerland). Agarose gel electrophoresis was performed with Wide Mini-Sub Cell GT Cell, Bio-Rad Labratories GmbH (München, Germany). ECL films results of Southern and Western analysis were exposed with PROTEC Processor Compact film-developing machine, Siemens (Erlangen, Germany). Centrifugations steps were performed with the Biogue pico, Biofuge fresco, Labofuge 400R Heraeus (Hanau, Germany) and 5804R, Eppendorf AG (Hamburg, Germany). The K96R K102R mutant constructs and the S129A mutant were generated by site-directed mutagenesis using Stratagene QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies).

2.1.1.1 *Saccharomyces cerevisiae* growth condition

Saccharomyces cerevisiae strains were grown in synthetic complete medium (SC) lacking the amino acid (uracil, histidine, tryptophan or leucine) corresponding to α-synuclein construct marker (SC-URA, SC-URA-HIS, SC-URA-HIS-TRP-LEU) at 30°C (Guthrie & Fink, 1991). Temperature sensitive yeast strains *smt3* and *ulp1* were grown at permissive temperature at 25°C, whereas down regulation of corresponding genes was achieved by shifting the cells to 30°C after being pre-grown at 25°C.

2.1.1.2 *Escherichia coli* growth condition

Escherichia coli strain was grown and harvested in Luria-Bertani (LB) medium (Bertani, 1951) containing, 1% bacto-trypton, 0.5% yeast extract and 1% NaCl with pH 7.5 at 37°C. In order to have solid medium, 2% agar was added. 100 mg/ml ampicillin was applied to the medium for selection.

2.1.2 Strains, plasmids and primers

Escherichia coli strain DH5α [F-, Φ80dΔ (lacZ) M15-1, Δ (lacZYA-argF) U169, recA1, endA1, hsdR17 (rK–, mK+), supE44, λ–, thi1, gyrA96, relA1] (Woodcock et al, 1989) was used for plasmid DNA preparation.

2.1.2.1 *Saccharomyces cerevisiae* strains

All yeast strains used in this study are presented in Table 2. Yeast backgrounds, which were used to construct strains with determined copies of α-synuclein integration in the yeast genome were Wild-type W303-1A (EUROSCARF, Frankfurt, Germany), *smt3* temperature sensitive mutant (*smt3*^{ts}) (Biggins et al, 2001), *ulp1* temperature sensitive mutant (*ulp1*^{ts}) (Hoege et al, 2002), *cdc5-DAmP* (cdc5 allele in DAmP collection) (Breslow et al, 2008). W303 served as parent in both corresponding temperature sensitive backgrounds.

Table 2. Yeast strains used in this study.

Strain	Genotype	Source
W303-1A	*MAT a; ura3-1; trp1-1; leu2-3_112; his3-11; ade2-1; can1-100*	EUROSCARF, (Frankfurt, Germany)
YBP206	*smt3*^{ts}*: MAT a; ura3-1; trp1-1; leu2-3_112; his3-1; ade2-1; can1-100 bar1Δ pGAL-HA3-SMT3:HIS3*	(Biggins et al, 2001)
RH3468	W303 containing 2 genomic copies *GAL1::SNCA*^{WT}*::GFP* in *URA3* locus	(Petroi et al, 2012)
RH3601	*smt3*^{ts} containing 2 genomic copies *GAL1::SNCA*^{WT}*::GFP* in *URA3* locus	This study
RH3602	W303 containing 2 genomic copies *GAL1::SNCA*$^{K96R\ K102R}$*::GFP* in *URA3* locus	This study
YBP5	*ulp1*^{ts}	(Hoege et al, 2002)

Table 2 continues.

RH3603	*ulp1*^{ts} containing YIp1ac211-ADH-His$_6$-Smt3 in *HIS3* locus	This study
RH3604	*RH3603* containing *GAL1::SNCA*^{WT} integrated in *TRP1* locus	This study
RH3605	*RH3603* containing *GAL1::SNCA*^{A30P} integrated in *TRP1* locus	This study
RH3606	*RH3603* containing *GAL1::SNCA*$^{K96R\ K102R}$ integrated in *TRP1* locus	This study
RH3607	*smt3*^{ts} containing 2 genomic copies *GAL1::SNCA*^{S129A}*::GFP* in *TRP1* locus	This study
	Cdc5-DAmP	(Breslow et al, 2008)
	Cdc5-DAmP containing 2 genomic copies *GAL1::SNCA*^{WT}*::GFP* in *TRP1* locus	AG Braus
	Cdc5-DAmP containing 2 genomic copies *GAL1::SNCA*^{A30P}*::GFP* in *TRP1* locus	AG Braus

2.1.2.1.1 Construction of yeast strains

Several strains were constructed in *smt3*^{ts}, W303 and *ulp1*^{ts} backgrounds. Wild-type (WT) α-synuclein encoding cDNA sequence (referred to as *SNCA*) or A30P mutant sequence were integrated in *TRP1* genomic locus of *ulp1*^{ts} background. The *ulp1*^{ts} yeast strains harboring sumoylation-deficient α-synuclein variants, were constructed by integrating K96R K102R synuclein or A30P K96R K102R synuclein in the *TRP1* genomic locus. Different *smt3*^{ts} yeast strains were constructed by integrating WT α-synuclein fused to GFP via linker (KLID) in the *URA3* genomic locus and S129A-synuclein fused to GFP via linker (KLID) in the *TRP1* genomic locus. Strains with two tandemic integrations of WT α-synuclein were selected for analysis. The W303 yeast strains were generated by integrating K96R K102R synuclein in *URA3* genomic locus. Strains with two tandemic integration of K96R K102R synuclein were selected for further analysis.

2.1.2.2 *Saccharomyces cerevisiae* plasmids

The yeast plasmids used in this study are listed in Table 3. In general, overexpression vector, pRS426 carrying *URA3* gene or pRS423 carrying *HIS3* gene

and integrative yeast plasmids, pRS306 carrying *URA3* gene or pRS304 carrying *TRP1* gene were used for yeast strains construction (Sikorski & Hieter, 1989). The cDNA of α-synuclein variants preceded by *GAL1*-promoter and followed by the *CYC1*-terminator were cloned into mentioned yeast vectors. α-synuclein variants were used as either tagged or untagged depending on the aim of the experiment. α-synuclein-KLID-GFP was used for live-cell fluorescence microscopy and α-synuclein-His6 was used for Ni^{2+}-NTA pull down. Untagged α-synuclein was used for Ni^{2+}-NTA pull-down of sumoylated proteins. The cDNA of *SNCA* gene fused to GFP via KLID linker was amplified by PCR from the genomic DNA of the yeast strain HiTox (Outeiro & Lindquist, 2003).

Human kinases GRK5 and PLK2 were cloned into the *Sma*I restriction site of pME2792 yeast vector proceeded by *GPD1* and *GAL* promoter, respectively.

Table 3. Yeast plasmids used in this study.

Plasmid	Description	Source
pME2795	pRS426-*GAL1-Promoter, CYC1-Terminator, URA3, 2µm, pUC origin, Amp^R*	(Petroi et al, 2012)
pME3760	pME2795 with *GAL1::SNCA^{WT}*	(Petroi et al, 2012)
pME3764	pME2795 with *GAL1::SNCA^{A30P}::GFP* (KLID linker)	(Petroi et al, 2012)
pME3759	pME2795 with *GFP*	(Petroi et al, 2012)
pME3945	pRS306 with *SNCA^{WT}::GFP* (KLID linker)*, CYC1-Terminator, URA3, integrative, pUC origin, Amp^R*	(Petroi et al, 2012)
pME3596	pRS304 with *GAL1-Promoter, CYC1-Terminator, TRP1, integrative, pUC origin, Amp^R*	This study
pME3597	pME3596 with *GAL1::SNCA^{WT}*	This study
pME3598	pME3596 with *GAL1::SNCA^{A30P}*	This study
pME4089	pME3596 with *GAL1:: SNCA $^{K96R\ K102R}$*	This study
pME4090	pME3596 with *GAL1:: SNCA$^{A30P\ K96R\ K102R}$*	This study

Table 3 continues.

pME4091	pRS306 with *GAL1:: SNCA $^{K96R\ K102R}$::GFP* (KLID linker), *CYC1-Terminator, URA3, integrative, pUC origin, Amp^{R}*	This study
pME2792	pRS423-*GAL1-Promoter, CYC1-Terminator, HIS3, 2µm, pUC origin, Amp^{R}*	This study
pME4092	pME2792 with *GAL::PLK2*	This study
pME4093	pME2792 with *GPD::GRK5*	This study
pME4094	pME3596 with *GAL1:: SNCA ^{S129A}::GFP* (KLID linker)	This study
pME4095	pME2795 with *GAL1::SNCA^{WT}::His6*	AG Braus
D1374	YIplac211-ADH-His-Smt3	(Hoege et al, 2002)
pME4099	pME2792 with *GAL1*:: *CDC5*	This study
pME4098	pME4099 with *GPD::GRK5*, destroyed *HIS3*	This study
pME4097	pME2795 with *GAL1::SNCA$^{K96R\ K102R}$::GFP* (KLID linker)	This study
pME4100	pME2795 with *GAL1*::*SNCA^{WT}*::*His6*	AG Braus
pME4101	pME2795 with *GAL1*::*SNCA^{A30P}*::*His6*	AG Braus
pME4102	pME2795 with *GAL1::SNCB::GFP* (KLID linker)	This study
pME4103	pME2795 with *GAL1*::*SNCG::GFP* (KLID linker)	This study

2.1.2.3 Primers

The list of oligonucleotides used in the current work are presented in Table 4.

Table 4. Oligonucleotides used in this study for plasmid construction.

Name	Sequence (5'- 3')	Use
BP10	TTA TTT GTA TAG TTC ATC CAT GC	KLID-GFP linker reverse no restriction site
BP15	AAG CTT ATC GAA AGT AAA GGA GAA GAA CTT	*SNCA* Forward for amplification of *SNCA-KLID-GFP* PCR fragment
BP16	ATC GAT AAG CTT GGC TTC AGG TTC GTA GTC	Reverse for amplification of *SNCA-KLID-GFP* PCR fragment
BP20	GTT AGA GCG GAT GTG GGG	*CYC* reverse primer used for sequencing
BP77	ATG TCG TTG GGT CCT CTT AAA G	Forward primer *cdc5* blunt end cloning
BP78	TTA ATC TAC GGT AAC AAT TGT GGA C	Reverse primer *cdc5* blunt end cloning
KanX forward	TTA ACC CGG GGA TCC TTT GTA C	KanX forward
KanX reverse	TAG ATT GTC GCA CCT GAT TGC C	KanX reverse
NTTP79	GCT GCA TAA CCA CTT TAA CTA	*GAL1* forward primer used for sequencing
Hsp1	ATG GAT GTA TTC ATG AAA GGA C	*SNCA* forward
Hsp18	CTG GCT TTG TCA GAA AGG ACC AGT TGG GCA GAA ATG AAG AAG G	$SNCA^{K96R\ K102R}$ forward primer for quick-change mutagenesis
Hsp19	CCT TCT TCA TTT CTG CCC AAC TGG TCC TTT CTG ACA AAG CCA G	$SNCA^{K96R\ K102R}$ reverse primer for quick-change mutagenesis
BP59	GCT TAT GAA ATG CCT GCC GAG GAA GGG TAT CAA G	$SNCA^{S129A}$ forward primer for quick-change mutagenesis
NTTP108	GTCGAAAGCTACATATAAGG AAC	*URA3* gene and using as a hybridization probe for the Southern analysis
NTTP109	AGTTTTGCTGGCCGCATCTT C	*URA3* gene and using as a hybridization probe for the Southern analysis
NTTP110	ATGTCTGTTATTAATTTCACA G	*TRP1* gene and using as a hybridization probe for the Southern analysis
NTTP111	CAGTAATAACCTATTTCTTAG C	*TRP1* gene and using as a hybridization probe for the Southern analysis

Table 4 continues.

BP60	CTT GAT ACC CTT CCT CGG CAG GCA TTT CAT AAG C	*SNCA*^{S129A} reverse primer for quick-change mutagenesis

2.2 Methods

2.2.1 Molecular biology and genetic manipulation methods

2.2.1.1 *Escherichia coli* transformation

Escherichia coli transformation was performed according to standard method (Inoue et al, 1990). *E. coli* DH5α competent cells (DSMZ, Braunschweig, Germany) were used for transformation. The competent cells were thawed on ice and 0.5 µg of the desired plasmid DNA or 10 µl of the ligation reaction were added to 200 µl competent cells. The cell mixture with DNA was incubated for 30 min on ice and was further subjected to heat shock at 42°C for 60 seconds. Cells were quickly put back on ice for 5 min. 800 µl of SOC medium (SOB + 20 mM glucose) was applied to the mixture and then incubated for 1 hour at 37°C. Cells were collected by centrifugation (Biofuge pico, Heraeus, Hanau, Germany) at 2500 rpm for 3 min and plated on LB agar plates containing respective antibiotic (ampicillin at final concentration of 100 µg/ml). Plates were incubated at 37°C overnight. Clones were picked and analyzed by polymerase chain reaction (PCR) (described later). In addition, sequencing analysis was performed for verification of the positive clones carrying the plasmid of interest in a correct orientation.

2.2.1.2 DNA sequence analysis

All constructs used in the current study were verified by DNA sequencing. DNA was sequenced at the Göttingen Genomic Laboratory. Sequences were analyzed with multiple sequence align tool (Corpet, 1988) also using the 4Peaks software (www.makentosj.com). The SGD (*Saccharomyces* Genome Database) online website (www.yeastgenome.org) was used to analyze yeast chromosomal sequences, which were used as DNA template amplification in this study.

2.2.1.3 Polymerase Chain Reaction (PCR)

PCR method was applied for DNA amplification according to established protocols (Saiki et al, 1988). The following polymerases were applied for PCR: *Taq* (FERMENTAS GmbH/THERMO FISHER SCIENTIFIC GmbH, St. Leon-Rot, Germany/Schwartz, Germany) and Phusion High-Fidelity (FINNZYMES/THERMO FISHER SCIENTIFIC GmbH, Vantaa, Finland/Schwartz, Germany). Phusion polymerase was applied for cloning purposes according to manufacturer instruction and *Taq* polymerase was used for checking the amplified sequences. PCR with *Taq* polymerase was programmed as following: initial denaturation step at 95°C for 2 min, continued by 33 cycles of denaturation at 95°C for 1 min, annealing at different temperatures for 45 seconds and extension for 1 min/kb at 72°C. The final step of PCR program was set at 72°C as final extension step for 10 min. All PCR programs were performed in Thermo cyclers (Eurofins MWG GmbH, Ebersberg, Germany).

2.2.1.4 Agarose gel electrophoresis

In order to separate DNA fragments by their size, agarose gel electrophoresis was performed in a Wide Mini-Sub Cell GT Cell (Bio-Rad Laboratories GmbH, München, Germany) at 90 V in TAE buffer (40 mM Tris, 10 mM acetic acid, 1 mM Na_2-EDTA). Loading dye (50 % glycerol, 0.4 % Bromophenol blue) was added to DNA of interest and loaded on agarose gel (1% agarose and 1 µg/ml ethidiumbromide in TAE buffer). DNA bands were detected with the Gel iX Imager (INTAS Science Imaging Instrument GmbH, Göttingen, Germany).

2.2.1.5 DNA isolation

QIAGEN Plasmid Mini Kit (QIAGEN GmbH, Hilden, Germany) was used to isolate plasmid DNA from *E. coli,* according to manufacture instruction. In order to extract DNA from gel, QIAquick Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany) was used following the producer protocol.

2.2.1.6 DNA digestions and ligations

According to identified cloning strategy, the PCR-amplified DNA sequences and the corresponding vector were digested with the appropriate restriction enzyme (MBI FERMENTAS, Vilnius, Lithuania). Per 0.5 µg DNA, 1-2 unit of enzyme in 10× reaction buffer were used. The reaction mixture was incubated at appropriate temperature (normally at 37°C) depending on the enzyme that was applied for 2-3 hours. The mixture was subjected to agarose gel electrophoresis and the restricted fragment was purified using the gel QIAquick Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany).

The linearized vector and respective insert were used in a molar ratio of 1:3 to be ligated. The T4 DNA ligase (MBI Fermentas, Vilnius, Lithuania) and 1×T4 ligation buffer were used for ligation. The reaction was incubated at 16°C overnight. The ligation reaction was inactivated at 65°C for 10 min.

2.2.1.7 Southern hybridization

Southern hybridization analyses were performed with several transformants to verify the integration of α-synuclein-GFP construct into the mutated genomic *ura3-1* locus (Southern, 1975). Isolation of genomic DNA from *S. cerevisiae* was performed according to standard procedures (Hoffman & Winston, 1987). 10 µg genomic DNA were subjected to restriction digestion with *Hin*d III (MBI Fermentas, Vilnius, Lithuania). The restriction fragments were resolved on a 1% agarose gel, transferred to a nitrocellulose membrane, cross-linked by UV irradiation for 5 min and hybridized to a *URA3* gene fragment probe. In order to prepare hybridization probe, 100 ng of DNA corresponding to *URA3* gene fragment were denatured at 95°C and followed by labeling with horseradish peroxidase using the ECL Direct Labeling and Detection System (GE Healthcare Limited, Buckinghamshire, United Kingdome). The membrane was washed several times and incubated with detection solution (GE Healthcare Limited, Buckinghamshire, United Kingdome). The membrane was exposed to ECL film (GE Healthcare Limited, Buckinghamshire, United Kingdome) in the dark and developed in the PROTEC Processor Compact film-developing machine (Siemens, Erlangen, Germany). Copy numbers of the integrated vector were estimated according to the profile of the restriction fragments. One copy corresponded to 2.7 kb + 4.7 kb and two copies to 2.7 kb + 4.7 kb + 6.2 kb.

For integration of α-synuclein-GFP into the mutated genomic *trp1-1* locus, 10 µg genomic DNA were subjected to restriction digestion with *EcoRI* (MBI Fermentas, Vilnius, Lithuania). One copy of the integrated vector corresponded to 1.9 kb + 4.2 kb restriction digestion fragments and two copies to 1.9 kb + 4.2 kb + 4.6 kb.

2.2.1.8 Quickchange Site-Directed Mutagenesis

In order to substitute α-synuclein lysine 96 (K96) and lysine 102 (K102) to arginine (K96R K102R) and serine 129 (S129) to alanine (S129A), Quickchange Site-Directed Mutagenesis was performed (Wang & Malcolm, 1999). DNA templates used for this study are listed in Table 5. Pair of complementary nucleotide primers containing the mutation of interest were designed (Table 4). The PCR amplification process was conducted according to the manufacture instruction (Agilent Technologies, Santa Clara, USA). Amplification of target DNA was carried out using thermostable high fidelity *PfuTurbo* C_x hotstart DNA polymerase (Agilent Technologies, Santa Clara, USA). In order to remove parental template DNA and select for the synthetized mutated DNA, 1 µl of *Dpn*I restriction enzyme (MBI Fermentas, Vilnius, Lithuania) was added directly to the amplification mixture and incubated for 1 hour at 37°C. 1 µl of digested reaction was subsequently transformed in *E. coli* DH5α. After isolation of the plasmid DNA (QIAGEN GmbH, Hilden, Germany), sequencing analysis was performed for verification of the positive mutants.

Table 5. Template DNA for Quickchange Site-Directed mutagenesis.

Template DNA	**Mutant of interest**
pME3596 with *GAL1::SNCA^{WT}*	pME3596 with *GAL1:: SNCA $^{K96R\ K102R}$*
pME3596 with *GAL1::SNCA^{A30P}*	pME3596 with *GAL1:: SNCA $^{A30P\ K96R\ K102R}$*
pRS306 with *SNCA^{WT}::GFP* (KLID linker), *CYC1-Terminator, URA3, integrative, pUC origin, Amp^{R}*	pRS306 with *SNCA$^{K96R\ K102R}$::GFP* (KLID linker), *CYC1-Terminator, URA3, integrative, pUC origin, Amp^{R}*
pRS304 with *GAL1::SNCA^{WT}::GFP* (KLID linker), *CYC1-Terminator, TRP1, integrative, pUC origin, Amp^{R}*	pRS304 with *GAL1::SNCA^{S129}::GFP* (KLID linker), *CYC1-Terminator, TRP1, integrative, pUC origin, Amp^{R}*

2.2.2 *Saccharomyces cerevisiae* methods

2.2.2.1 *S. cerevisiae* transformation

Yeast transformations were performed according to standard protocols (Gietz et al, 1992). Wild type background yeast cells were pre-grown overnight at 30°C in nutrient-rich YPD medium (Guthrie & Fink, 1991). Pre-grown cells were centrifuged at 3000 rpm for 3 min (Sigma 4K15C, Sigma Laboratory Centrifuges, Osterode am Harz, Germany) and transformed to fresh YPD medium for almost 5 hours. Fresh cells were washed with 10 ml 100 mM LiOAc/TE (5 ml 1 M Tris-Cl pH 8.0, 1 ml 0.5 M Na-EDTA pH 8.0, 100 mM LiOAc in a total volume of 50 ml H_2O) three times till being competent for transformation. 20 µl pre-warmed DNA (salmon sperm) was added to 200 µl competent cells. 1 µg of yeast 2-micron plasmid (2µ) was added to competent yeast cells. Integrative yeast plasmids were linearized prior to transformation. For targeting the *trp1* genomic locus, 10 µg of the DNA were digested for 2 h with *Stu*I (Fermentas, St.Leon-Rot, Germany) at 37°C. For targeting the *ura3* genomic locus, 10 µg of the DNA was digested for 2 h with *Eco*8II (Fermentas, St.Leon-Rot, Germany) at 37°C. The total 40 µl of the digestion were added to the yeast competent cells. 800 µl of 50% polyethylene glycol (PEG) in LiOAc/TE were added along with DNA to the competent cells. Cells with the particular DNA were incubated at 30°C at shaking platform for 30 min. Subsequently, heat shock at 42°C was applied to the cells for 20 min. Cells were smoothly centrifuged at 4000 rpm for 20 seconds and the cell pellet was re-suspended in 1 ml fresh YPD medium and incubated at 30°C for 2 hours. Finally, cells were centrifuged at 4000 rpm for 1 min and plated in SC solid medium lacking the selective amino acid. Cells carrying plasmid with *TRP1* marker were plated on SC-trp (lacking TRP amino acid) plates and cells carrying plasmid with *URA3* marker were plated on SC-ura (lacking URA amino acid). Plates were incubated at 30°C for 2-4 days. The positive transformants grown on the selective plates were isolated and re-streaked on a fresh plate.

In case of temperature sensitive strains, cells were pre-grown at 25°C and plates were incubated at 25°C at the end.

2.2.2.2 Isolation of *S. cerevisiae* genomic DNA

S. cerevisiae genomic DNA isolation was performed according to standard procedure (Hoffman & Winston, 1987). Cells were grown overnight in 10 ml YPD medium at 30°C (temperature sensitive yeast cells were grown at 25°C). Grown cells were collected by centrifugation. After washing the collected cells, 200 µl breaking buffer (2% V/V Triton X 100, 1% V/V SDS, 100 mM NaCl, 10 mM Tris-Hcl pH 8.0, 1 mM EDTA pH 8.0), 200 µl Phenol Choloroform Isomyl (25:24:1) and 0.45 mm glass beads were additionally added to the cell pellet. In order to break the cells, rigorous vortexing for 10 min at 4°C was performed. Mechanically broken cells were centrifuged for 5 min at 13000 rpm. Obtained supernatant was collected and mixed with 1 ml cold ethanol for DNA precipitation. Cells were spinned-down shortly and the pellet was incubated with 400 µl TE buffer (10 mM Tris-Hcl pH 7.5/8.0, 1 mM EDTA pH 8.0) and 3 µl RNase (Qiagen, Hilden, Germany) for 1 hour at 37°C. After adding 1 ml ethanol to the probes, they were centrifuged for 5 min at 13000 rpm. The DNA pellet was dried at room temperature. The DNA was dissolved in 50 µl TE buffer and stored at -20°C.

2.2.2.3 *S. cerevisiae* crude extract preparation

Yeast cells harboring DNA of interest were pre-grown in liquid SC medium lacking the selective amino acid, containing 2% raffinose overnight. Pre-grown cells were incubated in SC medium containing 2% galactose for *GAL1*-promoter induction to OD_{600} of 0.1. *GAL1*-promoter was induced for 6 hours and cells were cultivated by centrifugation at 4000 rpm for 3 min. Cell pellet was washed with 1 ml cold TE buffer (10 mM Tris-Hcl pH 7.5/8.0, 1 mM EDTA pH 8.0) and subsequently re-suspended in 200 µl R-buffer (150 µl 1 M Tris-Hcl pH 7.5, 6 µ 0.5 M EDTA, 150 µl 1 M DTT, 120 µl proteases inhibitor mixture (PIM) (Roche, Mannheim, Germany). In order to break the cells mechanically, 0.45 mm glass beads were added to the cell mixture and vigorously vortexed at 4°C for 10 min followed by centrifugation (Biofuge fresco, Heraeus, Hanau, Germany) at 13000 rpm for 10 min. The total protein crude extract was collected as a supernatant. Protein concentration was determined with a Bradford assay (Bradford, 1976).

2.2.2.4 Spotting test

For growth test on solid medium, yeast cells were pre-grown in SC medium containing 2% raffinose lacking the corresponding marker to mid-log phase. Cells

were normalized to equal densities, serially diluted 10-fold starting with an OD_{600} of 0.1, and spotted on SC-plates containing either 2% glucose or 2% galactose and lacking in corresponding marker. *Smt3*^{ts} mutant cells were incubated at permissive temperature (25°C) and restrictive temperature (30°C). W303 yeast cells were incubated only at 30°C. After 3 days incubation the plates were photographed.

2.2.2.5 Halo assay

Smt3^{ts} yeast cells harboring the gene of interest were grown to OD_{600} 0.1. 100 µl of the cell suspension was shifted to 10 ml 0.5 % liquid agar (30°C). The agar was poured onto SC medium plates lacking the selective marker. The filter pre-steriled disc paper soaked with 5 µl 30 % H_2O_2 (AppliChem GmbH, Darmstadt, Germany) was immediately placed onto the agar surface. Plates were incubated at permissive (25°C) and restrictive (30°C) temperatures for 2-3 days. The inhibition area was measured and the plates were photographed.

2.2.2.6 Promoter shut-off assay for aggregate clearance study

Yeast cells carrying α-synuclein or its mutants were pre-grown in SC selective medium containing 2% raffinose overnight at ambient temperature. Pre-grown cells were collected and shifted to 2% galactose SC selective medium to induce α-synuclein expression for 5 hours. In order to shut-off the *GAL1*-promoter, cells were shifted to SC medium containing 2% glucose.

2.2.2.6.1 Proteasome inhibition

According to previously described method (Liu et al, 2007) L-proline was used as nitrogen source instead of ammonium sulfate in growth medium. In addition 0.003% sodium dodecyl sulfate (SDS) was supplemented to the growth medium. In order to block the proteasome, Carbobenzoxyl-leucinyl-leucinyl-leucinal (MG132) dissolved in dimethyl sulfoxide (DMSO) was applied concomitantly to the cell suspension as described previously (Liu et al, 2007) in a final concentration of 75 µM. In parallel, equal volume of DMSO was added to the cells as a control. At several time points after promoter shut-off, cells were visualized by fluorescence microscopy.

2.2.2.6.2 Autophagy pathway inhibition

Phenylmethanesulfonyl fluoride (PMSF) dissolved in ethanol (EtOH) was supplemented to medium containing 2 % glucose in a final concentration of 1 mM. An equal volume of ethanol was added to the cells as a control (Lee & Goldberg, 1998). At several time points after promoter shut-off, cells were visualized by fluorescence microscopy. For experiments with temperature sensitive yeast strain *smt3*^{ts}, pre-incubation was performed at 25°C. Induction of α-synuclein expression and the promoter shut-off assay were performed at 25°C and 30°C. The reduction of number of cells displaying α-synuclein inclusions was recorded and plotted on a graph.

2.2.2.7 Protein stability

Promoter shut-off study was also performed for protein stability assay. Yeast cells were pre-grown in SC selective medium containing 2% raffinose overnight. Pre-grown cells were collected and transformed to SC selective medium containing 2% galactose for four hours *GAL1*-promoter induction. After four hours α-synuclein production, cells were collected. Collected cells were divided in two, half was subjected to protein extract purification (zero point) and the other half was shifted to SC selective medium containing 2% glucose to have the *GAL1*-promoter off. Cells were collected at indicated time points (3 hours, 6 hours, 18 hours).

2.2.3 Protein methods

2.2.3.1 Ni^{2+}-NTA affinity chromatography

Ni^{+2}-NTA affinity chromatography was conducted to purify His6-tagged recombinant protein expressed in *S. cerevisiae* (Porath et al, 1975). *Ulp1*^{ts} mutant cells carrying *GAL1-SNCA* integrations and His6-tagged Smt3 (His-Smt3) were pre-grown in 200 ml SC medium containing 2% raffinose at 30°C overnight. Total cells harvested by centrifugation were transferred to 2 liters YEPD liquid medium containing 2% galactose for 12 hours induction. Cells were collected by centrifugation at 4000 rpm for 20 min at 4°C in the Sorvall RC-3B Plus Refrigerated Centrifuge (Thermo Fisher Scientific Inc., Waltham, USA) and lysed by 25 ml 1.85 M NaOH containing 7.5 % ß-mercaptoethanol (Carl Roth GmbH & CO. KG, Karlsruhe, Germany) for 10 minutes on ice. Protein was precipitated with 25 ml 50% trichloroacetic acid (TCA) (Carl Roth

GmbH & CO. KG, Karlsruhe, Germany) and washed with 100% cold acetone. Proteins were suspended in 25 ml buffer A (6 M guanidine HCl, 100 mM sodium phosphate, 10 mM Tris/HCl, pH 8.0) and rotated for 1 hour at 25°C. The supernatant was cleared by centrifugation; the pH was adjusted to 7.0 by 1 M Tris base and supplemented with imidazole (AppliChem GmbH, Darmstadt, Germany) to final concentration of 20 mM. After equilibration of the His GraviTrap column (GE Healthcare Life Science, Buckinghamshire, United Kingdom) with 5 ml of buffer A containing 20 mM imidazole, proteins were applied to the column and the flow-through fraction was collected for analysis. The column was washed with buffer A supplemented with 20 mM imidazole then with buffer B (8 M Urea, 100 mM sodium phosphate, 10 mM Tris, pH 6.3). The column was washed with buffer C (50 mM Tris pH 8.0, 300 mM NaCl, 20 mM imidazole). Finally, the proteins were eluted four times with 1 ml of 200 mM imidazole resolved in buffer C. Protein concentration in the eluted fractions was determined with Bradford assay. To reuse the columns, they were first washed with 20 ml H_2O followed by 10 mL 0.2 M NaOH and another washing step with 20 ml H_2O. The columns were stored in 20% ethanol.

2.2.3.2 Immunoprecipitation

100 µg protein purified by Ni^{2+}-NTA was incubated with primary antibody (ubiquitin mouse monoclonal antibody, Milipore, MA, USA) at 4°C for 2 hours rotating in Immunoprecipitation (IP) buffer (50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 2 mM EDTA) with freshly added 6 mM protease inhibitor mixture (Roche, Mannheim Germany), 2 mM DTT, 0.1% phosphatase inhibitor (Roche, Mannheim Germany). The mixture was then incubated with pre-washed Protein A Sepharose beads (GE Healthcare Life Science, Buckinghamshire, United Kingdome) in IP buffer overnight rotating at 4°C. The mixture was centrifuged at 4°C for 1 min at 13000 rpm. After discarding the supernatant, the beads were washed three times with ice-cold IP buffer. The immunoprecipitated protein was dissolved from the beads by heating in 1x sample loading buffer at 95^{o}C for 10 min. The samples were subjected to Western hybridization analyses using rabbit α-synuclein polyclonal antibody (Santa Cruz Biotechnology, CA, USA).

2.2.3.3 Immunoblotting

Wild type (W303-1A) yeast cells harboring α-synuclein were pre-grown at 30°C in SC selective medium containing 2% raffinose. Cells were transferred to SC medium containing 2% galactose at OD_{600} of 0.1 to induce the *GAL1*-promoter for 5 hours.

Smt3^{ts} cells harboring α-synuclein were pre-incubated at 25°C and later transferred to either 25°C or 30°C. Total protein extracts were prepared as described before. The protein concentrations were determined with a Bradford assay. 10 µg of each protein was incubated with SDS loading buffer (250 mM Tris-HCl pH 6.8, 15% β-mercaptoethanol, 30% glycerol, 7% SDS, 0.3% bromphenol blue) and denatured at 95°C and then were subjected to 12% SDS-polyacrylamide gel electrophoresis. Separated proteins were transferred to a nitrocellulose membrane (Whatman Protran, Whatman GmbH, Dassel, Germany) by blotting for 1 hour at 100 V. The membrane was blocked in TBST buffer (100 mM Tris-HCl pH 8.0, 1.5 M NaCl, 0.5 % (v/v) Tween-20) containing 5% milk powder for at least 1 hour. Membrane was probed with primary antibody at 4°C. Primary antibodies used in this study were listed in Table 6. Membrane was washed with 1×TBST three times and then incubated with secondary antibody for 2 hours at room temperature. The secondary antibodies used in this study were (i) peroxidase-coupled goat anti-mouse or (ii) goat anti-rabbit immunoglobins G (Invitrogen GmbH, Karlsruhe, Germany). After washing the membrane with 1×TBST for at least three times, the proteins were detected with reagents from the Immobilon Western Chemiliminiscent HRP Substrate detection Kit (Millipore, Schwalbach, Germany). The membrane was exposed in the dark to ECL film (GE Healthcare Limited, Buckinghamshire, United Kingdome) and the film was developed in the PROTEC Processor Compact film-developing machine (Siemens, Erlangen, Germany).

Table 6. Primary antibodies used in this study.

Antibody	Animal	Type	Dilution	Source
Anti-α-synuclein	Mouse	Monoclonal	1:3000	AnaSpec, Fremont, CA, USA
Anti-β-synuclein	Rabbit	Monoclonal	1:5000	abcam, Cambridg, United Kingdome
Anti- α-synuclein S129 phosphorylated	Mouse	Monoclonal	1:5000	Wako, Osaka, Japan
Anti-ubiquitin	Mouse	Monoclonal	1:1000	Millipore, Billerica, MA, USA
Anti-SUMO	Rabbit	Polyclonal	1:1000	Rockland, Gilbertsville, USA
Anti- α-synuclein	Rabbit	Polyclonal	1:2000	abcam, Cambridge, United Kingdome

2.2.3.4 Quantification of Western hybridizations

Pixel density values for Western quantification were obtained from TIFF files generated from digitized X-ray films (KODAK) and analyzed with the ImageJ software (Abramoff et al, 2004). Before comparison, sample density values were normalized to the corresponding loading control. The adjusted density values were standardized to the control lane to get fold increase. The significance of differences was calculated using Students t-test or one-way ANOVA test. P value < 0.05 was considered to indicate a significant difference.

2.2.3.5 Tandem Ubiquitin Binding Entities (TUBEs)

Determination of poly-ubiquitinated proteins can be achieved with certain ubiquitin binding associated domains (UBAs). Agarose Tandem Ubiquitin Binding Entities (TUBEs) (LifeSensors, Inc. Malvern, USA) facilitate “one-step” pull-down of poly ubiquitinated proteins. 500 µg of the protein crude extract, isolated from yeast cells were subjected to TUBEs pull-down. Equilibration of Agarose TUBEs was performed according to manufacture instruction.

Initially, 500 µg of total protein was diluted to equal volume of 200 µl final in R-buffer (50 mM Tris-Hcl pH 7.5, 1 mM EDTA, 50 mM DTT, 1× Proteases Inhibitor Mixture (PIM)). 20 µl of TUBEs was added to each sample and the pull-down was followed by the suggested protocol from the manufacture. Untreated Agarose beads were applied as negative control. Supernatants were stored as unbound-fraction and applied to Western hybridization analysis in parallel with the pull-down proteins purified from TUBEs. The purified samples and the supernatants were mixed in SDS loading buffer (250 mM Tris-HCl pH 6.8, 15% β-mercaptoethanol, 30% glycerol, 7% SDS, 0.3% bromphenol blue) and denatured at 95°C. The samples were further analyzed with Western hybridization (Laemmli, 1970).

2.2.4 Fluorescence microscopy and quantifications

Wild type (W303-1A) yeast cells harboring α-synuclein were grown in SC selective medium containing 2% raffinose at 30°C and *smt3*^{ts} mutant cells at 25°C overnight. Pre-grown cells were transferred to 2% galactose containing medium for induction of α-synuclein expression for 6 hours. S*mt3*^{ts} mutant cells were induced at 25°C and 30°C. Fluorescent images were obtained with Zeiss Observer (Zeiss, Göttingen, Germany). Z1 microscope equipped with CSU-X1 A1 confocal scanner unit

(YOKOGAWA), QuantEM: 512SC (Photometrics) digital camera and SlideBook 5.0 software package (Intelligent Imaging Innovations). For quantification of α-synuclein aggregation at least 300 cells were counted per strain and per experiment. The number of cells presenting inclusions was referred to the total number of cells counted. The values are mean of at least three independent experiments.

3 Results

3.1 Post-translational modifications of α-synuclein in yeast model of Parkinson's disease

3.1.1 Sumoylation of α-synuclein

3.1.1.1 α-synuclein is sumoylated in yeast

Sumoylation is a rapid and reversible protein modification that plays an important role to change and regulate its substrate protein stability, localization, interactions and function. SUMO protein is involved in various neurodegenerative diseases such as Huntington's disease, multiple system atrophy and Parkinson's disease (Dorval & Fraser, 2006; Krumova et al, 2011). α-synuclein protein, which is implicated in Parkinson's disease pathology, was shown to be mono-sumoylated in mammalian cells (Krumova et al, 2011). However, the impact of sumoylation on α-synuclein-mediated toxicity remains to be elucidated.

Here, we addressed the question if α-synuclein sumoylation is conserved from yeast to higher eukaryotic cells. Yeast cells harbor the essential gene *SMT3* homologue to human *SUMO1* (Takahashi et al, 1999). We first analyzed whether α-synuclein is sumoylated in yeast cells. The dynamics of between sumoylation and de-sumoylation reactions impede accurate studies. The *ulp1^{ts}* strain, defective in SUMO de-conjugation enzyme, was transformed with wild type (WT) α-synuclein and A30P mutant and the empty vector (control) driven under *GAL1* promoter and integrated in the *TRP1* genomic locus. The pre-grown cells at permissive temperature (25°C) were shifted to restrictive temperature (30°C), which enabled the down regulation of SUMO de-conjugation enzymes. *Ulp1^{ts}* cells expressing WT α-synuclein and A30P mutant were examined by Western hybridization to choose the best candidate for further investigation (Figure 7A). Western hybridization analysis with SMT3 antibody revealed that down regulation of the ULP1 protease activity at non-permissive 30°C resulted in enrichment of SUMO-conjugated proteins in *ulp1^{ts}* yeast background compared to the control (W303) (Figure 7B). In order to detect α-synuclein conjugated to SMT3, the *ulp1^{ts}* strain expressing WT α-synuclein and A30P were co-transformed with His-Smt3 followed by Ni^{2+} affinity chromatography. Total sumoylated proteins were pulled down by Ni^{2+}-NTA. The modified α-synuclein was successfully detected from the total sumoylated proteins by western hybridization

with α-synuclein specific antibody (Figure 7C). Sumoylated WT α-synuclein and A30P mutant migrated approximately to 35 kDa, whereas unmodified protein migrates around at 17 kDa. This data indicate that WT α-synuclein and its mutant A30P are sumoylated *in vivo* by the yeast homologue of human SUMO, which support the conserved function of α-synuclein sumoylation from yeast to mammalian organisms.

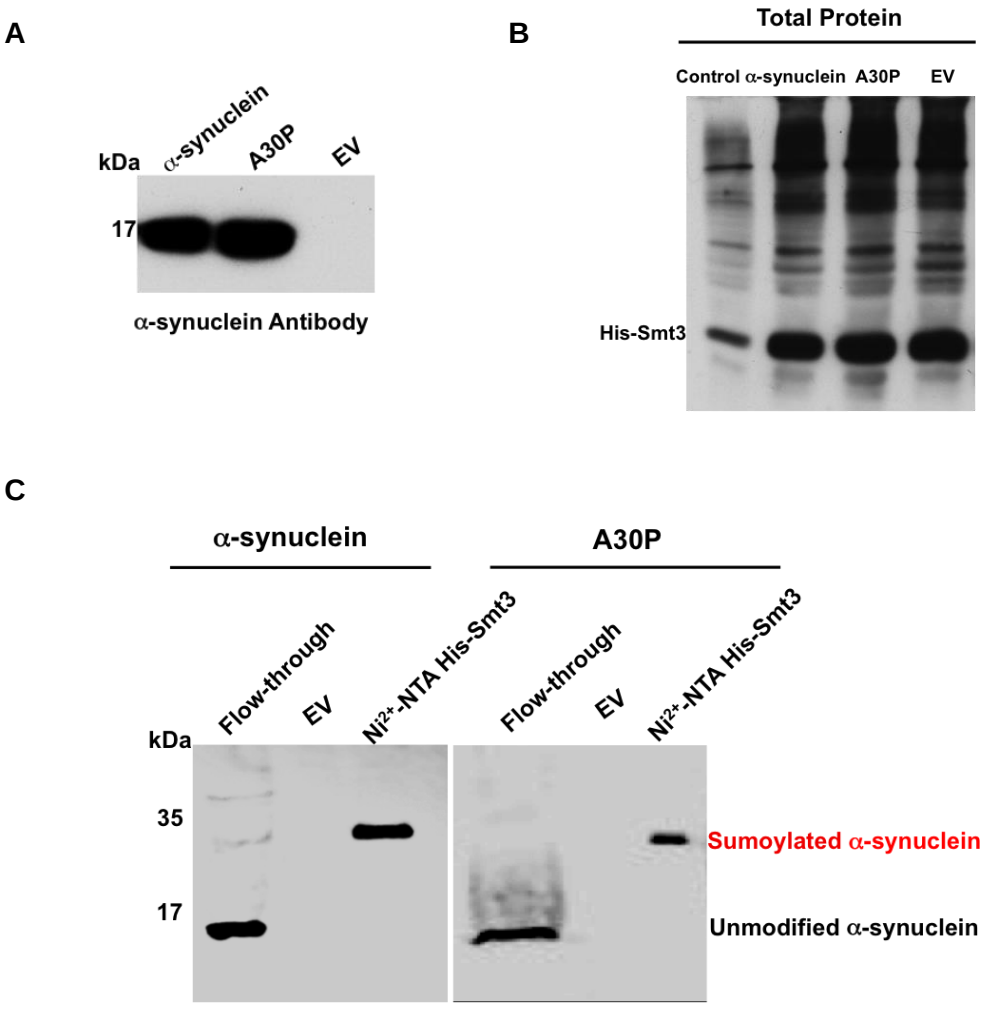

Figure 7. α-synuclein is sumoylated in *Saccharomyces cerevisiae*.

A. Total protein extracts of *ulp1*^{ts} cells expressing α-synuclein, A30P mutant and empty vector (EV) were subjected to Western hybridization analyses. α-synuclein was detected using specific α-synuclein antibody. **B.** Total protein extract of *ulp1*^{ts} yeast cells, defective in SUMO-de-conjugation, co-expressing α-synuclein and His6-tagged Smt3, A30P and His6-tagged Smt3 and EV and His6-tagged Smt3. Enriched sumoylated proteins in the *ulp1*^{ts} strain in comparison with the control WT yeast background (W303) were detected by Western hybridization with anti-Smt3 antibody. **C.** Nickel pull-down of His6-tagged Smt3 (His-Smt3) in *ulp1*^{ts} cells co-expressing α-synuclein. Sumoylated α-synuclein was detected in the pull-down fractions with α-synuclein antibody. Unmodified α-synuclein was detected in flow-through. Yeast cells transformed with empty vector were used as a control.

3.1.1.2 Lysines 96 and 102 are the major α-synuclein sumoylation sites in yeast as in human

α-synuclein contains two SUMO consensus acceptor sites, K96 and K102, and eleven-nonconsensus lysines (K6, K10, K12, K21, K23, K32, K34, K43, K45, K58 and K60) (Figure 8). It had been shown that K96 and K102 serve as key sumoylation sites of α-synuclein in human cells (Krumova et al, 2011).

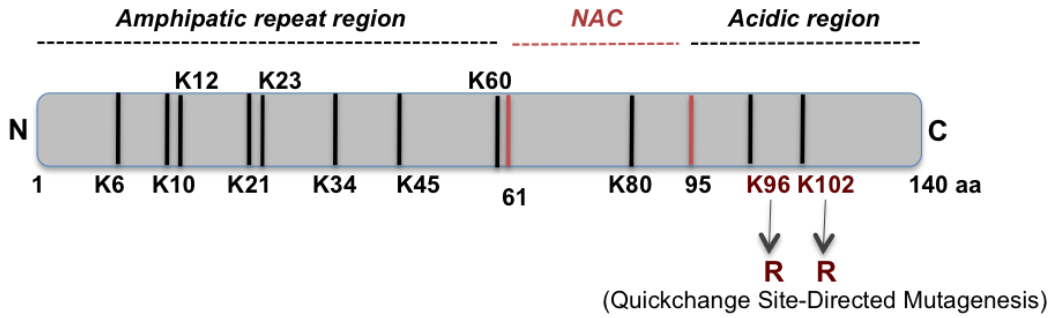

Figure 8. α-Synuclein SUMO acceptor sites.

Scheme of α-synuclein sumoylation sites. α-synuclein displays in total eleven non-consensus lysine (K) residues of which 8 lysines in the N-terminal amphipathic region, 1 lysine in the hydrophobic NAC region, and the 2 lysines in the C-terminal acidic region. K96 and K102 are identified as the major α-synuclein sumoylation sites (Krumova et al, 2011). Lysine 96 (K96) and lysine 102 (K102) were substituted to arginine (K96R K102R) with Quickchange Site-Directed Mutagenesis in this study.

In order to verify if K96 and K102 are conserved as major sumoylation sites of α-synuclein in yeast, the codon for the residues K96 and K102 in WT α-synuclein and A30P-synuclein were substituted to arginine codon using Quickchange Site-Directed Mutagenesis method (Figure 8). The newly generated constructs were verified and sequenced. The proved K96R K102R-synuclein and A30P K96R K102R-synuclein codon replacement constructs were transformed in *ulp1*^{ts} strain. The strains that expressed appropriate amounts of α-synuclein variants (K96R K102R-synuclein or A30P K96R K102R-synuclein) were co-transformed with His-Smt3 in order to purify the sumoylated proteins by Ni^{2+}-NTA. After purifying all substrates conjugated to His-Smt3, Western hybridization analyses with α-synuclein antibody were performed. A significant reduction of the sumoylation of K96R K102R variant and a complete sumoylation abolishment in the A30P variant carrying substitutions in K96 and K102 were observed (Figure 9). This result suggests that α-synuclein SUMO acceptor sites are conserved in yeast.

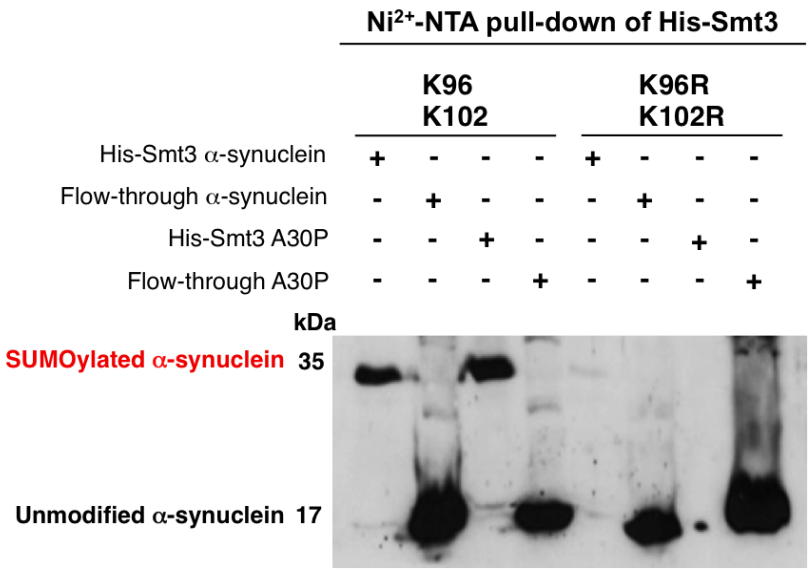

Figure 9. Major sumoylation residues of α-synuclein are conserved in yeast.

Lysines 96 and 102 to arginine codon substitutions (K96R K102R) resulted in decreased α-synuclein sumoylation. Not mutated (K96 K102) and mutated (K96R K102R) WT α-synuclein and A30P were transformed in *ulp1*^{ts} yeast cells expressing His-Smt3. His6-tagged SUMO-conjugates were pulled down by Ni^{2+}-NTA and α-synuclein was detected by Western hybridization using α-synuclein antibody.

3.1.1.3 Sumoylation protects yeast cell against α-synuclein-mediated cytotoxicity and aggregate formation

The importance of sumoylation in Parkinson's pathology arises the question how sumoylation might affect α-synuclein-mediated cytotoxicity in yeast cells. It has been shown that inhibiting α-synuclein from sumoylation leads to neuronal cell death in the rat model of Parkinson's disease (Krumova et al, 2011) though it has been reported that accumulation of sumoylated α-synuclein resulting from proteasome inhibition leads to cell death (Kim et al, 2011). Hence, further investigations are essential to lighten the road of sumoylation impact in Parkinson's disease. *Saccharomyces cerevisiae,* temperature sensitive *smt3* mutant, conditionally defective in the yeast SUMO gene, was used in this study to investigate the effect of sumoylation impairment on α-synuclein toxicity. Different sumoylation status can be achieved by growing temperature sensitive yeast cells at permissive temperature (25°C) and at restrictive temperature (30°C) when sumoylation is down regulated. *Smt3*^{ts} strain was transformed with WT α-synuclein-KLID-GFP from integrative plasmid and A30P-KLID-GFP with high-copy plasmid expression. GFP expressing cells were used as a control. The number of integrated copies was determined by Southern hybridization

and *smt3*^{ts} strain expressing two copy integrations of WT α-synuclein were selected for analysis (Figure 12). It has been shown that expression of WT α-synuclein from two copies is under the threshold for yeast growth inhibition (Petroi et al, 2012).

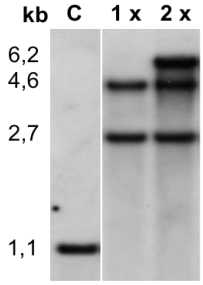

Figure 10. Determination of gene copy number of α-synuclein, integrated in the genome.

Southern hybridization of *smt3*^{ts} strains expressing WT α-synuclein using labeled *URA3* as probe. Integrated α-synuclein-GFP genes correspond to 2.7 kb + 4.7 kb (1 x) and 2.7 kb + 4.7 kb + 6.2 kb (2 x) according to the Southern hybridization strategy. C (control): genomic locus without vector integration; corresponds to 1.1 kb.

Growth of the *smt3*^{ts} yeast cells expressing WT α-synuclein and A30P mutant were compared in the presence or absence of SUMO at permissive (25°C) and restrictive (30°C) temperatures. At the permissive temperature (25°C), when sumoylation is not impaired, all strains grew equally well (Figure 11). Spotting assays revealed that at the restrictive temperature (30°C), when sumoylation is down regulated, expression of wild-type α-synuclein resulted in growth inhibition in comparison to cells expressing GFP as a control. Similar results were obtained for A30P, where high-copy plasmid expression normally does not impair yeast growth (Petroi et al, 2012), whereas defects in sumoylation resulted in a drastic growth inhibition (Figure 11). This suggests that SUMO modification has a protective role in α-synuclein expressing yeast cells.

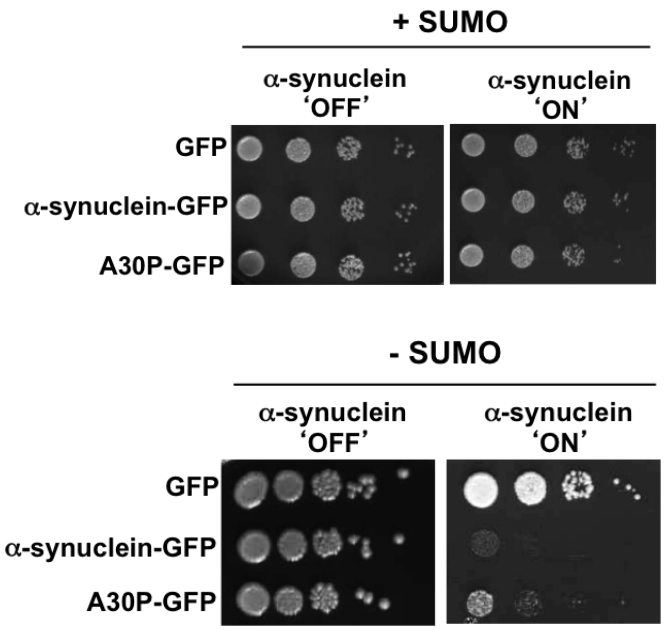

Figure 11. Sumoylation protects against α-synuclein-mediated toxicity.

Spotting assay of *smt3*^{ts} mutant strains expressing α-synuclein-GFP or A30P-GFP at permissive (25°C; + SUMO) or restrictive temperature (30°C; - SUMO). *GAL1*-driven α-synuclein-GFP is expressed from two genomically integrated copies. *GAL1*-driven A30P-GFP is expressed from a 2 µm plasmid. GFP, expressed from the same promoter, is used as a control. Yeast cells were spotted in 10-fold dilutions on selection plates containing glucose (α-synuclein 'OFF') or galactose (α-synuclein 'ON').

Furthermore, to study the correlation of sumoylation-mediated α-synuclein cytotoxicity and α-synuclein aggregate formation, live cell fluorescence microscopy was conducted. Cells were visualized after five hours of α-synuclein protein induction. Quantification of the number of *smt3*^{ts} cells displaying α-synuclein inclusions revealed significant increases in cells displaying α-synuclein inclusions when sumoylation is down regulated. To check for possible temperature effects, the wild type yeast background (W303), *smt3*^{ts} parental strain, was employed for comparison. The temperature did not affect the number of W303 cells with α-synuclein aggregates (Figure 12), which excluded that the difference in the number of cells with inclusions is due to a temperature shift. This further suggests a protective role of sumoylation against α-synuclein aggregate formation. Our data strengthen the idea of an α-synuclein sumoylation protective effect against α-synuclein-mediated cytotoxicity and aggregate formation, which is conserved from yeast to higher model systems like rat (Krumova et al, 2011).

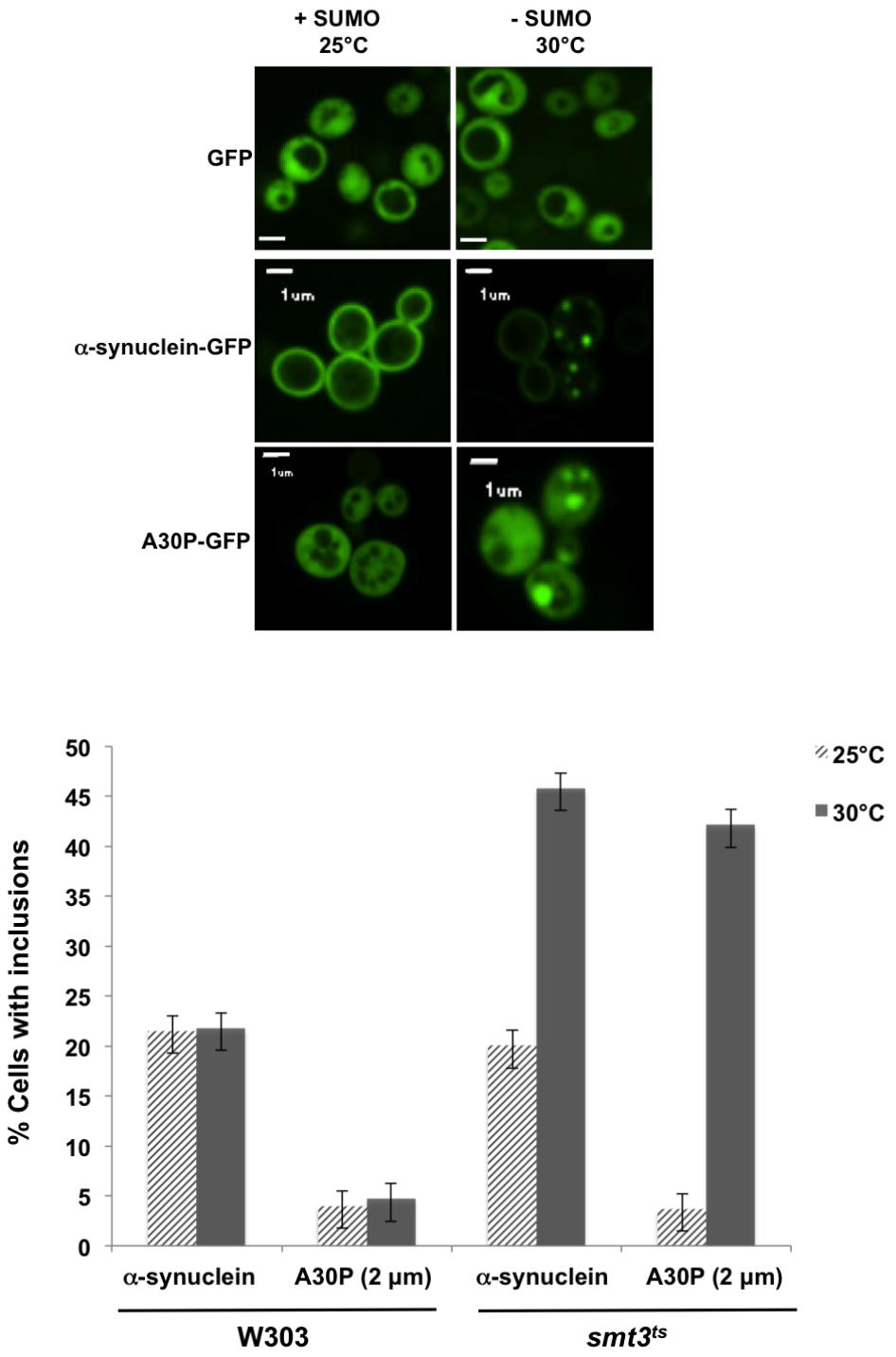

Figure 12. Sumoylation impairment increases α-synuclein aggregate formation.

Fluorescence microscopy of *smt3*^{ts} cells expressing α-synuclein-GFP or A30P-GFP at permissive (25°C; + SUMO) or restrictive temperature (30°C; - SUMO). Cells expressing GFP alone showed as control. Scale bar, 1 µm, (up). Quantification of the percentage of cells displaying α-synuclein inclusions. At least 300 cells were counted per strain and experiment (below).

3.1.1.4 Protective function of SUMO requires direct α-synuclein modification

In order to analyze whether the protective function of SUMO requires direct sumoylation of α-synuclein, W303 yeast strain was transformed with α-synuclein-deficient sumoylation variant (K96R K102R-synuclein). A yeast strain carrying two copies of K96R K102R-KLID-GFP has been determined by Southern analysis (Figure 13) and subjected to growth assay (Figure 14A). In addition, the number of cells carrying sumoylation-deficient α-synuclein aggregates were quantified after visualization by fluorescence microscopy (Figure 14B). Cells expressing K96R K102R-synuclein displayed an increased number of cells with inclusions compared to WT α-synuclein. Thus, blocking the key α-synuclein sumoylation residues resulted in higher α-synuclein-mediated cytotoxicity and aggregate formation, which further supports the direct protective role of sumoylation against α-synuclein-mediated toxicity.

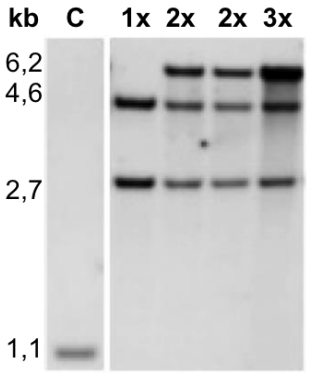

Figure 13. Southern hybridization using labeled *URA3* as probe.

Integrated α-synuclein-GFP genes correspond to 2.7 kb + 4.7 kb (1x) and 2.7 kb + 4.7 kb + 6.2 kb (2 x) and 2.7 kb + 4.7 kb + 6.2 kb (higher intensity) (3 x). C (control): genomic locus without vector integration; corresponds to 1.1 kb.

Expression of the K96R K102R-KLID-GFP (two copies) resulted in growth inhibition in contrast to WT α-synuclein (two copies), which did not impair yeast growth (Figure 14A).

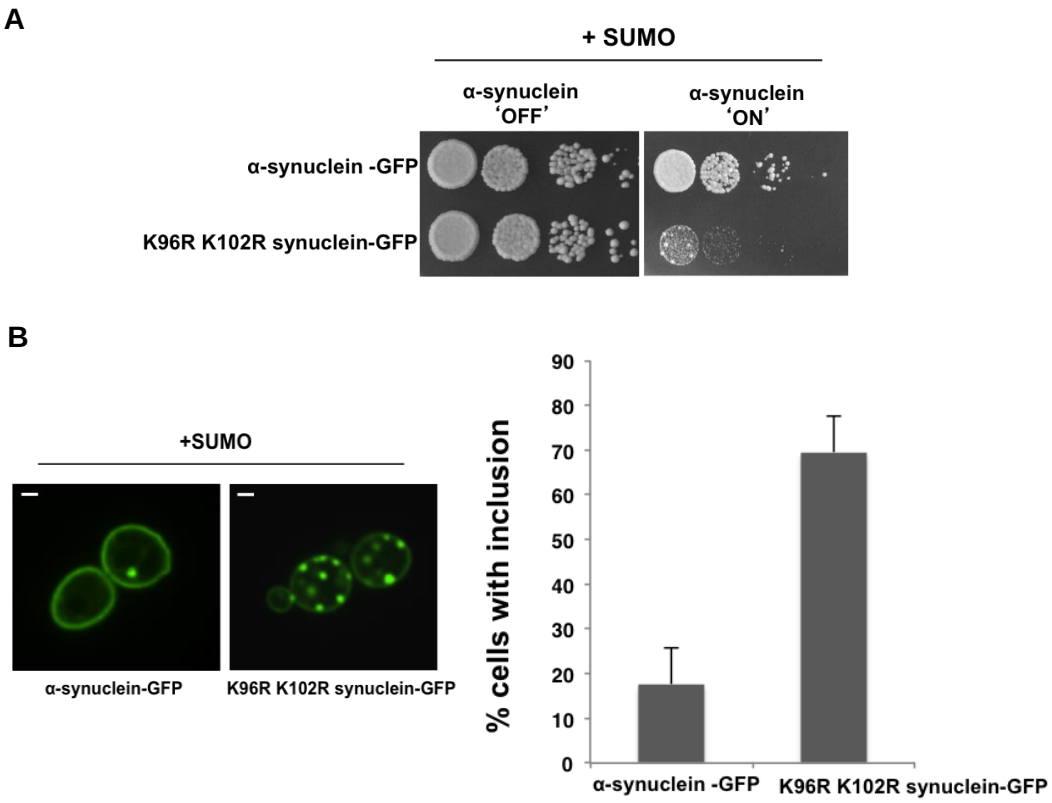

Figure 14. Sumoylation deficient α-synuclein (K96R K102R synuclein) is toxic to yeast and forms aggregates.

A. Spotting assay of W303 yeast cells, carrying two copies of *GAL1*-driven α-synuclein-GFP and K96R K102R synuclein-GFP. Yeast cells were spotted in 10-fold dilutions on selection plates containing glucose (α-synuclein 'OFF') or galactose (α-synuclein 'ON'). **B.** Live-cell fluorescence microscopy of W303 cells expressing K96R K102R synuclein. Scale bar 1 µm (left). Quantification of the percentage of cells displaying α-synuclein inclusions (right).

3.1.2 Phosphorylation of α-synuclein

3.1.2.1 α-synuclein is phosphorylated in yeast

The major goal of this study was to investigate a possible cross-talk between two main post-translational modifications of α-synuclein; sumoylation and phosphorylation. α-synuclein deposited in Lewy bodies is highly phosphorylated. 90% of α-synuclein is phosphorylated at S129 within Parkinson's disease Lewy bodies, whereas in the healthy brain, less than 5% of α-synuclein is phosphorylated

at S129 (Anderson et al, 2006; Fujiwara et al, 2002). The effects of phosphorylation on α-synuclein-induced toxicity are complex with reports supporting negative as well as positive impacts on cells (Azeredo da Silveira et al, 2009; Chen & Feany, 2005; Freichel et al, 2007; Gorbatyuk et al, 2008; Hasegawa et al, 2002a; Hasegawa et al, 2002b). Therefore, we next investigated the interplay between α-synuclein sumoylation and phosphorylation by examining how changes in sumoylation affect α-synuclein phosphorylation and whether this impacted on α-synuclein toxicity.

3.1.2.2 Overexpression of yeast endogenous kinase Cdc5 is toxic to yeast cells

Various kinases have been implicated in phosphorylation of α-synuclein at S129. Serine/threonine Polo like kinases family was shown to contribute more than other kinases (Waxman & Giasson, 2011). These kinases are highly conserved from yeast to human. *Saccharomyces cerevisiae* has a single endogenous Polo-like kinase; Cdc5, which can phosphorylate α-synuclein in yeast (Gitler et al, 2009; Wang et al, 2012). We started analyzing the effect of Cdc5 on α-synuclein toxicity in yeast. We used *cdc5-DAmP* strain in which the Cdc5 expression is downregulated in the presence of geneticine (G418). Further *cdc5-DAmP* strains were generated that expressed WT α-synuclein or A30P synuclein from two genomically integrated copies by synthetic genetic array technology (B. Popova, unpublished). Growth test of *cdc5-DAmP* cells expressing WT α-synuclein and A30P mutant revealed that when Cdc5 is down regulated yeast cells expressing WT α-synuclein or A30P mutant grow equally to the control cells, where α-synuclein(s) were not expressed (Figure 15).

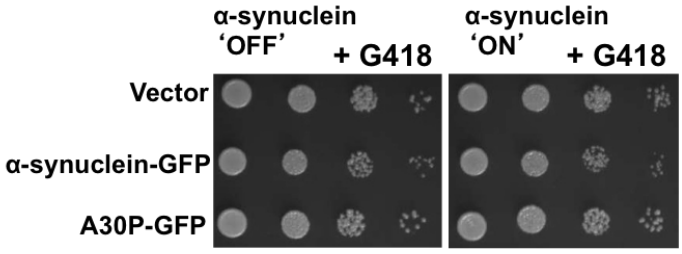

Figure 15. Down regulation of Cdc5 has no impact on α-synuclein mediated toxicity.

Spotting assay of DAmP (Decreased Abundance by mRNA Perturbation) allele of Cdc5, carrying two copies of *GAL1*-driven α-synuclein-GFP or A30P-GFP. Decreased Abundance by mRNA Perturbation (DAmP) allele of *cdc5* was used to assess the effect of down-regulation of the Cdc5 activity. Empty vector is used as a control. *Cdc5-DAmP* cells were spotted in 10-fold dilutions on selection plates containing glucose (α-synuclein 'OFF') or galactose (α-synuclein 'ON') with geneticin (*G418)*.

This data suggested that down regulation of Cdc5 did not affect α-synuclein-related behavior. In order to investigate whether overexpression of Cdc5 has an effect on α-synuclein expressing cells, the *smt3*^{ts} cells expressing WT α-synuclein from two genomically integrated copies were co-transformed with *GAL1*-driven Cdc5. Cells expressing GFP and Cdc5 were taken as control. Spotting assay showed that overexpression of Cdc5 resulted in growth impairment in cells, co-expressing GFP and Cdc5 either at permissive 25°C or restrictive temperature 30°C. Remarkably, overexpression of Cdc5 was lethal to yeast cells co-expressing α-synuclein(s) and Cdc5, which suggested that higher level of Cdc5 might result in disruption of other molecular pathways in yeast which made it lethal to the cells (Figure 16).

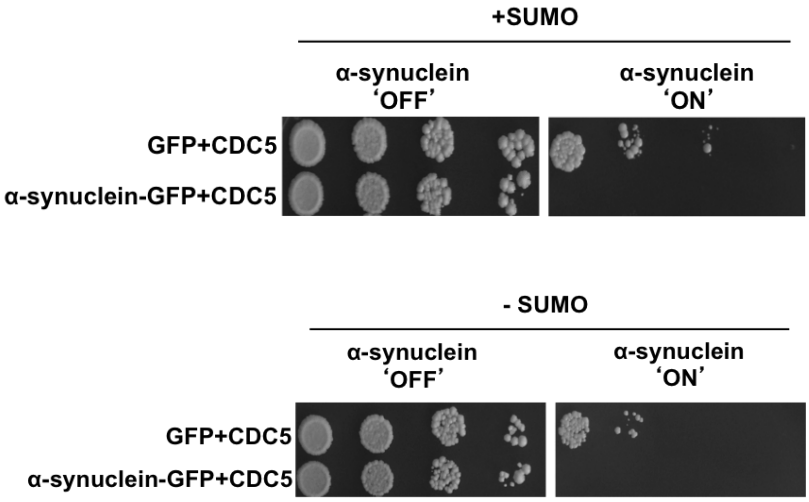

Figure 16. Overexpression of CDC5 is toxic to yeast cells.

Spotting assay of *smt3*^{ts} cells co-expressing α-synuclein-GFP from two genomically integrated copies with *GAL1*-CDC5, expressed form a 2 μm plasmid, either at permissive (25°C; + SUMO) or restrictive temperature (30°C; - SUMO). Yeast cells were spotted in 10-fold dilutions on selection plates containing glucose (α-synuclein 'OFF'; kinase 'OFF') or galactose (α-synuclein 'ON'; kinase 'ON').

3.1.2.3 Expression of human kinases GRK5 and PLK2 elevates α-synuclein phosphorylation level

α-synuclein is constitutively phosphorylated in low levels at S129 in normal brains and predominantly in Lewy bodies extracted from Parkinson's disease patient brains (Fujiwara et al, 2002). Different human kinases are involved in α-synuclein phosphorylation (Ellis et al, 2001; Fujiwara et al, 2002; Inglis et al, 2009; Mbefo et al, 2010; Okochi et al, 2000; Pronin et al, 2000; Sakamoto et al, 2009; Waxman & Giasson, 2008; Waxman & Giasson, 2011). Among them, PLK2 and GRK5 are

shown to be the most efficient kinases in phosphorylating α-synuclein at S129 (Inglis et al, 2009; Pronin et al, 2000; Salvi et al, 2012). To see whether human kinases GRK5 and PLK2 affect α-synuclein phosphorylation level at S129 in yeast, GRK5 and PLK2 were cloned in yeast high-copy plasmid (2 µm) under *GPD* and *GAL1* promoter, respectively. *Smt3*^{ts} mutant strains carrying two copies of WT α-synuclein were co-transformed with *GPD and GAL1*-driven GRK5 or PLK2 and for comparison a vector control (EV). Western hybridization analyses with α-synuclein phosphorylated at S129 specific antibody (αSyn pS129) revealed that heterologous expression of kinases GRK5 or PLK2 resulted in increased phosphorylation of α-synuclein at S129 in comparison to control cells without additional kinase activity (EV) (Figure 17).

In the absence of human kinases α-synuclein is phosphorylated at S129 by the Polo-like endogenous kinase Cdc5 (Wang et al, 2012), which is detected here in the control lane when no heterologous kinases were present (Figure 17).

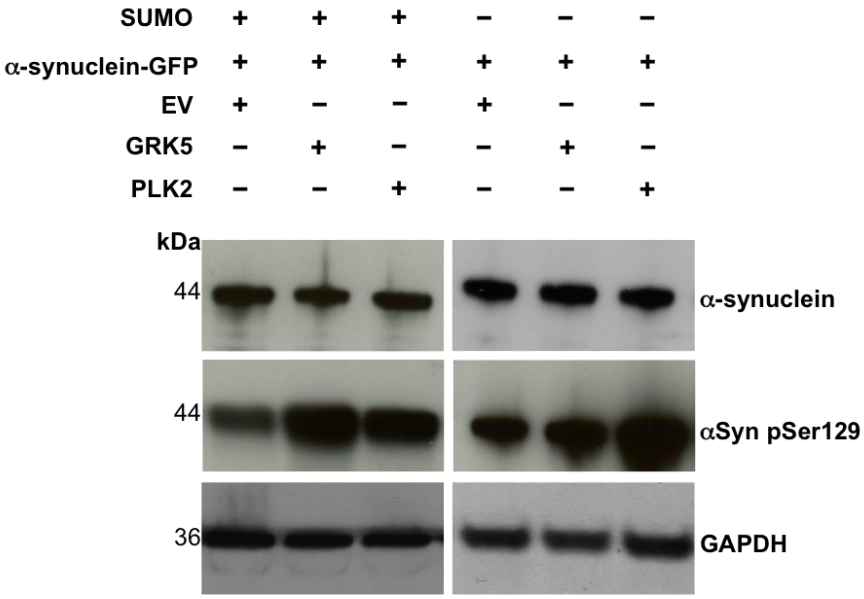

Figure 17. Expression of the human kinases GRK5/PLK2 increases α-synuclein S129 phosphorylation in yeast.

*Smt*3^{ts} mutant cells co-expressing α-synuclein and GRK5 or PLK2 at permissive (25°C; + SUMO) or restrictive temperature (30°C; - SUMO). α-synuclein expression was detected using α-synuclein specific antibody. The phosphorylation level of α-synuclein on S129 was detected by α-synuclein S129 phosphorylation specific antibody (αSyn pSer129) when expressed either alone (α-synuclein–GFP + empty vector (EV)) or in the presence of GRK5 or PLK2. GAPDH served as loading control.

3.1.2.4 GRK5 phosphorylates α-synuclein constitutively, whereas PLK2 prefers non-modified α-synuclein

Proteins can be subjected to various post-translational modifications, which can interplay directly or indirectly to regulate their substrates function (Fink, 2005). With the previous results we showed that α-synuclein is sumoylated in yeast at the same sites as in human cells. In addition, the expression of human kinases PLK2 and GRK5 resulted in higher α-synuclein phosphorylation at S129 in yeast cells. Further, we aimed to investigate whether there is a cross-talk between α-synuclein sumoylation and α-synuclein S129 phosphorylation. We addressed this question by investigating whether sumoylation affects α-synuclein S129 phosphorylation levels. Several studies demonstrated that phosphorylation of some proteins (for example heat shock factors) is dependent on their sumoylation status (Hietakangas et al, 2006; Yao et al, 2011). We explored the effect of sumoylation on α-synuclein phosphorylation at S129 by lowering the total cellular SUMO level and comparing the α-synuclein S129 phosphorylation level in the presence of GRK5 and PLK2. The constructed *smt3*^{ts} strain harboring two genomic copies of WT α-synuclein (Figure 10) were transformed with the human kinase PLK2 or GRK5. To analyze how the sumoylation profile alters α-synuclein phosphorylation at S129 in the presence of PLK2 or GRK5, yeast cells were pre-grown at permissive temperature (25°C) and shifted in parallel to both 25°C and 30°C in 2% galactose inducing media for 5 hours α-synuclein production. The total protein content of yeast cells was extracted and subjected to Western hybridization analysis. In order to quantify the phosphorylated α-synuclein at S129, four independent clones were investigated. Quantifying increased α-synuclein S129 (Figure 18) phosphorylation level in both conditions (with or without SUMO) revealed that GRK5 leads to higher phosphorylation level of α-synuclein at S129 independently from sumoylation statues, suggesting that GRK5 can use sumoylated as well as unmodified α-synuclein as substrate to phosphorylate S129. PLK2 promotes α-synuclein phosphorylation level at S129 even more, when sumoylation is down regulated at 30°C and the total cellular sumoylated proteins were reduced. These results suggest differences in substrate specificity for the two kinases. PLK2 kinase is further stimulated to phosphorylate S129 when the target protein is unmodified, whereas GRK5 seems to be less specific in phosphorylating S129 of sumoylated or unmodified α-synuclein. Overall, these data suggest an interplay between α-synuclein sumoylation and phosphorylation.

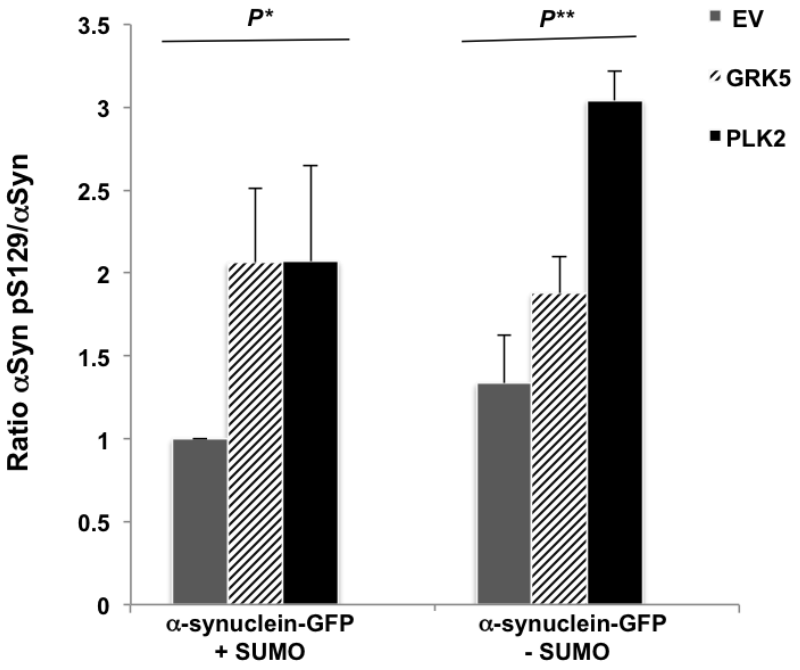

Figure 18. Interplay between sumoylation and α-synuclein phosphorylation.

Quantification of α-synuclein S129 phosphorylation level in the presence or absence of GRK5 and PLK2, respectively at permissive (25°C; + SUMO) or restrictive temperature (30°C; - SUMO). Densitometric analysis of the immunodetection of αSyn pSer129 was normalized to the total amount of α-synuclein and relative to α-synuclein + EV at permissive temperature (25°C; + SUMO). Significance of differences was calculated with one-way ANOVA test (*P* < 0,01; P** < 0.001*, n=4).

3.1.2.5 α-synuclein is sumoylated in the presence of GRK5

Our previous findings support an interplay between sumoylation and α-synuclein phosphorylation (Figure 18). In order to get more insight into the interplay between these two modifiers, we examined whether increased α-synuclein S129 phosphorylation levels by GRK5 changed its sumoylation level. *Ulp1^{ts}* cells co-expressing WT α-synuclein and His-Smt3 were co-transformed with GRK5 and the kinase backbone empty vector (EV) as control. The total sumoylated proteins were purified by Ni^{2+}-NTA. To get reliable quantifications, three independent experiments were performed. The result revealed that α-synuclein is sumoylated when co-expressed with GRK5, however no significant differences could be identified in the level of sumoylated α-synuclein (Figure 19).

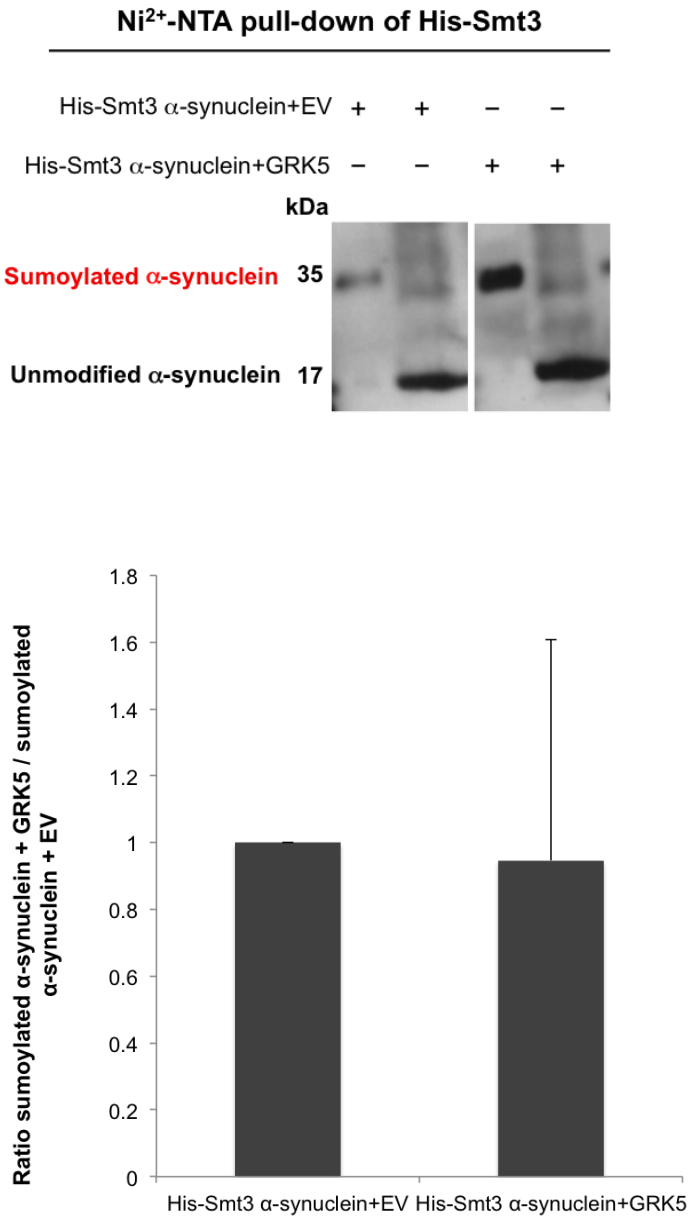

Figure 19. α-synuclein is sumoylated in the presence of GRK5.

Nickel pull-down of His6-tagged Smt3 (His-Smt3) in *ulp1*^{ts} cells co-expressing α-synuclein and GRK5 or empty vector (EV). Sumoylated α-synuclein was detected in the pull-down fractions with α-synuclein antibody. Unmodified α-synuclein was detected in flow-through (upper panel). α-synuclein sumoylation level in the presence and absence of GRK5 was quantified and presented as ratio to the control (input α-synuclein) (below panel).

3.1.2.6 Higher α-synuclein phosphorylation levels at S129 by GRK5 and PLK2 expression alleviate induced cytotoxicity and inclusion formation in a SUMO-deficient yeast strain

To further study the interplay between sumoylation and α-synuclein S129 phosphorylation, the effect of increased α-synuclein S129 phosphorylation by GRK5 or PLK2 expression on α-synuclein-mediated cytotoxicity associated with sumoylation impairment was questioned. The effect of GRK5 and PLK2 on α-synuclein-mediated toxicity associated with sumoylation impairment was tested by spotting assays. Cells expressing two copies of WT α-synuclein already presented significant growth inhibition when sumoylation is impaired at 30°C (Figure 11) but in the presence of human kinases yeast cells rescued from α-synuclein-mediated toxicity in the absence of SUMO (Figure 20). We found that GRK5 suppressed the growth defect associated with impaired sumoylation whereas PLK2 expression resulted in a less pronounced improvement of growth in comparison to cells expressing GRK5. Cells grew equally well to the control when sumoylation was not impaired and the kinases were expressed.

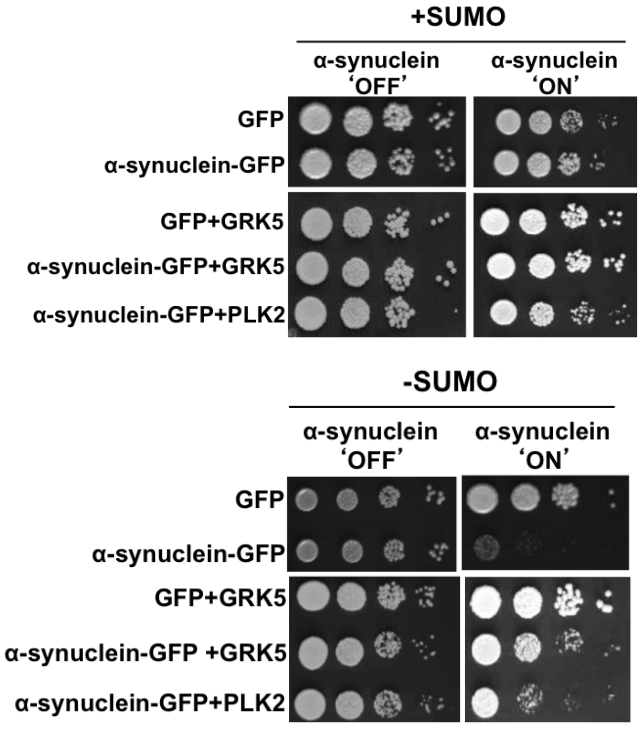

Figure 20. Increased α-synuclein S129 phosphorylation level by GRK5/PLK2 expression alleviates the toxicity.

Spotting assay of *smt3*^{ts} cells co-expressing α-synuclein-GFP with GRK5 or PLK2 either at permissive (25°C; + SUMO) or restrictive temperature (30°C; - SUMO). Yeast cells were spotted in 10-fold dilutions on selection plates containing glucose (α-synuclein 'OFF'; kinases 'OFF') or galactose (α-synuclein 'ON'; kinases 'ON').

Yeast cell survival correlated with reduced numbers of cells displaying inclusions in the presence of human kinases (Figure 21). The previous results have shown that in the absence of SUMO, cells displaying inclusions increased (Figure 12), whereas the higher phosphorylation level of α-synuclein at S129 in the presence of GRK5 and PLK2 resulted in significant decreases in the number of cells presenting inclusions in the absence of SUMO. The percentage of cells with inclusions at permissive temperature was affected by kinase expression (Figure 21). These results suggest that higher α-synuclein phosphorylation at S129 rescues the cells from α-synuclein-mediated cytotoxicity and inclusion formation associated with sumoylation impairment in a kinase-dependent manner.

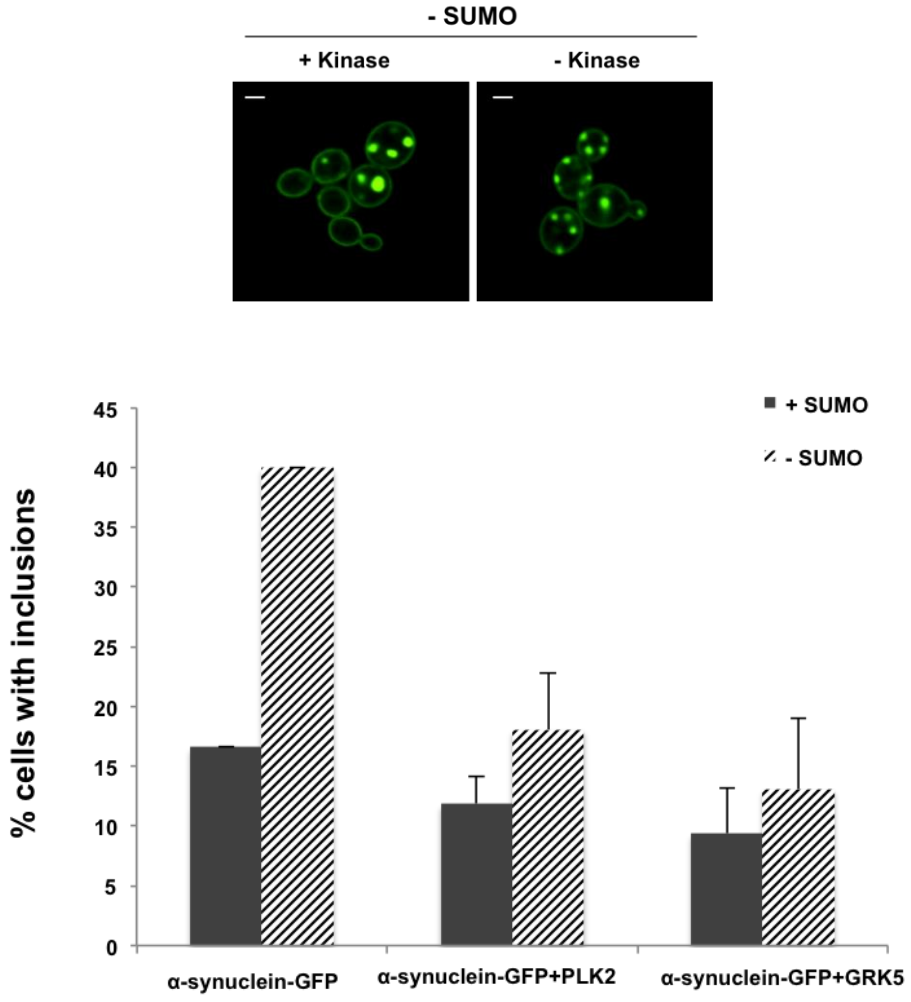

Figure 21. Increased α-synuclein S129 phosphorylation levels by GRK5/PLK2 reduces inclusions associated with impaired sumoylation in *smt3*^{ts} cells.

Fluorescence microscopy of *smt3*^{ts} cells expressing α-synuclein in the presence or absence of GRK5 or PLK2. Scale bar 1 μm (up). Quantification of percentage of cells displaying α-synuclein inclusions in the presence or absence of the kinases (below).

3.1.2.7 Overexpression of GRK5 reduces the oxidative stress-mediated sensitivity associated with sumoylation deficiency in α-synuclein expressing cells

Our observations revealed that higher α-synuclein phosphorylation at S129 by overexpression of GRK5 alleviated the α-synuclein cytoxicity associated with sumoylation impairment (Figure 20). In order to explore more probable rescue effect of higher α-synuclein S129 phosphorylation on α-synuclein-mediated toxicity in yeast cells impaired in sumoylation, we examined oxidative stress influence. Several reports over the past years implicated involvement of oxidative stress in a number of disease states, including Parkinson's disease (Giasson et al, 2000; Jenner, 2003; Kikuchi et al, 2002; Sherer et al, 2002; Souza et al, 2000). The exact mechanistic role of oxidative stress in Parkinson's disease progression is yet to be cleared because it is linked to other components of the degenerative process. It is difficult to determine whether oxidative stress directly leads to cells death, or is the consequence of this event. We treated *smt3*^{ts} yeast strain expressing WT α-synuclein (two genomically copies) with hydrogen peroxide, which results in the accumulation of reactive oxygen species (ROS), in the presence and absence of GRK5 at permissive or restrictive temperature. Cells expressing GFP alone were used as control. Halo assay analyzes showed less sensitivity of α-synuclein cells to ROS in the absence of cellular SUMO when GKR5 was overexpressed (Figure 22). These data suggest that GRK5-mediated α-synuclein phosphorylation advocates α-synuclein expressing cells from harmful factors such as oxidative stress in the absence of SUMO modifier. This further supports that higher α-synuclein phosphorylation at S129 can compensate the sumoylation impairment-associated impact on α-synuclein expressing cells.

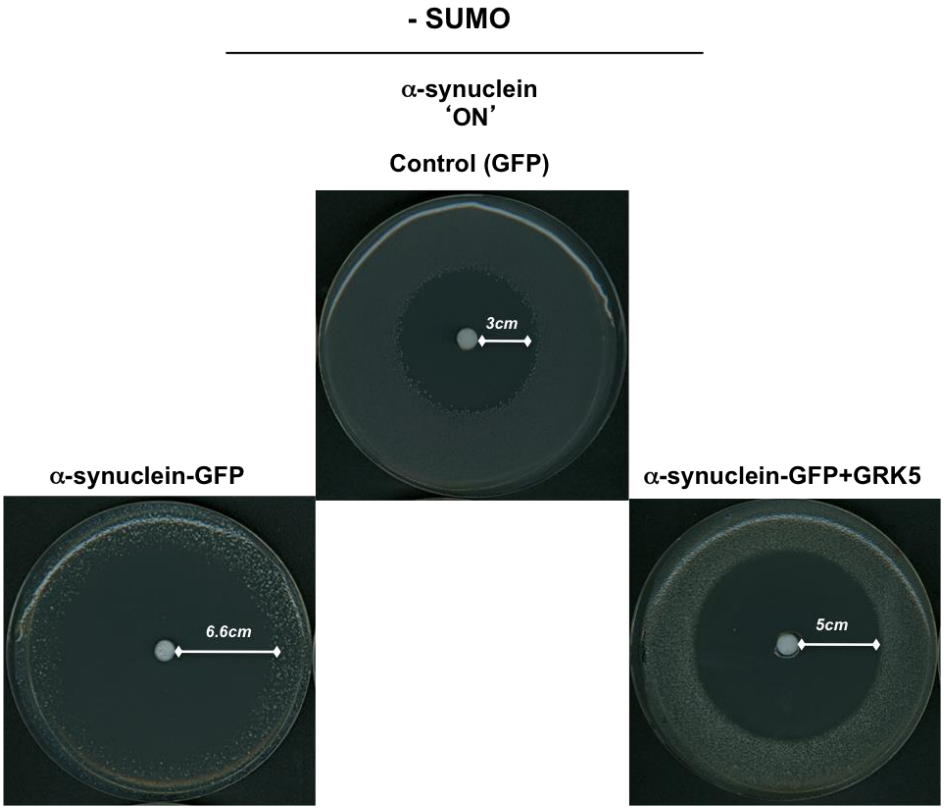

Figure 22. GRK5 overexpression reduces the sensitivity of α-synuclein-expressing cells to oxidative stress in the absence of SUMO.

Halo assay of *smt3*^{ts} cells expressing WT α-synuclein from two genomically integrated copies at restrictive temperature (30°C; - SUMO) alone or co-expressed with GRK5. Cells expressing GFP alone considered as control. 5 µl of 30% H_2O_2 was applied at the sterile disc positioned in the middle of SC-selective marker plates containing 2% galactose (α-synuclein 'ON'). The cells sensitivity to oxidative stress was measured after two days incubation at restrictive temperature (30°C). The diameter of the inhibition area, where the cells were not able to grow, was considered as measure for oxidative stress sensitivity.

3.1.2.8 Increased α-synuclein S129 phosphorylation cannot attenuate yeast growth impairment mediated by higher α-synuclein concentration

To learn more about the effect of increased phosphorylation levels of α-synuclein at S129 on α-synuclein-mediated cytotoxicity, W303 yeast cells expressing three copies of WT α-synuclein, which is shown to be a threshold for cytotoxicity in yeast (Petroi et al, 2012), were transformed with the human kinases GRK5 and PLK2. Spotting tests revealed that α-synuclein-mediated cytotoxicity has not been reduced by

overexpression of either kinase (Figure 23A). The same results were observed when the kinases were co-expressed in yeast cells with WT α-synuclein from episomal 2 µm plasmid (Figure 23B). These results suggest that the rescue effect of higher α-synuclein phosphorylation levels at S129 can not directly reduce α-synuclein toxicity but only compensate sumoylation impairment, which further supports the interplay between these two post-translational modifications.

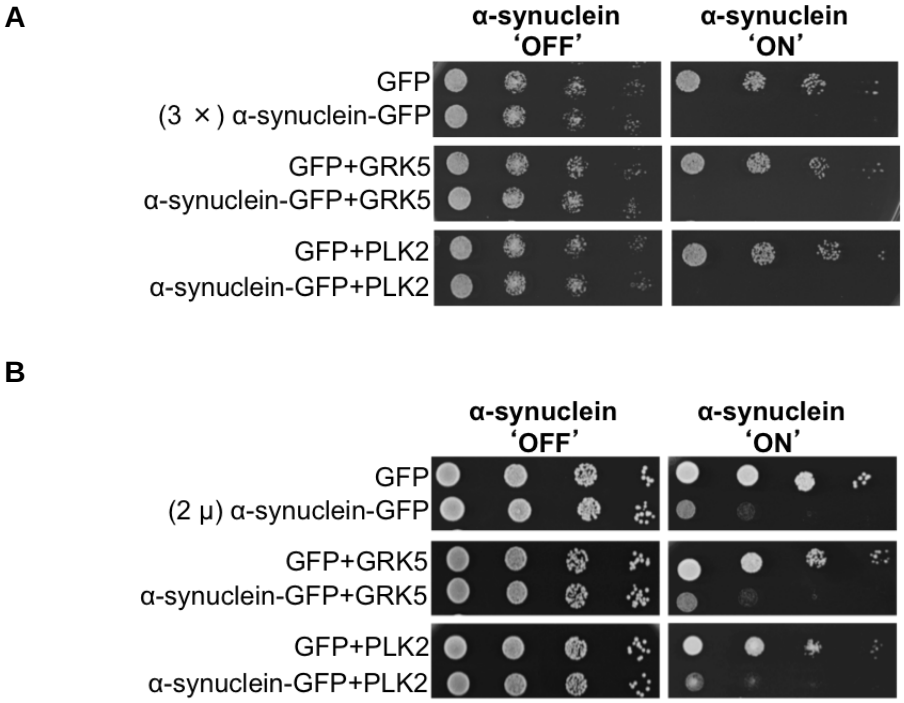

Figure 23. Increased level α-synuclein-mediated toxicity is not attenuated by kinases.

A. Spotting assay of W303 strain co-expressing α-synuclein-GFP with kinases (GRK5/PLK2). *GAL1*-driven α-synuclein-GFP was expressed from three genomically integrated copies. GFP, expressed from the same promoter, was used as a control. **B.** Spotting assay of W303 strain co-expressing α-synuclein-GFP with kinases (GRK5/PLK2). *GAL1*-driven α-synuclein-GFP was expressed from a 2 µm plasmid. Yeast cells were spotted in 10-fold dilutions on selection plates containing glucose (α-synuclein 'OFF') or galactose (α-synuclein 'ON').

3.1.2.9 Rescue of SUMO defect is S129 specific

α-synuclein contains several phosphorylation sites including S129, S87, Y125, Y133 and Y136 (Ellis et al, 2001; Kim et al, 2006; Nakamura et al, 2001; Okochi et al, 2000) (Figure 4). α-synuclein was shown to be predominantly phosphorylated at the S129 residue (Anderson et al, 2006). To further analyze the rescue effect of GRK5

and PLK2 against α-synuclein toxicity associated with sumoylation impairment, S129 specificity has been questioned. For this purpose, serine 129 was substituted to alanine by Quickchange Site-Directed Mutagenesis method. The specificity of phosphorylation of GRK5 or PLK2 on S129 was analyzed in greater detail by integrating two copies of an S129A mutant form of α-synuclein in the genome. *Smt3*^{ts} strain with two genomically integrated copies of S129A synuclein were constructed (Figure 24A). These cells were co-transformed with GRK5 and PLK2 kinases. In order to evaluate the α-synuclein S129 antibody specificity, protein purified from cells co-expressing S129A synuclein and GRK5/PLK2 were subjected to Western hybridization analysis. Treating the membrane with α-synuclein S129 antibody showed the complete abolishment of phosphorylated α-synuclein when the S129 is mutated to alanine (Figure 24B).

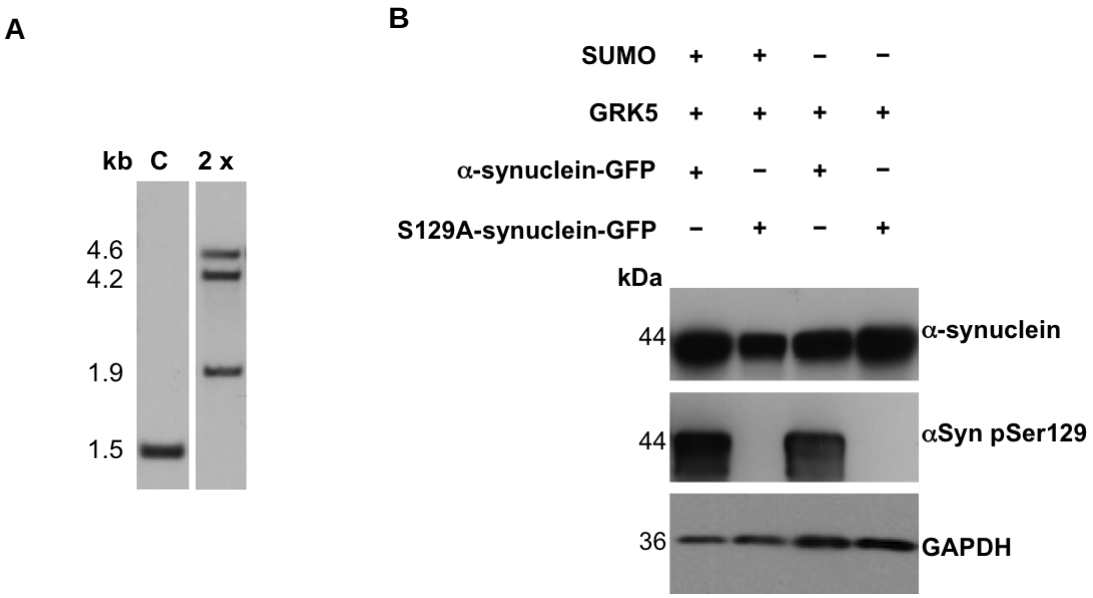

Figure 24. Substitution of serine to alanine results in abolishment of α-synuclein S129 phosphorylation by GRK5.

A. Southern hybridization using labeled *TRP1* as probe. Integrated genes for α-synuclein-GFP correspond to 1.9 kb + 4.2 kb + 4.6 kb (2 x). C (control): genomic locus without vector integration; corresponds to 1,5 kb. **B.** *Smt*3^{ts} mutant cells co-expressing α-synuclein with GRK5 and S129A-synuclein-GFP with GRK5 at permissive (25°C; + SUMO) or restrictive temperature (30°C; - SUMO). α-synuclein levels were detected with α-synuclein specific antibody and α-synuclein phosphorylation at S129 was detected by α-synuclein S129 phosphorylation specific antibody (αSyn pSer129). GAPDH was used as loading control.

Growth tests were conducted to analyze α-synuclein S129 specificity in rescue effect mediated by kinases expression in the absence of SUMO. In the presence of functional SUMO, co-expression of S129A with GRK5 had the same growth phenotype as that observed for cells co-expressing WT α-synuclein with GRK5 (Figure 25). Slight growth retardation was observed by co-expression of S129A and PLK2 (Figure 25). In the absence of functional SUMO, neither kinase could rescue the growth defect of the mutant α-synuclein, where the phosphorylation site was missing (Figure 25). These data indicate that the SUMO dependent effect of GRK5 or PLK2 expression on yeast growth depends on the phosphorylation of α-synuclein at S129.

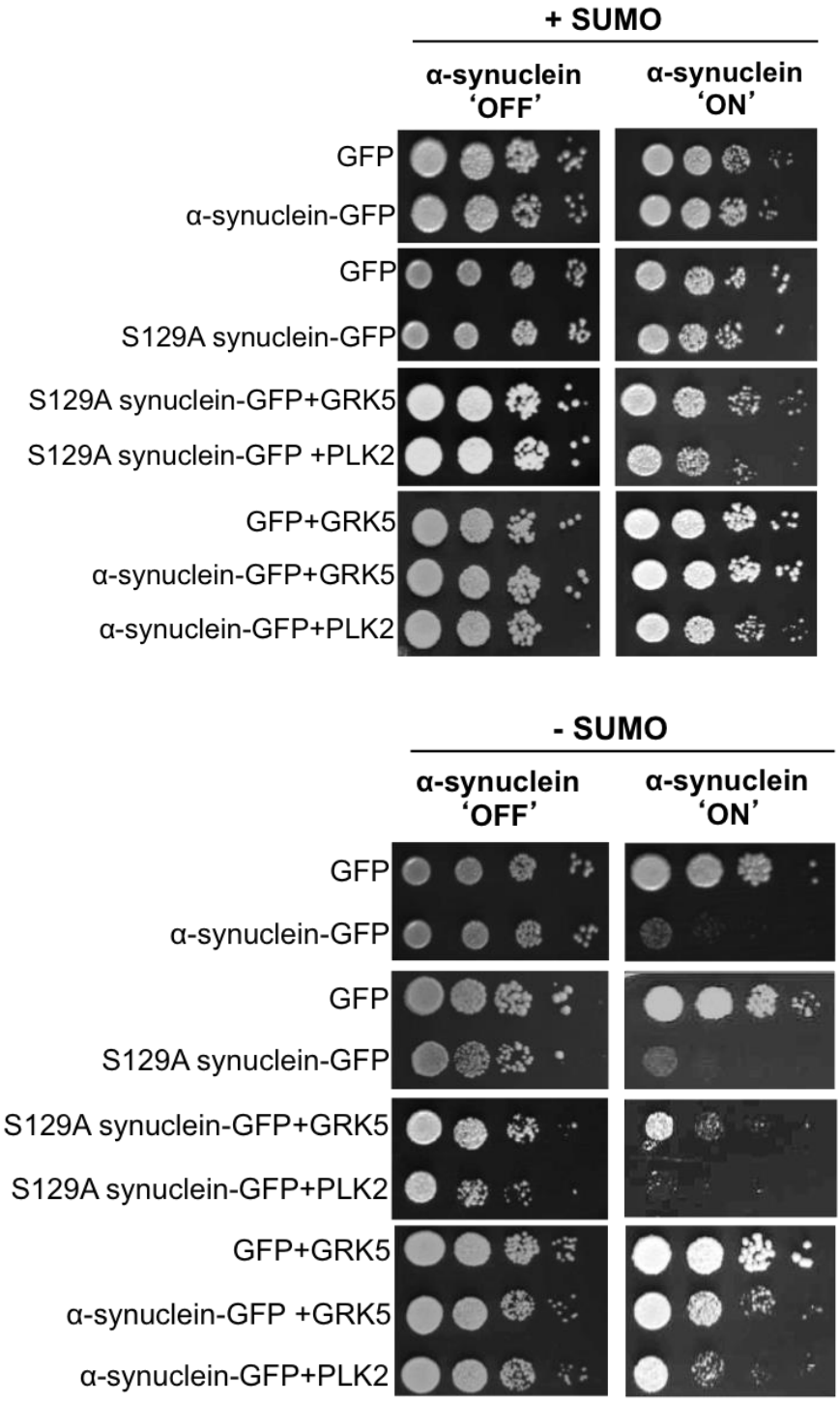

Figure 25. Rescue of SUMO defect is S129 specific.

Spotting assay of *smt3*^{ts} cells co-expressing α-synuclein-GFP or S129A synuclein-GFP with GRK5 or PLK2 either at permissive (25°C; + SUMO) (up) or restrictive temperature (30°C; - SUMO) (below), The cells growth was compared to α-synuclein-GFP with GRK5 or PLK2 either at permissive (25°C; + SUMO) or restrictive temperature (30°C; - SUMO). Yeast cells were spotted in 10-fold dilutions on selection plates containing glucose (α-synuclein 'OFF'; kinases 'OFF') or galactose (α-synuclein 'ON'; kinases 'ON').

Furthermore, decreased accumulation of α-synuclein inclusions in the absence of cellular SUMO and presence of GRK5 or PLK2 (Figure 21) was S129 dependent, since co-expression of S129A mutant with either kinase did not reveal decreased accumulation of α-synuclein inclusions in the absence of SUMO (Figure 26). These results suggest that increased levels of the α-synuclein S129 phosphorylation can suppress the α-synuclein-induced cytotoxicity in SUMO-deficient mutant strain of yeast.

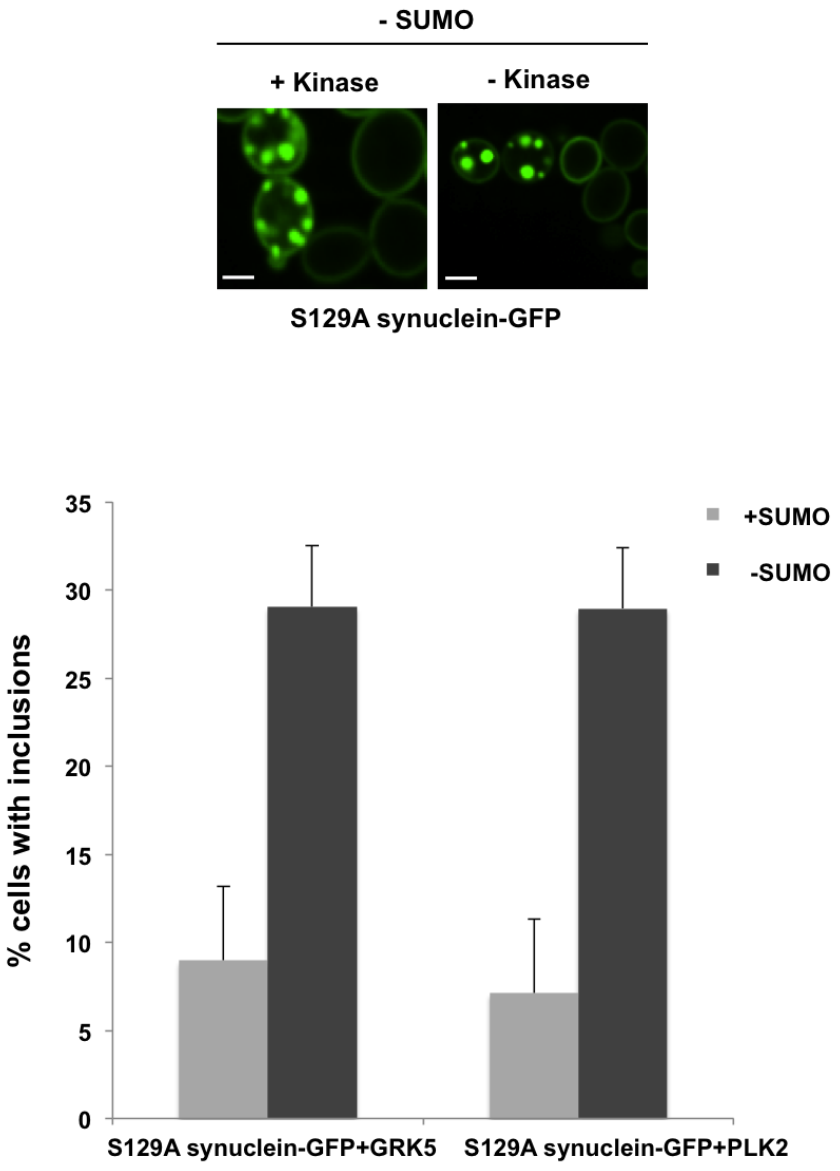

Figure 26. Inclusions reduction in SUMO deficient cells is S129 specific.

Fluorescence microscopy of SUMO deficient cells expressing S129A synuclein-GFP in the presence or absence of GRK5 or PLK2 (Kinase) (up). Scale bar 1 µm. Quantification of percentage of cells displaying S129A synuclein-GFP inclusions in the presence or absence of GRK5 or PLK2 (below).

3.1.2.10 GRK5 has direct effect on sumoylation-deficient α-synuclein mediated cytotoxicity, whereas PLK2 displays an indirect effect

To obtain more insights into the interplay of α-synuclein sumoylation and phosphorylation, the major sumoylation sites of α-synuclein (K96 and K102) were blocked. The direct or indirect effect of each kinase on sumoylation-deficient α-synuclein mediated cytotoxicity was evaluated. To this aim, we used W303 cells expressing two copies of K96R K102R mutant, blocked in two major sumoylation residues (Figure 13). GRK5 and PLK2 kinases were transformed in the strain and growth assays were performed. Co-expression of GRK5 and K96R K102R mutant resulted in a striking recovery of growth (Figure 27). This suggests that GRK5 directly suppresses the sumoylation defect of α-synuclein. In contrast, expression of PLK2 did not significantly influence yeast growth. This suggests an indirect effect on α-synuclein toxicity caused by down regulation of the sumoylation activity, which then allows a partial growth recovery by PLK2 expression.

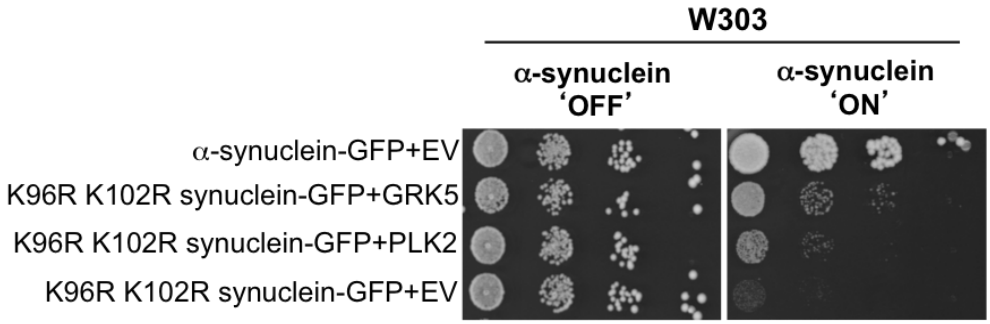

Figure 27. Reduction of sumoylation-deficient synuclein-mediated cytotoxicity in a kinase dependent-manner.

Spotting assay of W303 yeast cells, carrying two copies of *GAL1*-driven α-synuclein-GFP and K96R, K102R synuclein-GFP in the presence of GRK5 and PLK2 or empty vector (EV). Yeast cells were spotted in 10-fold dilutions on selection plates containing 2 % glucose (α-synuclein 'OFF'; kinases 'OFF') or 2 % galactose (α-synuclein 'ON'; kinases 'ON').

3.2 SUMO-deficiency and aggregate clearance of α-synuclein

Although several studies explored the degradation mechanisms involved in α-synuclein aggregate clearance, the mechanisms regulating α-synuclein partitioning between the main degradation pathways remains arguable (Chu et al, 2009; Ebrahimi-Fakhari et al, 2011; Furukawa et al, 2002; Hara et al, 2006; McLean et al, 2001; Petroi et al, 2012; Rideout et al, 2001). In this chapter, we addressed the question whether interplay between specific post-translational modifications of α-synuclein modulates the processing of the inclusions through degradation by autophagy and proteasome pathways.

3.2.1 Sumoylation supports aggregate clearance of α-synuclein by autophagy

α-synuclein aggregates are mainly cleared by autophagy pathway, whereas there is a minor contribution of the proteasome in clearing α-synuclein aggregates in yeast cells (Petroi et al, 2012). The previous results showed that sumoylation has a protective role against α-synuclein aggregate formations (Figure 12). The next aim was to study how sumoylation might affect the α-synuclein aggregate clearance in yeast. We studied autophagy contribution in aggregate clearance of α-synuclein when sumoylation is inhibited by performing *GAL1* promoter shut-off experiments. We analyzed the impact of blocking the autophagy pathway by chemical treatments. Phenylmethylsulfonyl fluoride (PMSF) was used to block the activity of vacuolar proteases. *Smt3*^{ts} strain harboring two copy integrations of WT α-synuclein was first pre-grown overnight at permissive temperature and then shifted to induction media containing 2% galactose at both permissive and restrictive temperature for four hours. The *GAL1* promoter was switched off by transferring cells to media containing 2% glucose and supplemented with PMSF dissolved in ethanol to inhibit autophagy pathway. In parallel, cells were treated with ethanol as control. Life-cell fluorescence microscopy was performed two hours after promoter shut-off and the number of cells displaying aggregates was quantified. At permissive temperature when sumoylation is not down regulated, cells were unable to clear aggregates two hours after promoter shut-off, whereas cells expressing α-synuclein at restrictive temperature, when sumoylation is down regulated, cleared aggregates upon promoter shut-off in the same manner as the control (Figure 28). In order to validate this result genetically

and study the aggregate clearance of sumoylation deficient α-synuclein directly, W303 cells expressing two copies of K96R K102R variant were subjected to promoter shut-off study with PMSF drug treatment. After four hours production of K96R K102R variant, cells were shifted to 2% glucose containing media supplemented with PMSF. Quantification of number of the cells after promoter shut-off revealed that cells expressing K96R K102R mutant cleared inclusions in a similar manner to that observed with vehicle only (ethanol) (Figure 28). These results suggest that defect in sumoylation interferes with the aggregate clearance of α-synuclein through the autophagic degradation pathway. Thus, sumoylation supports the autophagy-dependent clearance of α-synuclein.

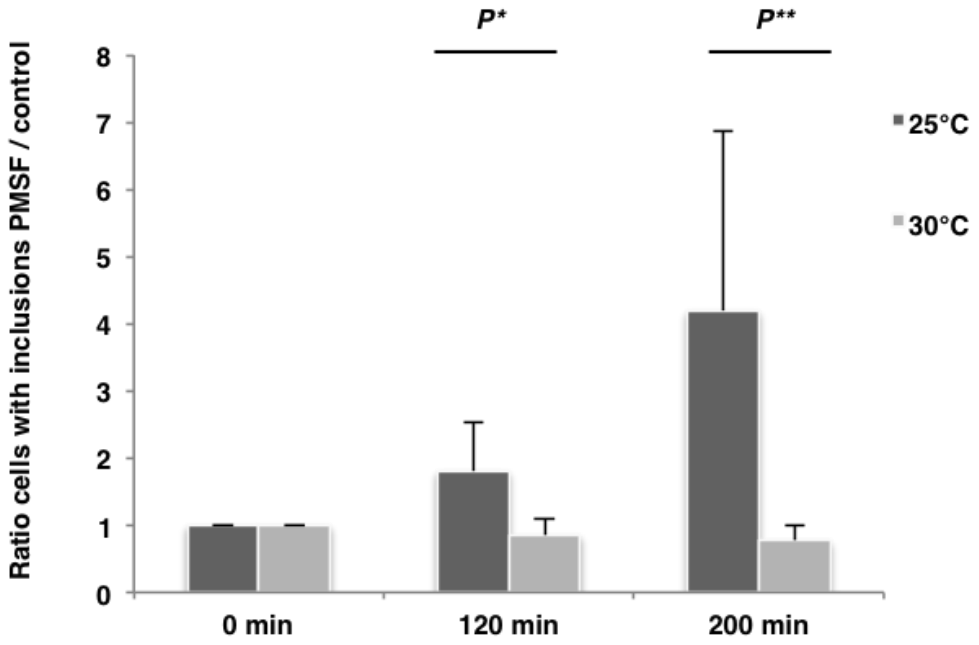

Figure 28. Sumoylation supports α-synuclein aggregate clearance through autophagy pathway.

Quantification of aggregates in *smt*3^{ts} cells expressing α-synuclein-GFP, two times integrated in the genome upon *GAL1*-promoter shut off at indicated time points. Cells were incubated in 2% galactose containing media for four hours at permissive (25°C) and restrictive temperature (30°C). Then the cells were shifted to 2% glucose containing media supplemented with 1 mM PMSF dissolved in EtOH and only EtOH as a control. Cells with aggregates were counted at the indicated time points and presented as a ratio to the control (EtOH). Significance of differences was calculated with t- test (*P*, P** < 0.05*, n=3).

3.2.2 Phosphorylation partially supports SUMO-deficient α-synuclein aggregate clearance via the autophagy pathway

The role of phosphorylation at S129 on aggregate clearance of SUMO deficient cells was addressed. To this aim, W303 cells co-expressing two genomically integrated copies of SUMO-deficient α-synuclein (K96R K102R mutant) and human kinases GRK5/PLK2 were studied upon promoter shut-off experiment with PMSF treatment. After four hours inducing K96R K102R mutant, cells were shifted to 2% glucose containing media supplemented with PMSF. Fluorescence microscopy and aggregation quantification after 2 hours promoter shut-off revealed that expression of kinases prevented the clearance of aggregates when autophagy pathway is blocked, whereas cells expressing SUMO-deficient α-synuclein without the kinase expression were able to clear aggregates in the absence of autophagy mechanism (Figure 29). These data suggest that expression of GRK5 or PLK2 can partially rescue the aggregate clearance through autophagy pathway.

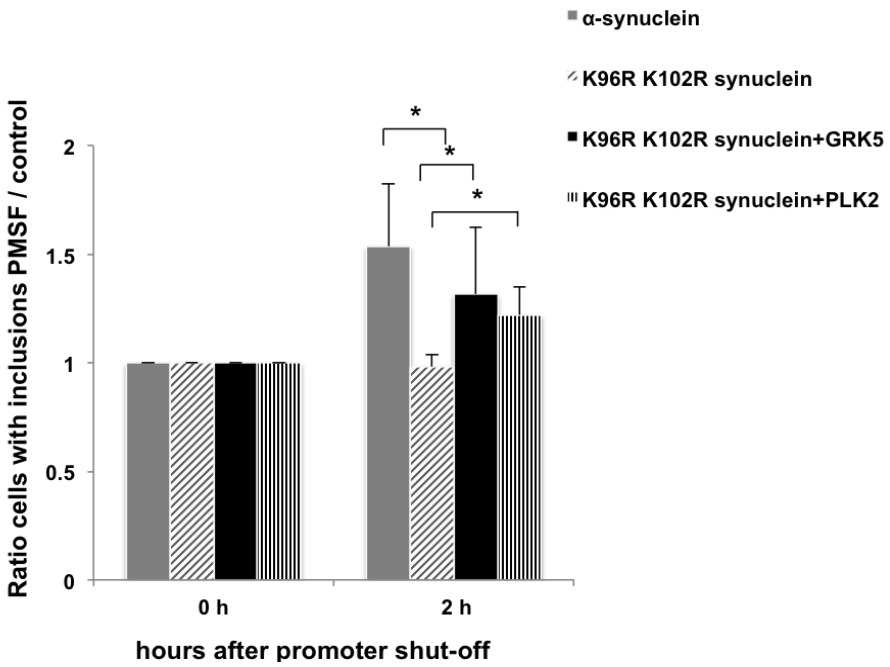

Figure 29. α-synuclein aggregate clearance upon promoter shut-off and inhibition of autophagy.

Inhibition of the vacuolar degradation pathway by PMSF. Quantification of cells expressing α-synuclein-GFP, K96R K102R synuclein-GFP and K96R K102R synuclein-GFP, expressing GRK5 or PLK2, respectively. α-synuclein-GFP and K96R K102R synuclein-GFP were expressed from two genomically integrated *GAL1*-promoter driven copies. After four hours induction of the protein expression in galactose medium, cells were shifted to glucose medium supplemented with 1 mM PMSF dissolved in ethanol (EtOH) or only EtOH as a control. Quantification of the reduction of inclusions was done 2 hours after the promoter shut-off. Cells with inclusions were counted and presented as a ratio to the control (EtOH). Significance of differences was calculated with t-test (*P* < 0.05, n=3).*

3.2.2.1 α-synuclein is ubiquitinated in yeast

Accumulated proteins can be degraded via different degradation pathways (Goldberg, 2003; Wong & Cuervo, 2010). According to our previous observations in this study, sumoylation deficient α-synuclein is not degraded by autophagy, which suggests involvement of another degradation pathway in α-synuclein aggregate clearance. Proteasome contributes to degrade proteins in ubiquitin dependent or independent manner (Smith et al, 2011). One of the most important post-translational modifications of α-synuclein involved in Parkinson's disease pathology is ubiquitination. α-synuclein has been shown to be mono- and di-ubiquitinated *in vitro* and *in vivo* (Ebrahimi-Fakhari et al, 2011; Nonaka et al, 2005). To get more insight into the mechanism involved in α-synuclein aggregate clearance, we tested whether α-synuclein is ubiquitinated in yeast cells and how higher phosphorylation might affect α-synuclein ubiquitination status. For this purpose, immunoprecipitation (IP) analysis was performed. In order to enrich α-synuclein protein, W303 cells overexpressing α-synuclein-His6 and A30P-His6 were subjected to Ni^{2+} affinity chromatography. Enriched α-synuclein protein pulled down by Ni^{2+}-NTA was immunoprecipitated with ubiquitin antibody (Figure 30).

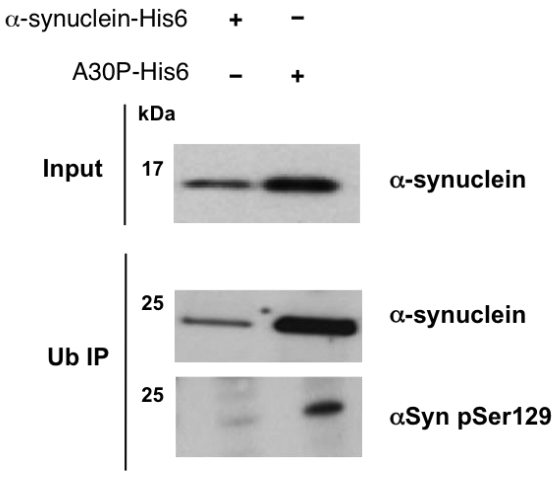

Figure 30. α-synuclein is ubiquitinated and phosphorylated simultaneously in yeast cells.

α-synuclein-His6 and A30P-His6 protein was purified by Ni^{2+} pull-down. α-synuclein purified from Ni^{2+} pull-down was detected with α-synuclein specific antibody and then subjected to immunoprecipitation with ubiquitin antibody. The ubiquitinated and phosphorylated α-synuclein was detected by α-synuclein and αSyn pS129 specific antibody, respectively.

Western hybridization analysis after ubiquitin IP with α-synuclein specific antibody detected the mono-ubiquitinated α-synuclein and its A30P mutant, which migrated to 22 kDa (Figure 30). Treating the membranes with phospho-specific antibody (αSyn pSer129) in parallel indicated that α-synuclein is simultaneously phosphorylated at S129. This result revealed that the preference of monomeric α-synuclein to be mono-ubiquitinated is conserved from yeast to higher organisms.

3.2.1 Ubiquitination of α-synuclein depends on its phosphorylation and sumoylation status

We showed that α-synuclein is mono-ubiquitinted in yeast (Figure 30). We also showed that the ubiquitinated α-synuclein is phosphorylated at S129 at the same time (Figure 30). Next goal was to investigate the influence of phosphorylation and sumoylation on α-synuclein ubiquitination profile. The *smt3*^{ts} yeast cells expressing α-synuclein-His6 were co-expressed with GRK5 and PLK2. The empty vector of the kinase was used as control. Cells were pre-grown at permissive temperature and then shifted to induction medium (2% galactose) in presence or absence of SUMO at different temperatures. Then pull-down of the enriched α-synuclein via Ni^{2+}-NTA were applied. α-synuclein eluted from columns was subjected to ubiquitin immunoprecipitation. Immunoblotting of the immunoprecipitated protein with α-synuclein antibody revealed different pattern of ubiquitinated proteins, migrating at molecular mass range of 22-36 kDa. When no kinases were expressed, we observed a single molecular band at around 29 kDa (Figure 31). Interestingly, in the presence of the kinases, we observed multiple distinct bands, the major one migrating at 22 kDa. In addition, we observed a smear pattern of the modified α-synuclein to higher molecular weights, which was more pronounced when sumoylation is down regulated (Figure 31). Judging from the size and from previous reports, we hypothesize that α-synuclein underwent mono- (22 kDa), di- (29 kDa) and tri-ubiquitination (Hasegawa et al, 2002c). Expression of GRK5 revealed more profound effect on the ubiquitination pattern of α-synuclein in comparison with PLK2, especially in the absence of SUMO.

The results indicate that the ubiquitination pattern of sumoylated and non-sumoylated α-synuclein changes in a kinase-specific manner. GRK5, which phosphorylates α-synuclein independently of its sumoylation status, promotes multi-ubiquitination of α-synuclein and the effect is more profound when the protein is not sumoylated. PLK2,

which preferentially phosphorylates S129 of non-sumoylated α-synuclein, induces mainly mono-ubiquitination. The findings suggest a cross-talk between sumoylation, phosphorylation and ubiquitination of α-synuclein in yeast.

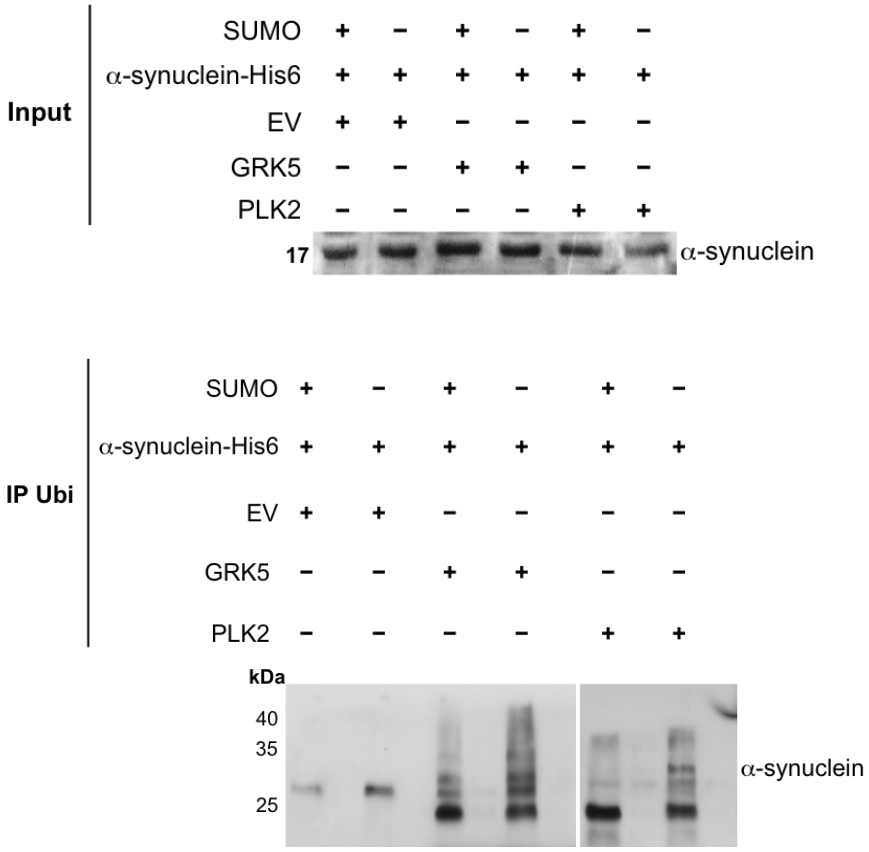

Figure 31. Sumoylation and α-synuclein S129 phosphorylation affect the ubiquitination pattern of α-synuclein.

*Smt*3^{ts} cells expressing α-synuclein-His6 co-transformed with GRK5 or PLK2 and empty vector of the kinases (EV) as a control at permissive (25°C; + SUMO) or restrictive temperature (30°C; - SUMO). The purified α-synuclein protein from Ni^{2+} pull-down was subjected to ubiquitin immunoprecipitation. The ubiquitinated α-synuclein was analyzed by Western hybridizationting with α-synuclein antibody.

α-synuclein is highly mono- or di-ubiquitinated at multiple residues in Lewy bodies (Anderson et al, 2006; Hasegawa et al, 2002c; Sampathu et al, 2003) (Figure 4). Observing multi-ubiquitinated α-synuclein in the presence of the kinases raised the question whether α-synuclein undergoes poly-ubiquitination. To investigate the nature of multi-ubiquitinated α-synuclein, we used Tandem Ubiquitin Binding Entities (TUBEs) (Hjerpe et al, 2009), which enable the identification of poly-ubiquitinated proteins. The total protein crude extracts were purified from s*mt3*^{ts} yeast cells co-

expressing α-synuclein-His6 with GRK5 or PLK2 and the empty vector of the kinase as control, in presence or absence of SUMO at different temperatures. In order to control the TUBEs purification specificity, total protein crude extract from s*mt3*^{ts} yeast cells co-expressing α-synuclein-His6 with GRK5 at permissive or restrictive temperatures were incubated with agarose beads as negative control reactions. The expression of α-synuclein was detected with α-synuclein specific antibody (Figure 32). Equal amounts of the total protein from each strain were subjected to TUBEs analysis. Immunoblotting analysis of the precipitated proteins revealed no poly-ubiquitinated α-synuclein (Figure 32 (middle), no signal), which is in line with previous evidences. Western hybridization analysis of the purified proteins from TUBEs with ubiquitin specific antibody showed multiple bands in precipitated proteins whereas no signal was observed in negative control reactions (Figure 32 (below)). These data suggest that α-synuclein is multi-ubiquitinated in the presence of the kinases at several residues rather than poly-ubiquitinated (ubiquitination-chain) at a specific lysine residue.

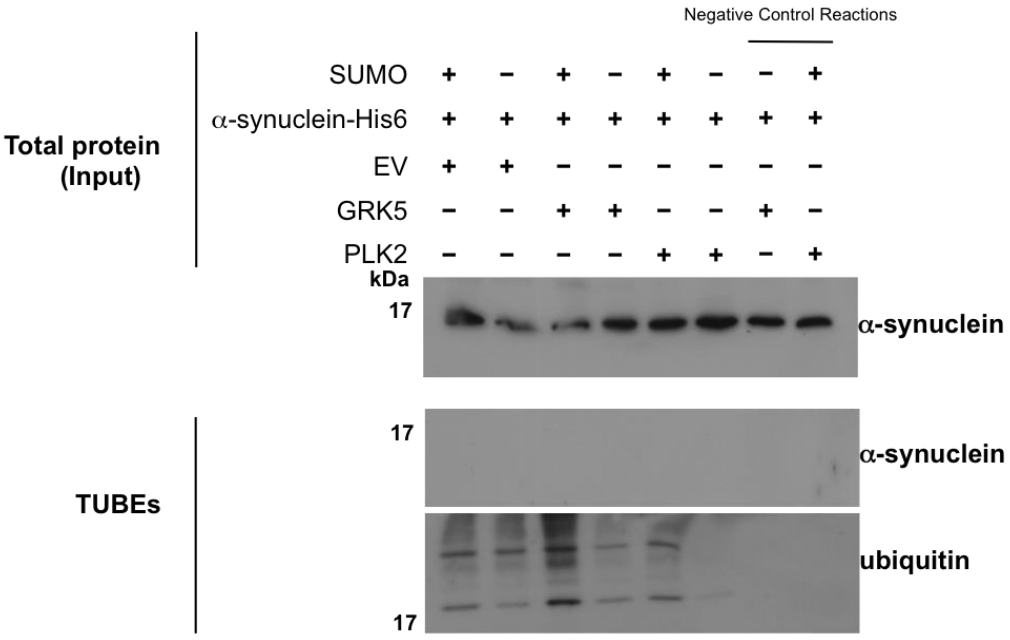

Figure 32. TUBEs do not show poly-ubiquitinated α-synuclein in yeast.

Western hybridization analysis of total protein crude extract from s*mt3*^{ts} yeast cells co-expressing α-synuclein-His6 with GRK5 or PLK2 and the empty vector in the presence or absence of SUMO at different temperature (Input). The last two samples are untreated total protein crude extract from α-synuclein-His6 with GRK5 at permissive and restrictive temperatures, used as TUBEs Negative Control Reactions. α-synuclein was detected with α-synuclein specific antibody (Upper). Western hybridization analysis of poly-ubiquitinated α-synuclein after the TUBEs purification with α-synuclein specific antibody (middle). Western hybridization analysis of poly-ubiquitinated proteins after the TUBEs purification with ubiquitin specific antibody (below).

3.2.2 The proteasome degradation pathway plays a major role in SUMO-deficient α-synuclein aggregate clearance

Observation of α-synuclein ubiquitination status alteration and the supportive effect of sumoylation on α-synuclein aggregate clearance via autophagy pathway promoted us to analyze how sumoylation and phosphorylation affect the α-synuclein aggregate clearance by the proteasome. We assessed the study of the ubiquitin proteasome system by blocking the proteasome activity by drug treatment. For this reason, the drug MG132 was used to inhibit proteasomal activity (Lee & Goldberg, 1998; Liu et al, 2007). Impermeability of yeast chitin cell wall hampered the use of proteasome inhibitors in *S. cerevisiae*. A new method to overcome this difficulty is to use L-proline instead of ammonium sulfate as nitrogen source in growth medium and addition of small amount of sodium dodecyl sulfate (0.003%SDS), which leads to transient opening of the cell wall (Liu et al, 2007). W303 cells expressing two genomically integrated copies of K96R K102R mutant were pre-grown in corresponding media and further studied after promoter shut-off. In parallel, W303 cells expressing two copies of WT α-synuclein were used as control. After inducing the α-synuclein expression for four hours, cells were shifted to 2% glucose containing media supplemented with MG132 dissolved in DMSO. As control, K96R K102R-synuclein expressing cells were treated with DMSO. Quantification of cells presenting aggregates 2 hours after promoter shut-off revealed equal aggregate clearance of WT α-synuclein in MG132-treated cells when compared with the control (DMSO) (Figure 33). In contrast, in the presence of proteasome inhibitor, cells were unable to clear aggregates when α-synuclein major sumoylation sites were blocked (K96R K102R) (Figure 33). This suggests a major contribution of the proteasome in α-synuclein aggregate clearance when α-synuclein is sumoylation-deficient. Our data indicate that sumoylated α-synuclein is primarily targeted to the autophagy pathway and non-sumoylated α-synuclein primarily to the proteasome. Inhibition of sumoylation results in inefficient autophagy-mediated aggregate clearance and directs α-synuclein to the proteasome.

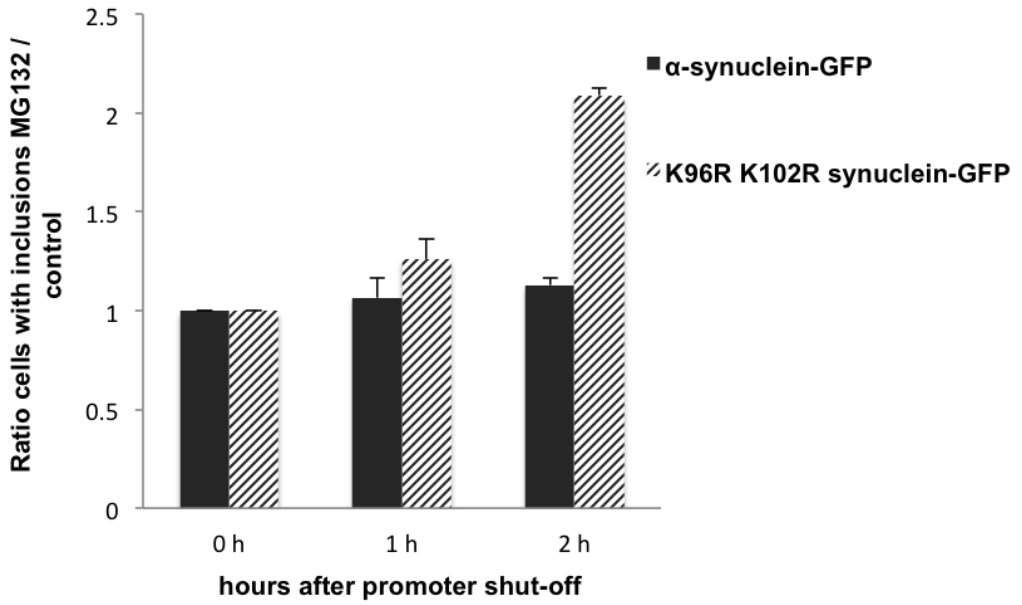

Figure 33. α-synuclein aggregate clearance upon promoter shut-off and proteasome inhibition.

Quantification of cells expressing α-synuclein-GFP, K96R K102R synuclein-GFP from two genomically integrated *GAL1*-driven copies. After four hours inductions of the protein expression in galactose medium, cells were shifted to glucose medium supplemented with 75 µM MG132, dissolved in DMSO or only DMSO as a control. Quantification of the reduction of inclusions was done 2 hours after the promoter shut-off. Cells with inclusions were counted and presented as a ratio to the control (DMSO).

3.2.3 Phosphorylation supports proteasome pathway in α-synuclein aggregate clearance in kinase-dependent manner

We have shown that GRK5 and PLK2 expression stimulates α-synuclein ubiquitination status when sumoylation is impaired (Figure 31). In addition, promoter shut-off studies revealed the partial contribution of autophagy in aggregate clearance of SUMO-deficient α-synuclein when kinases are present (Figure 29). To further study the interplay between these modifiers in context of α-synuclein aggregate clearance, we analyzed the effect of GRK5 or PLK2 expression and sumoylation on α-synuclein aggregate clearance via proteasome degradation pathway. To this aim, promoter shut-off studies were performed with W303 co-expressing SUMO-deficient α-synuclein (K96R K102R synuclein) and each of the kinases by inhibiting the proteasome with MG132. Cells expressing two genomically integrated WT α-synuclein driven by the *GAL1*-promoter were analyzed as control. As previously

explained, α-synuclein expression was induced for four hours and the cells were shifted to glucose containing media. Two hours after promoter shut-off the cells with aggregates were counted. The results indicated that in the presence of the kinases the aggregates were directed to the proteasome pathway when α-synuclein is impaired in sumoylation in kinase-dependent manner (Figure 34). This suggests a major contribution of the proteasome in α-synuclein aggregate clearance when the α-synuclein is sumoylation-deficient. Cells were unable to clear inclusions when α-synuclein sumoylation (K96R K102R) and the proteasome (MG132) were blocked simultaneously. Expression of GRK5 and the sumoylation-deficient mutant promoted the proteasome-dependent clearing of inclusions and, accordingly, MG132 treatment resulted in an increased percentage of cells with inclusions. Again, PLK2 had only a minor impact on inclusion clearance by the proteasome in comparison to GRK5.

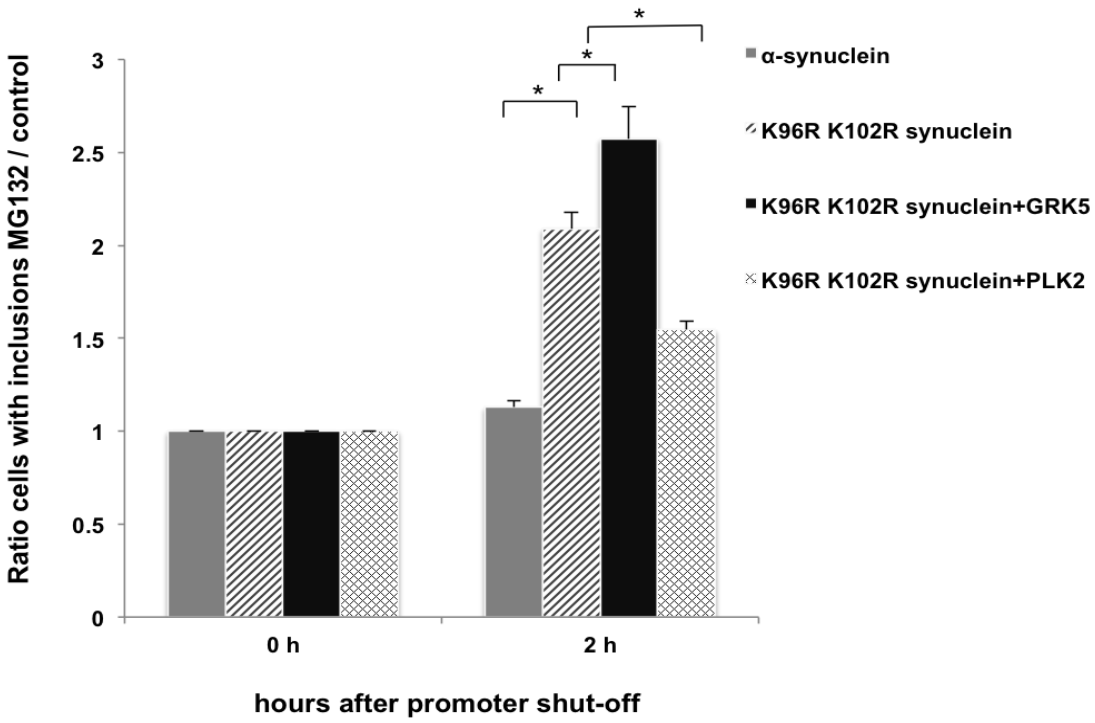

Figure 34. Phosphorylation promotes aggregate degradation of sumoylation-deficient α-synuclein by the proteasome.

Quantification of cells expressing α-synuclein-GFP, K96R K102R synuclein-GFP and K96R K102R synuclein-GFP, expressing GRK5 or PLK2, respectively. α -synuclein-GFP and K96R K102R synuclein-GFP were expressed from two *GAL1*-promoter drivengenomically integrated copies. After four hours inductions of the protein expression in galactose medium, cells were shifted to glucose medium supplemented with 75 µM MG132 dissolved in DMSO or only DMSO as a control. Quantification of the reduction of inclusions was done 2 hours after the promoter shut-off. Cells with inclusions were counted and presented as a ratio to the control (DMSO). Significance of differences was calculated with t-test (*P*< 0.05, n=3)*.

3.2.4 Sumoylation impairment does not alter α-synuclein protein stability in yeast

Sumoylation is also known to modulate its target proteins activity/stability (Bologna & Ferrari, 2013). Sumoylation regulates CHFR tumor suppressor stability negatively and promotes its degradation in UPS-dependent manner (Bae et al, 2013). To explore the effect of sumoylation on α-synuclein stability, we performed *GAL1* promoter shut-off experiment with *smt3*^{ts} mutant cells expressing WT α-synuclein from two genomically integrated gene copies. Expression of α-synuclein was induced in 2% galactose-containing medium for four hours at permissive (25^{o}C) and restrictive (30^{o}C) temperature and the cells were then shifted to glucose medium, which represses the promoter. Cells were harvested at indicated time points (0, 3, 6, 18 h) and the whole protein extract subjected to Western hybridization analysis with α-synuclein antibody. Immunoblotting analysis revealed that α-synuclein protein was stable with no changes in its protein level either in presence or absence of SUMO at different temperatures (Figure 35A). To further validate our finding and investigate whether direct inhibition of α-synuclein sumoylation by blocking the major sumoylation sites (K96R K102R) will affect the protein stability over the time, promoter shut-off study was performed in parallel with W303 yeast cells, expressing K96R K102R synuclein from two genomically integrated copies and cells expressing WT α-synuclein. *GAL1* promoter was shut-off four hours after induction and cells collected at indicated time points. Similarly as observed with *smt3*^{ts} mutant cells expressing WT α-synuclein, Western hybridization analysis of the protein extracts at the indicated times after promoter shut-off with α-synuclein antibody revealed that sumoylation deficient α-synuclein (K96R K102R) is equally stable as WT α-synuclein (Figure 35B). Altogether, our stability analysis performed by promoter shut-off approaches indicated that sumoylation-deficient α-synuclein exhibited similar stability to that of its WT form, therefore suggesting that SUMO-deficient α-synuclein is stable in yeast cells and this modification did not alter α-synuclein stability.

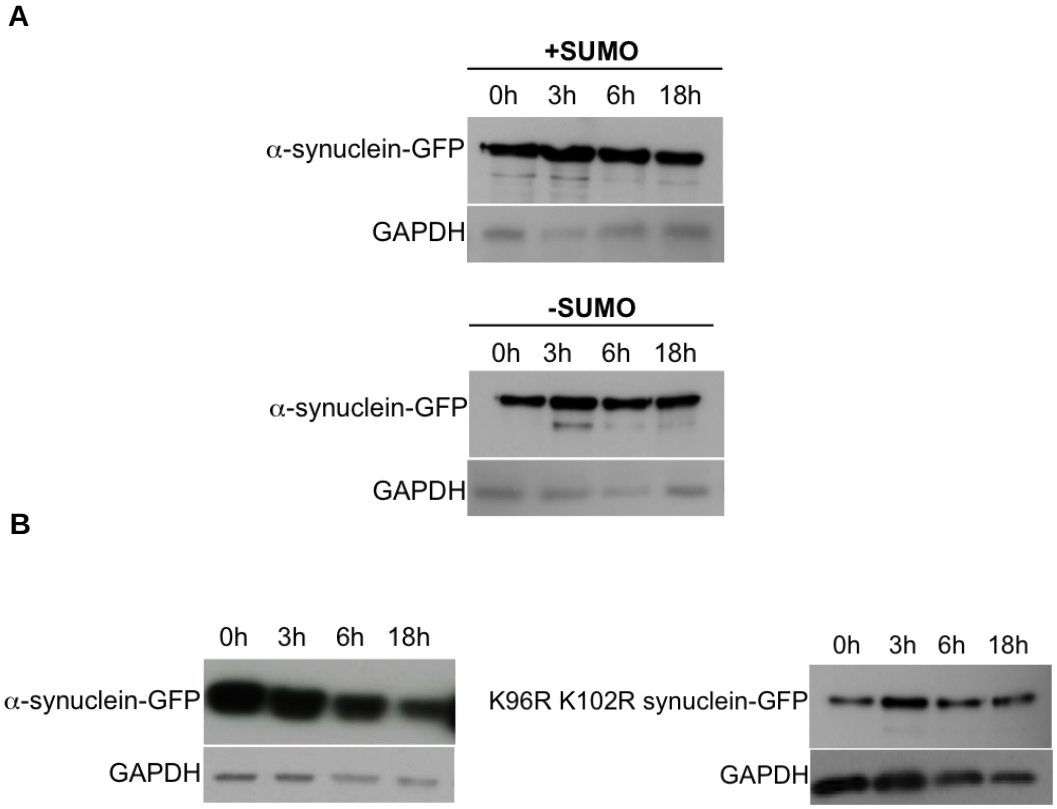

Figure 35. Sumoylation does not alter α-synuclein protein stability.

A. *GAL1* promoter shut-off studies. *Smt3*^{ts} cells expressing α-synuclein were induced for four hours in galactose (α-synuclein "on") at permissive (25°C; + SUMO) or restrictive temperature (30°C; - SUMO) and then transferred to glucose containing medium (α-synuclein "off"). Immunoblotting analysis was performed at the indicated time-points after promoter shut-off with α-synuclein antibody and GAPDH antibody as loading control. **B.** Western hybridization analysis of *GAL1* promoter shut-off in W303 yeast cells expressing α-synuclein-GFP (left panel) and K96R K102R synuclein-GFP at the indicated time-points with α-synuclein antibody and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody as loading control.

3.2.5 SUMO-deficient α-synuclein stability reduces when its phosphorylation level on S129 is higher by GRK5 and PLK2

Among different regulatory mechanisms, where phosphorylation plays a role, tuning the protein stability was shown to be an important phenomenon in different cellular pathways including protein degradation (Xu et al, 2009). Having defined that phosphorylation of α-synuclein by GRK5 and PLK2 changes its ubiquitination status in the presence and absence of SUMO (Figure 31), we aimed to address the

question whether sumoylation-deficient α-synuclein protein stability is changed by expression of the kinases. To this end, promoter shut-off study was done with W303 cells carrying two genomically integrated copies of SUMO-deficient α-synuclein (K96R K102R), co-expressed GRK5 or PLK2. K96R K102R synuclein protein was produced for four hours and the promoter was suppressed by shifting cells to media containing 2% glucose. Total protein extracts were isolated from cells harvested at indicated time points (0, 3, 6, 18 h) after promoter shut-off. The probes were subjected to Western hybridization analysis and sumoylation-deficient α-synuclein protein levels were detected by α-synuclein specific antibody (Figure 36). Immunoblotting analysis indicated that α-synuclein higher phosphorylation at S129 when its major SUMO sites are blocked affected its turnover and the protein level declined over the time. These data indicate that expression of the kinases affects α-synuclein stability when its major sumoylation sites are impaired, which corroborate our findings that expression of kinases increases the ubiquitination level of α-synuclein, thus promoting the degradation of the protein by the proteasome.

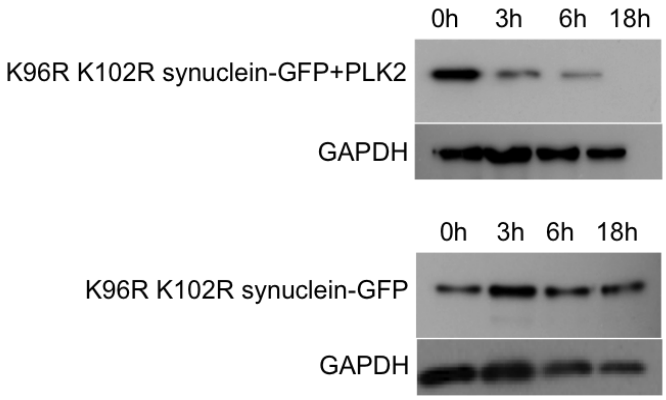

Figure 36. GRK5 and PLK2 decrease sumoylation-deficient α-synuclein protein stability.

Western hybridization analysis of *GAL1* promoter shut-off studies. W303 yeast cells co-expressing K96R K102R synuclein-GFP with GRK5 or PLK2 were induced for four hours in galactose (α-synuclein “on”) and then transferred to glucose containing medium (α-synuclein “off”). α-synuclein protein levels were detected with α-synuclein specific antibody. GAPDH antibody was used as control.

3.3 The yeast model for β and γ-synuclein aggregation and toxicity

3.3.1 β-synuclein overexpression is toxic to yeast cells whereas γ-synuclein does not have any impact

Recently, the other two member of the synuclein family, β- and γ-synuclein, beside α-synuclein considered as neurodegeneration-inducing proteins, which are associated with Parkinson's disease progression. However their exact contribution in Parkinson's disease pathology remains elusive (Ninkina et al, 2009; Nishioka et al, 2010; Sung & Eliezer, 2007; Taschenberger et al, 2013; von Bohlen Und Halbach, 2004). In this study we aimed to characterize β- and γ-synuclein mediated cytotoxicity in yeast as model of Parkinson's disease. The human β-synuclein encoding cDNA sequence, *SNCB*, and γ-synuclein cDNA sequence, *SNCG*, were C-terminally GFP-tagged via a KLID linker and further cloned into a high-copy yeast vector (2 μ) under control of galactose-inducible promoter *GAL1*. The attachment of GFP tag via a linker to α-synuclein was shown to be mandatory to preserve the toxic effect of the untagged version of the protein in yeast model of Parkinson's disease (Petroi et al, 2012). The growth of WT yeast (W303) carrying each variant of synuclein was analyzed with spotting test. Overexpression of β-synuclein caused severe growth inhibition similar to WT α-synuclein expressing cells (Figure 37A), suggesting the toxic effect of β-synuclein in yeast. In comparison, γ-synuclein expressing cells grew uninhibited as the control (GFP expressing cells) (Figure 37A).

One of the major aims of this study was to decipher the role of the post-translational modifications such as sumoylation in Parkinson's disease pathology. So far, we showed that sumoylation protects yeast cells against α-synuclein toxicity and inclusions (Figure 11 and 14). In order to learn more about β- and γ-synuclein associated impact and broaden our investigation regarding sumoylation, we aimed to study the mediated effect of β- and γ-synuclein in presence and absence of cellular SUMO in yeast. *Smt3*^{ts} yeast strain was transformed with β-synuclein-KLID-GFP and γ-synuclein-KLID-GFP high-copy plasmid vectors. *Smt3*^{ts} strain expressing WT α-synuclein from two genomically integrated gene copies (Figure 10) was used for comparison. GFP expressing cells were used as a control. Cells harboring β-synuclein-KLID-GFP, γ-synuclein-KLID-GFP, GFP alone and WT α-synuclein from two genomically integrated gene copies were subjected to growth test in the presence and absence of SUMO at permissive (25°C) and restrictive (30°C)

temperatures. Cells expressing β-synuclein-KLID-GFP presented growth inhibition at both temperatures (Figure 37B). In addition, their growth was more inhibited when the cellular SUMO was impaired at restrictive temperature (30°C). In comparison, γ-synuclein-KLID-GFP expressing cells grew equally well like the control at both permissive and restrictive temperatures (Figure 37B). These data suggested that β-synuclein-mediated toxicity might be regulated by sumoylation, which needs to be further investigated.

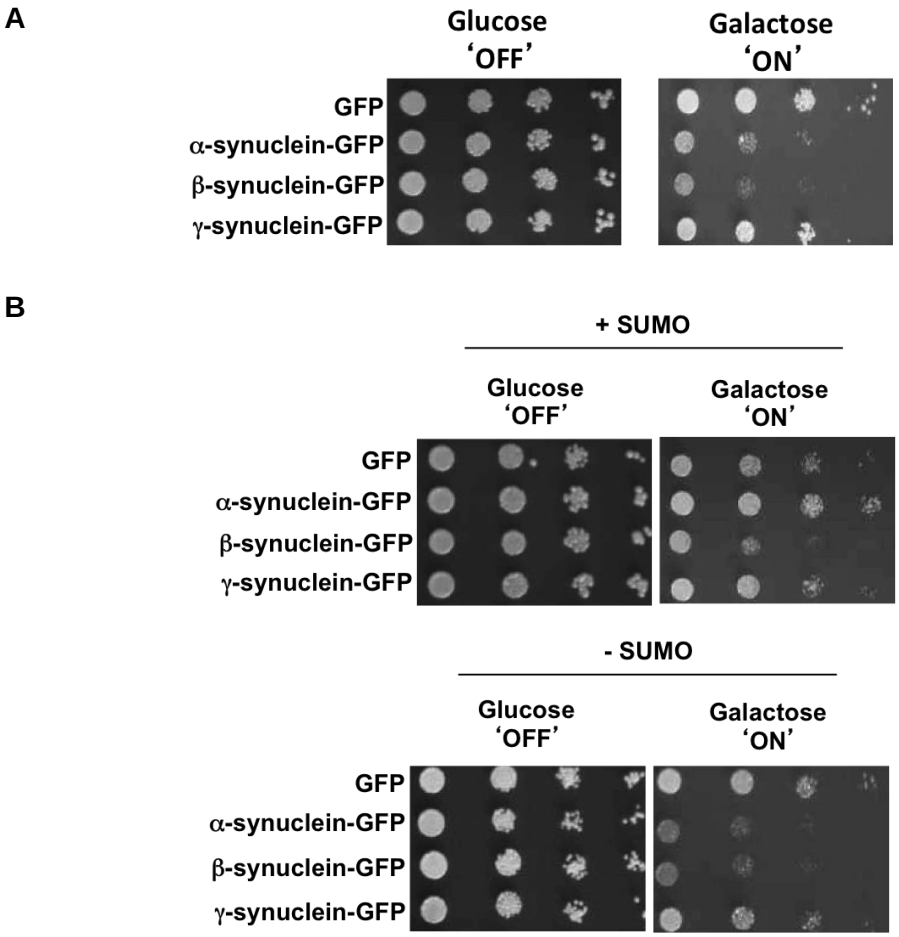

Figure 37. β-synuclein is toxic to yeast cell unlike γ-synuclein.

A. WT yeast cells (W303) were transformed with a high copy plasmid carrying α-synuclein-KLID-GFP, β-synuclein-KLID-GFP and γ-synuclein-KLID-GFP under the control of *GAL1.* GFP expressing cells, expressed from the same promoter, served as control. Yeast cells were spotted in 10-fold dilutions on selection plates containing glucose (*GAL1* promoter 'OFF') or galactose (*GAL1* promoter 'ON'). **B**. Spotting assay of *smt3*^{ts} mutant strain expressing WT α-synuclein-KLID-GFP from two genomically integrated gene copies, β-synuclein-KLID-GFP and γ-synuclein-KLID-GFP at permissive (25°C; + SUMO) or restrictive temperature (30°C; - SUMO). *GAL1*-driven synucleins are expressed from a 2 µm plasmid. GFP, expressed from the same promoter, is used as a control. Yeast cells were spotted in 10-fold dilutions on selection plates containing non-inducing (glucose) and inducing (galactose) solid medium.

3.3.2 β-synuclein and γ-synuclein overexpression results in aggregate formation in yeast

In order to study the localization of β- and γ-synuclein in yeast cells and follow their pattern, we visualized the GFP fusion β- and γ-synuclein in yeast expressing cells. It has been previously reported that α-synuclein toxicity is correlated with its aggregate formation in yeast (Petroi et al, 2012). To examine whether the β-synuclein-mediated cytotoxicity is correlated with its aggregate formation, the *smt3*^{ts} yeast strain expressing β-synuclein-KLID-GFP in presence and absence of SUMO was subjected to live cell fluorescence microscopy after 5 hours induction of β-synuclein-KLID-GFP expression.

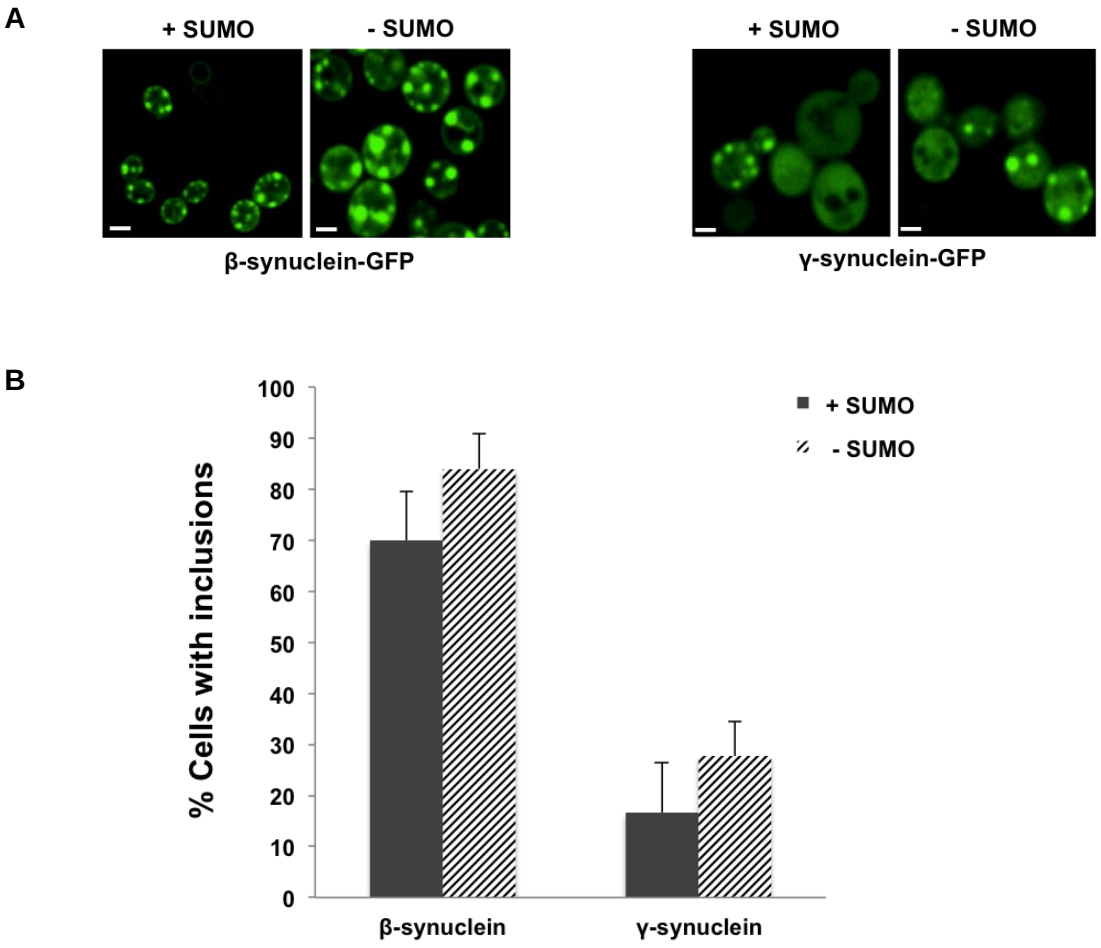

Figure 38. β-synuclein cytotoxicity correlates with its aggregate formation.

A. Live-cell fluorescence microscopy of *smt3*^{ts} cells harboring β-synuclein-KLID-GFP (left) and γ-synuclein-KLID-GFP (right) in the presence (+SUMO) and absence (-SUMO) of cellular SUMO. Cells were induced for 5 hours in 2% galactose containing medium and then subjected to fluorescence microscopy. Scale bar 1 μm. **B.** Quantification of number of *smt3*^{ts} cells harboring β-synuclein-KLID-GFP and γ-synuclein-KLID-GFP in the presence (+SUMO) and absence (-SUMO) displaying aggregate formation. Quantification was done after more than biological replications.

The results revealed that the toxic β-synuclein forms aggregates irregardless of cellular SUMO status, which shows the correlation between β-synuclein cytotoxicity and aggregate formation (Figure 38A). Quantification of numbers of cells displaying β-synuclein aggregates showed that, when sumoylation is down regulated the number of cells with β-synuclein aggregates were increased (Figure 38B). *Smt3*^{ts} yeast cells were monitored after 5 hours expressing γ-synuclein-KLID-GFP in the presence and absence of SUMO. γ-synuclein showed aggregate formation as well as cytoplasmic localization (Figure 38A). The number of cells displaying γ-synuclein aggregates was lower at permissive temperature than at restrictive temperature, suggesting regulatory effect of SUMO in γ-synuclein-mediated aggregate formation (Figure 38B).

4 Discussion

Due to the increasing life expectancy, study of human neurodegenerative disorders such as Parkinson's disease is getting more important. The aggregation of α-synuclein has emerged as the most important player in Parkinson's disease process (Luk & Lee, 2014). α-synuclein propensity to aggregate can be altered with several factors including post-translational modifications.

Baker's yeast *Saccharomyces cerevisiae* serves as a valuable cellular tool to underline different mechanisms explaining α-synuclein pathology in Parkinson's disease (Tenreiro et al, 2013). α-synuclein is toxic to yeast in a gene dosage dependent manner and the α-synuclein-mediated cytotoxicy is correlated with the number of cells showing inclusions (Outeiro & Lindquist, 2003; Petroi et al, 2012). In addition, the main degradation pathway that contributes to α-synuclein aggregate clearance in yeast is autophagy (Petroi et al, 2012).
We used *Saccharomyces cerevisiae* as a model to investigate the molecular interplay between sumoylation and phosphorylation in the clearance of α-synuclein aggregates. We uncovered a complex cross-talk between these post-translational modifications which impacts on ubiquitination and influences the degradation of α-synuclein by both autophagy and the 26S proteasome. The differential processing of α-synuclein by these two modifications systems interferes with inclusion formation and cytotoxicity.

4.1 Cross-talk between α-synuclein sumoylation and S129 phosphorylation

4.1.1 α-synuclein sumoylation in yeast model

α-synuclein undergoes numerous post-translational modifications such as phosphorylation, ubiquitination, nitration, acetylation, O-glycosylation or sumoylation (Figure 4). α-synuclein was found to be a SUMO target in cultured human cells and in a rat animal model of Parkinson's disease (Dorval & Fraser, 2006; Krumova et al, 2011). The number of sumoylation studies of α-synuclein is very limited in comparison to those on other post-translational modification publications. Therefore limiting our understanding of the implications of sumoylation on α-synuclein biology are still puzzling.

The first goal of this study was to investigate whether there is α-synuclein sumoylation in yeast. Furthermore, the regulatory influence of this modification on α-synuclein-mediated cytotoxicity was analyzed. We showed that both wild type α-synuclein and the A30P mutant are sumoylated *in vivo* in yeast at K96 and K102, two sumoylation sites that are conserved in eukaryotes including humans (Dorval & Fraser, 2006; Krumova et al, 2011) (Figure 7C and 11). By decreasing the cellular SUMO pool, growth analysis revealed a protective effect of sumoylation against α-synuclein-mediated cytotoxicity and inclusion formation in yeast cells (Figure 11 and 14). SUMO is one of the most soluble proteins known (Marblestone et al, 2006). The alteration in solubility of aggregate-prone proteins is connected to their pathological tendency to form intercellular aggregates. The lack of overlapping between SUMO and α-synuclein immunoreactivity in post-mortem studies of human brains suggested that α-synuclein aggregations are not SUMO-modified in Parkinson's patients (Pountney et al, 2005). Previously, sumoylation was suggested to keep α-synuclein in solution, which results in decreased α-synuclein aggregation (Krumova et al, 2011). This indicates the direct impact of SUMO on α-synuclein solubility. Recently an interesting investigation reported the SUMO's impact on STAT1 protein. STAT proteins are dimeric transcription factors, which modulate the biological effect of cytokines in human cells. Sumoylation of STAT1 regulates its activity indirectly by increasing the solubility of STAT1 paracrystals (Droescher et al, 2011a; Droescher et al, 2011b). The reduction of SUMO levels in *Drosophila melanogaster* model of Huntington's disease promoted the neuropathology, resulting in neuronal cell loss (Steffan et al, 2004). Similarly, sumoylation was found to modulate the solubility of transcriptional regulator DJ1, androgen receptor and ataxin 7 that also reduced the toxicity of these proteins in other degenerative diseases (Janer et al, 2010; Mukherjee et al, 2009; Shinbo et al, 2006). Moreover, α-synuclein sumoylation abolishment by mutating the codons for the major SUMO sites of α-synuclein, K96 and K102, revealed that these two major lysine residues are conserved from yeast to human (Krumova et al, 2011). This in addition confirmed the direct defensive influence of sumoylation against α-synuclein-mediated cytotoxicity in yeast (Figure 14A). Consistently, impairment of sumoylation in yeast as well as expression of sumoylation-deficient α-synuclein resulted in a significant increase in the number of cells displaying α-synuclein inclusions (Figure 14B). This further supports the beneficial regulatory role of sumoylation in inhibiting α-synuclein inclusion formation *in vivo*.

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4.1.2 α-synuclein phosphorylation in yeast

Non-pathologic α-synuclein is phosphorylated at low levels (Anderson et al, 2006; Fujiwara et al, 2002; Kahle et al, 2002; Waxman & Giasson, 2008), whereas hyper-phosphorylation of α-synuclein at S129 was observed in pathological inclusions of postmortem human brain samples (Anderson et al, 2006; Fujiwara et al, 2002; Kahle et al, 2002; Neumann et al, 2002; Nishie et al, 2004; Waxman & Giasson, 2008). The finding of 90% insoluble α-synuclein phosphorylated at S129 in Lewy bodies led to extensive studies of this key α-synuclein post-translational modification. Study of the levels of pathologic form of phosphorylated α-synuclein revealed that soluble non-phosphorylated α-synuclein decreases over the Parkinson's disease time course (Zhou et al, 2011). Though the initial findings suggested that the α-synuclein pervasive S129 phosphorylation is a significant pathogenic event (Chen & Feany, 2005; Freichel et al, 2007), the exact impact of this modifier remains elusive. In this study we explored yeast as model to investigate the molecular interplay between α-synuclein sumoylation and S129 phosphorylation. We examined the effect of efficient human kinases as GRK5 (Arawaka et al, 2006; Pronin et al, 2000) or PLK2 (Inglis et al, 2009; Salvi et al, 2012) on α-synuclein S129 phosphorylation in yeast. Initially we checked CDC5, the yeast ortholog of PLK2 in hampered yeast. Overexpression of CDC5 was lethal to yeast cell, which consequently stopped further investigations Figure (15 and 19). Our observations confirmed the potent effect of human kinases GRK5 and PLK2 in elevating α-synuclein S129 phosphorylation level in yeast (Figure 17). Furthermore, our data revealed a significant increase of PLK2-mediated α-synuclein phosphorylation at S129 in SUMO deficient cells, when compared to that observed in cells with intact sumoylation machinery (Figure 18). PLK2 seems to be especially efficient on non-sumoylated α-synuclein. In contrast, GRK5 is less specific and can increase the level of S129 phosphorylation independently of the cellular SUMO pool (Figure 18). It is reported that PLK2 phosphorylates α-synuclein at S129 in yeast cells and increases α-synuclein inclusions independently from the phosphorylation level at S129 (Basso et al, 2013).

Sumoylation and phosphorylation are both reversible dynamic processes, which can actively interfere with each other and modulate the behavior of their substrates. Several examples have been reported in the literature where phosphorylation depends on the sumoylation profile of target proteins (Hietakangas et al, 2006; Ugrinova et al, 2011). Sumoylation can modulate the specific interaction with kinases or phosphatases, by changing substrate surfaces and activity. In particular,

sumoylation of protein-tyrosine phosphatase 1B has been shown to reduce the catalytic activity and, therefore, change the phosphorylation status of substrates (Dadke et al, 2007). The systematic study of the cellular phosphoproteome upon SUMO inhibition revealed that the reduction in sumoylation resulted in less activity of the casein kinase II. This leads to less phosphorylation of the substrates and modulate the cell cycle progression (Yao et al, 2011). Furthermore, identification of phosphorylation-dependent sumoylation motifs (PDSMs) in proteins such as heat shock factors facilitates the explanation of interplay between protein phosphorylation and sumoylation (Hietakangas et al, 2006).

The cross-talk between sumoylation and other post-translational modifications is best shown by the tumor suppressor p53. The last 30 amino acids of human p53 are heavily modified by sumoylation, phosphorylation, acetylation, neddylation, ubiquitination and methylation (Kruse & Gu, 2008; Wu & Chiang, 2009). Although it remains unclear whether serine 392 phosphorylation stimulate p53 lysine 386 sumoylation, it is shown that p53 lysine 386 sumoylation enhances its serine 392 phosphorylation by PKR (Bennett et al, 2012). We observed that α-synuclein is sumoylated in the presence of GRK5 (Figure 19), though no significant differences in α-synuclein sumoylation level could be identified.

These data corroborate an interconnection between sumoylation and kinase activity to regulate α-synuclein S129 phosphorylation. Though the exact molecular mechanisms that might be involved in this regulation needs further studies.

A better understanding of the molecular mechanisms of the α-synuclein-related influence on Parkinson's disease pathology will provide novel therapeutic strategies. Accumulation evidence suggests that α-synuclein post-translational modifications (sumoylation, ubiquitination and phosphorylation) modulate α-synuclein-mediated toxicity and aggregate formation. The protective effect of α-synuclein sumoylation against its aggregate formation in cell-based study as well as rat model of Parkinson's disease (Dorval & Fraser, 2006; Krumova et al, 2011) support this findings. Suppression and enhancing impact of α-synuclein phosphorylation on its aggregate properties (Chen & Feany, 2005; Gorbatyuk et al, 2008; Paleologou et al, 2008) and the regulatory mechanism of α-synuclein ubiquitination on its aggregation propensity and degradation fate (Rott et al, 2011; Rott et al, 2008) are other pronounced findings in this concept. However, there is still no consensus on the effects of different modifications on α-synuclein aggregation and toxicity (Azeredo da Silveira et al, 2009; Basso et al, 2013; Kim et al, 2011; Krumova et al, 2011; Oueslati

et al, 2013; Smith et al, 2005). In addition, the presence of different α-synuclein residues (Figure 4) that are potential factors for several post-translational modifications, highlight the importance of studying the interplay between the different modifiers.

Here, we focused on the interplay between α-synuclein sumoylation and S129 phosphorylation. Whereas earlier studies did not observe effects of α-synuclein phosphorylation at S129 on α-synuclein-mediated toxicity and aggregation (Azeredo da Silveira et al, 2009; McFarland et al, 2009), protective roles of α-synuclein S129 phosphorylation were described in a strain-specific manner in yeast (Sancenon et al, 2012). Therefore, the specific genetic context was proposed to determine the sensitivity to changes in α-synuclein phosphorylation. This suggests a complex and subtle cross-talk between different modifications that can change features of the target protein including inclusion formation, stability and the affinity to the autophagic or the proteasome degradation pathways. We showed that increased α-synuclein S129 phosphorylation induced by GRK5 was able to rescue yeast cells from α-synuclein-mediated cytotoxicity associated with sumoylation impairment (Figure 20). Alleviation of α-synuclein-mediated cytotoxicity in SUMO deficient cells correlates with a decreased number of cells presenting α-synuclein intracellular inclusions (Figure 21). Expression of GRK5 induced a strong improvement on yeast growth when the sumoylation was impaired. Our finding showed that there is no rescue in cells expressing S129-phosphorylation blocked α-synuclein (S129A) in the presence of GRK5 when cellular SUMO pool is inhibited (Figure 25). This suggested a direct cross-talk between increased α-synuclein phosphorylation at S129 and sumoylation. These data were further supported by the results with the sumoylation-deficient α-synuclein (K96R K102R synuclein) (Figure 27). Environmental factors such as oxidative stress are also shown to be involved in Parkinson's disease pathology (Hauser & Hastings, 2013; Michel et al, 2013). Accumulation of reactive oxygen spices (ROS) in cells resulted in up-regulation of global sumoylation, which further affect the mitochondrial processes (Manza et al, 2004). Increased phosphorylation level of alpha subunit of eIF2 facilitates the adaption of cells to oxidative stress (Koromilas & Mounir, 2013). Increased ROS-mediated H_2O_2 in the absence of cellular SUMO resulted in a severe growth inhibition in yeast cells expressing α-synuclein. Overexpression of GRK5 in the absence of SUMO reduced α-synuclein expressing cells sensitivity to oxidative stress (Figure 22). Overall our observation suggested that increased α-synuclein phosphorylation at S129 with GRK5 could

compensate the α-synuclein-mediated cytotoxicity associated with sumoylation impairment.

Despite increased α-synuclein phosphorylation levels at S129 in the absence of SUMO, overexpression of PLK2 led to improve cell growth from α-synuclein-mediated toxicity associated with sumoylation impairment (Figure 20). This further correlated with decreased numbers of cells displaying α-synuclein inclusions (Figure 21). PLK2 might cause additional effects on other targets than α-synuclein in yeast, or on other phosphorylation sites of α-synuclein. These data are in agreement with a recent study, where a specific role of PLK2 on α-synuclein inclusion formation and toxicity in yeast is reported, independent of the level of α-synuclein phosphorylation on S129 (Basso et al, 2013).

4.2 α-synuclein aggregate clearance

The dynamic process of α-synuclein aggregate formation depends on the equilibrium between synthesis and degradation, which determines the protein level of α-synuclein. An important question is how α-synuclein degradation is distributed between the ubiquitin-proteasome (UPS) system and the autophagy-lysosome/vacuole pathway (ALP).

4.2.1 Contribution of post-translational modification network in α-synuclein aggregate clearance

The current study addresses the involvement of post-translational modifications in α-synuclein aggregate clearance. We previously found that autophagy represents the major pathway for aggregate clearance in yeast after shut-off of protein biosynthesis, allowing cells to recover from α-synuclein toxicity (Petroi et al, 2012).

One of the major findings of this study is that sumoylation of α-synuclein promotes aggregate clearance by autophagy. α-synuclein clearance is impaired when sumoylation is inhibited either by reducing the cellular SUMO pool or by amino acid substitutions of the SUMO target sites of α-synuclein (Figure 28). At low levels, α-synuclein seems to be preferentially degraded by the UPS, whereas increased α-synuclein expression stimulates autophagy as the main degradation pathway (Ebrahimi-Fakhari et al, 2011). Sumoylation and ubiquitination can act as competitors

to target the same substrate protein. The impaired sumoylation might facilitate higher ubiquitination of the target protein and affect its fate of degradation. Interestingly, sumoylation was indicated as a targeting signal for ubiquitination and ubiquitin-dependent degradation (Cheng et al, 2007; Uzunova et al, 2007). Reduction of cellular SUMO pool might enhance ubiquitination authority of α-synuclein, which lead the protein to UPS for degradation (Ebrahimi-Fakhari et al, 2012), though, the interplay between sumoylation, ubiquitination and degradation pathways involved in protein clearance is more complex than anticipated (Kim et al, 2011; Tatham et al, 2011).

Another major finding is that phosphorylation of α-synuclein by GRK5 or PLK2 can partially support autophagy and compensate sumoylation impairment effect (Figure 29). Sumoylation and phosphorylation are two post-translational modifications of α-synuclein that protect against α-synuclein-induced toxicity (Krumova et al, 2011; Paleologou et al, 2008; Waxman & Giasson, 2008). However, they represent distinct signals for the processing of α-synuclein by different degradation pathways (Oueslati et al, 2013). Whereas sumoylation primarily targets α-synuclein for autophagy, phosphorylation by kinases such as GRK5 has a dual effect because it partially rescues the autophagy pathway but also promotes increased ubiquitination and a reduced half-life of the protein (Figure 31 and 36). Phosphorylation is a well-known priming reaction for ubiquitination (Hasegawa et al, 2002c; Hershko & Ciechanover, 1998). We found that enriched WT α-synuclein expressed in WT yeast background is mono-ubiquitinated (Figure 30) in agreement with evidence showing that α-synuclein purified from Lewy bodies is mainly mono- and di-ubiquitinated (Tofaris et al, 2003), whereas α-synuclein higher phosphorylation at S129 lead to promoted ubiquitination by altering the ubiquitination profile of α-synuclein when sumoylation is inhibited (Figure 31). MG132 treatment impaired the sumoylation-deficient α-synuclein aggregate clearance more compared to the reduction of aggregate clearance with PMSF in the presence of GRK5 (Figure 33 and 37). These data suggest that UPS degradation pathway contributes more to the clearance of sumoylation-deficient α-synuclein with increased S129 phosphorylation, induced by GRK5. This further supports the idea that phosphorylation promotes the degradation of α-synuclein by the proteasome.

The protective role of PLK2, which can form a complex with α-synuclein and can also induce the autophagy pathway, seems to be more complicated and might include additional phosphorylation target proteins. Recently, *in vivo* study suggested that induction of PLK2 activity mediate α-synuclein turnover via enhancing its clearance

by the autophagy pathway (Oueslati et al, 2013). Inhibition of both cellular degradation pathways chemically resulted in the same contribution of each system in aggregate clearance of sumoylation-deficient α-synuclein when PLK2 kinase was overexpressed (Figure 33 and 37).

A dual modification that is interdependent allows a subtle fine-tuning as molecular mechanism to selectively control α-synuclein turnover in response to sumoylation or phosphorylation input signals. Sumoylation might induce structural and conformational changes in α-synuclein and thus modulate the interaction with different kinases, which have various effects in the channeling to distinct degradation pathways. Post-translational modifications of α-synuclein seem to orchestrate the harmonic interplay to maintain the balance of protein removal. This network relies on complex cross-talk between molecular mechanisms that interact directly/indirectly to subject the pathogenic protein for efficient degradation.

4.3 β-synuclein is toxic to yeast

There are high similarities between α- and β-synuclein in amino acid sequences identity (> 50 %) in their N-terminus (Lavedan, 1998) and their sub-cellular localization (Clayton & George, 1998). Several studies revealed a role of β-synuclein role in the progress of Parkinson's disease. In the present study we explored β-synuclein-mediated effects on yeast growth. We showed that overexpression of β-synuclein is inhibiting yeast growth severely (Figure 37A). The toxic effect of β-synuclein on yeast cells correlated with increased number of yeast cells presenting inclusions (Figure 38B). Only limited investigations focused on the function of β-synuclein Parkinson's disease pathology. Early studies showed the potential propensity of β-synuclein to inhibit α-synuclein aggregate formation. It was suggested that β-synuclein ameliorate α-synuclein neurotoxicity (Hashimoto et al, 2001; Park & Lansbury, 2003; Uversky et al, 2002). A recent study demonstrated that β-synuclein is aggregating in dopaminergic neurons and is as neurotoxic as α-synuclein to those cells (Taschenberger et al, 2013). This is in line with our observations in the yeast model system. Furthermore, the sumoylation impact on β-synuclein-mediated toxicity in yeast cells was addressed. β-synuclein toxic effect was increased in sumoylation deficient yeast cells (at restrictive temperature), was correlated with increased number of cells displaying inclusions (Figure 38B). Protective effect of sumoylation against α-synuclein-mediated toxicity suggested the same mechanism that might be

involved in β-synuclein pathobiology. It would be interesting to investigate post-translational modifications of β- and γ-synuclein such as sumoylation and phosphorylation in yeast model system. These might provide a key to uncover the complex pathology of Parkinson's disease.

5 Conclusion

α-synuclein post-translational modifications play an intrinsic role in Parkinson's disease pathology. The susceptibility of post-translational modifications to interplay affects α-synuclein-mediated consequences in Parkinson's disease progression.

This study provides evidence that the degree of switching between autophagic and proteasomal degradation of α-synuclein is linked to a molecular cross-talk between sumoylation and phosphorylation. Sumoylation preferentially directs α-synuclein towards autophagy and phosphorylation can shift the fate of α-synuclein to increased ubiquitination and proteasome degradation. Ultimately, a deeper understanding of this cross-talk will enable the design of effective strategies for directing α-synuclein for processing by the desired degradation machinery and may, therefore, constitute the basis for novel therapeutic strategies in Parkinson's disease and other synucleinopathies.

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Abbreviations

A ..... alanine

ampR ..... ampicillin resistance

ATP ..... adenosine triphosphate

bp ..... base pair

cDNA ..... complementary DNA

C ..... control

CA ..... Callifornia

cm ..... centimeter

C-terminus ..... carboxy terminus

D ..... aspartic acid

Da ..... Dalton

DLB ..... dementia with Lewy bodies

DMSO ..... Dimethyl sulfoxide

DNA ..... deoxyribonucleic acid

DTT ..... dithiothreitol

E ..... glutamic acid

ECL ..... enhanced chemiluminescence

EDTA ..... 2,2',2'',2'''-(Ethane-1,2-diydinitrilo) tetraacetic acid

EtOH ..... Ethanol

EV ..... empty vector

G ..... glycine

GADPH ..... glyceraldehyde 3-phosphate dehydrogenase

GAL..... galactose

GBA ..... glucocerebrosidase

GFP ..... green-fluorescent protein

GmbH ..... Geselschaft mit beschränkter Haftung

H ..... histidine (His)

H ..... hour(s)

K ..... lysine

kan^R ..... kanamycin resistance

kb..... kilobase(s)

kDa ..... kilo Dalton

L..... liter

LBs..... Lewy bodies

LRRK2 ..... Leucine-rich repeat kinase 2

IP ..... immuneprecipitation

LB ..... lysogeny broth (Luria-Bertani medium)

mg..... milli-gramm

min..... minute(s)

ml..... milli-liter

MM..... minimal medium

mM..... milli-molar

mRNA ..... messenger RNA

NAC ..... non-Aβ component domain

NaOH..... Sodium hydroxide

NTA..... nitrilotriacetic acid

N-terminus ..... amino terminus

P ..... proline

PAGE..... polyacrylamide gel electrophoresis

PCR ..... polymerase chain reaction

PDSM ..... phosphorylation-dependent sumoylation motif

PIM ..... protease inhibitor mix

PINK1 ..... PTEN-induced putative kinase 1

PMSF..... phenylmethanesulfonyl fluride

PTM ..... post-translational modification

RNA ..... ribonucleic acid

S ..... serine

S ..... second(s)

SAE..... SUMO activity enzyme

SC..... synthetic complete

SDS ..... sodium dodecyl sulfate

SENP ..... Sentrin/Sumo-specific protease

SIM ..... Sumo interaction motif

SNARE..... Soluble N-ethylmaleimide sensitive fusion Attachment Protein

STAT..... signal transducer and activator of transcription

SUMO ..... small ubiquitin-like modifier

SUP ..... SUMO-specific protease

T..... time

T..... threonine

TCA..... trichloroacetic acid

Tris..... 2-Amino- 2-hydroxymethyl-propane-1, 3-diol

TRP..... tryptophan

TUBEs..... Tandem Ubiquitin Binding Entities

tRNA ..... transfer RNA

UBA ..... ubiquitin binding associated domain

UBC ..... ubiquitin conjugating enzyme

UCH-L1..... ubiquitin carboxyl terminal hydrolase L1

Ulp ..... ubiquitin-like protein-specific protease

USA ..... United States of America

UTR ..... un-translated region

UV..... ultra-violet

WT ..... wild type

YEPD ..... Yeast Extract Peptone Dextrose

Acknowledgments

One of the joys of an accomplishment is to look back and recall all people that made it possible. I would love to express my appreciation to all who have been along with me during this journey.

First and most I would like to express my gratitude to Prof. Gerhard H. Braus for giving me the opportunity to join his group and carry out my Ph.D. thesis under his supervision. I am grateful for his advisement, encouragement, patience, and high vision of science, which drove me to learn more every day to fulfill my goals.

My very special thank goes to Dr. Blagovesta Popova for her scientific trainings and appreciate her time to proof read my thesis. I am grateful for her endless kindness, patience and encouragements, which supported me during this time.

I would like to thank my thesis committee members: Prof. Stefanie Pöggeler for her useful comments and remarks during my Ph.D. work and to read and evaluate my thesis in a short period of time, Prof. Heike Krebber for supporting my research with various constructive suggestions and advice.

I would like to thank Profs. Tiago Outeiro and Paul Fraser for thoughtful and detailed comments and invaluable insights to develop my Ph.D. work.

I would like to thank Ms. Maria Mayer for her technical assistance and patience to provide me with any unexpected experimental material needed.

I am very grateful to Dr. Rebekka Harting to proof read of my thesis and helpful discussions.

I would like to thank my colleagues Alexandra Juckert and Drs. Christoph Sasse and Jennifer Gerke to proof read my thesis.

I would love to thank my dear friend Dr. Katayoun Mansouri, since high school, for her moral and academic support despite geographical distances for almost 20 years.

My gratitude goes to members of lab 110: Chi-jan Lin, Dr. Henriette Irmer, Dr. Blagovesta Popova and Alexandra Juckert, who have been very supportive during this time in scientific and personal situations. Your kindness, thoughtfulness and patience is priceless and made the lab my second home.

My gratefulness goes to Sabine Reen for her time and patience to help me with the Thesis Word file formatting.

I would like to thank Department of Prof. Gerhard Braus, who gave me a home far away from home with their extreme humanity and sympathy.

A very special thank goes to my dear friend, Tara Hosseinizad, who has been cheering me on at all time like a sister and a moral support during difficult times.

I am also appreciative to Mrs. Farzane Farshad and Mr. Reza Jahanshiri for their carrying and kindness during this journey.

I would like to take the opportunity to thank CNMPB (Nanoscale Microscopy and Molecular Physiology of the Brain) for providing productive meetings and workshops. Also CNMPB seminars helped me to broaden my knowledge of understanding the Parkinson's disease pathobiology.

I am grateful to Göttingen Graduate School for Neurosciences, Biophysics, and Molecular Biosciences (GGNB) to grant me with "Travel awards" to present my results in various national as well as international conferences.

I would like to thank GGNB office especially, Ms. Christin Schröter for her continuous support. I would love to take the chance to thank Ms. Heidi Northemann and Ms. Nicole Scheiter for their endless administrative support.

My absolute appreciation goes to my family. I would love to express my gratitude to my dear parents (Zahra Nozari and Dr. Hossein Shahpasandzadeh) for their outmost love and support that without them this mission would never be succeeded. I would love to express my deepest appreciation to my lovely sisters (Dr. Mahdieh Shahpasandzadeh, Homa Shahpasandzadeh and Mina Shahpasandzadeh), who without their love, encouragement and support this journey would not be ended.

Curriculum Vitae

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Scientific background

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2011 – Member of the "Göttingen Graduate School for Neurosciences and Molecular Biosciences" **(GGNB)**.

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Publications

Shahpasandzadeh H, Popova B, Juckert A, Fraser PE, Outeiro TF, Braus GH (2014) Interplay between sumoylation and phosphorylation for protection against alpha-synuclein inclusions. *J Biol Chem* M114.559237

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To Make Something Special, You Just Have To Believe It Is Special.

Hedieh