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CHAPTER 7

Role of post-translational modifications in modulating the structure, function and toxicity of α-synuclein: implications for Parkinson's disease pathogenesis and therapies

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Abstract: A better understanding of the molecular and cellular determinants that influence the pathology of Parkinson's disease (PD) is essential for developing effective diagnostic, preventative and therapeutic strategies to treat this devastating disease. A number of post-translational modifications to α-syn are present within the Lewy bodies in the brains of affected patients and transgenic models of PD and related disorders. However, whether disease-associated α-syn post-translational modifications promote or inhibit α-syn aggregation and neurotoxicity *in vivo* remains unknown. Herein, we summarize and discuss the major disease-associated post-translational modifications (phosphorylation, truncation and ubiquitination) and present our current understanding of the effect of these modifications on α-syn aggregation and toxicity. Elucidating the molecular mechanisms underlying post-translation modifications of α-syn and the consequences of such modifications on the biochemical, structural, aggregation and toxic properties of the protein is essential for unravelling the molecular basis of its function(s) in health and disease. Furthermore, the identification of the natural enzymes involved in regulating the post-translational modifications of α-synuclein will yield novel and more tractable therapeutic targets to treat PD and related synucleinopathies.

Keywords: α-synuclein; post-translationnal modification; phosphorylation; truncation; ubiquitination

Introduction

A better understanding of the molecular and cellular determinants that influence the pathology of Parkinson's disease (PD) is essential for developing effective diagnostic, preventative and

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therapeutic strategies to treat this devastating disease. Current strategies offer little more than transient symptomatic relief. Evidence from genetics, pathology, animal modelling, cell culture and *in vitro* biochemical and biophysical studies supports the hypothesis that α-synuclein (α-syn) plays a central role in the pathogenesis of PD and several other neurodegenerative diseases, collectively referred to as 'synucleinopathies'. Although α-syn has emerged as a viable therapeutic target, the molecular and cellular determinants involved in initiating and/or propagating α-syn aggregation and toxicity and the relationship between these processes and disease progression remain poorly understood. In PD, several post-translational modifications of α-syn are associated with the formation of Lewy bodies (LB) in the brains of affected patients and of transgenic (TG) models of PD and other synucleinopathies. However, whether disease-associated α-syn post-translational modifications such as phosphorylation, truncation, ubiquitination and nitration promote or inhibit α-syn aggregation and neurotoxicity *in vivo* remains unknown. This understanding is critical to elucidate the role of α-syn in the pathogenesis of PD and to develop therapeutic strategies for PD. Therefore, characterizing the molecular mechanisms underlying post-translation modifications of α-syn and the consequences of such modifications on the biochemical, structural, aggregation and toxic properties of the protein is essential for unravelling the molecular basis of its function(s) in health and disease. Furthermore, identification of the natural enzymes (e.g. kinases and phosphatases) involved in regulating α-syn post-translational modifications will yield novel and more effective therapeutic targets to treat PD and related synucleinopathies.

Structural and biochemical properties of α-syn

The sequence of α-syn can be divided into three domains with different features (Fig. 1a). The

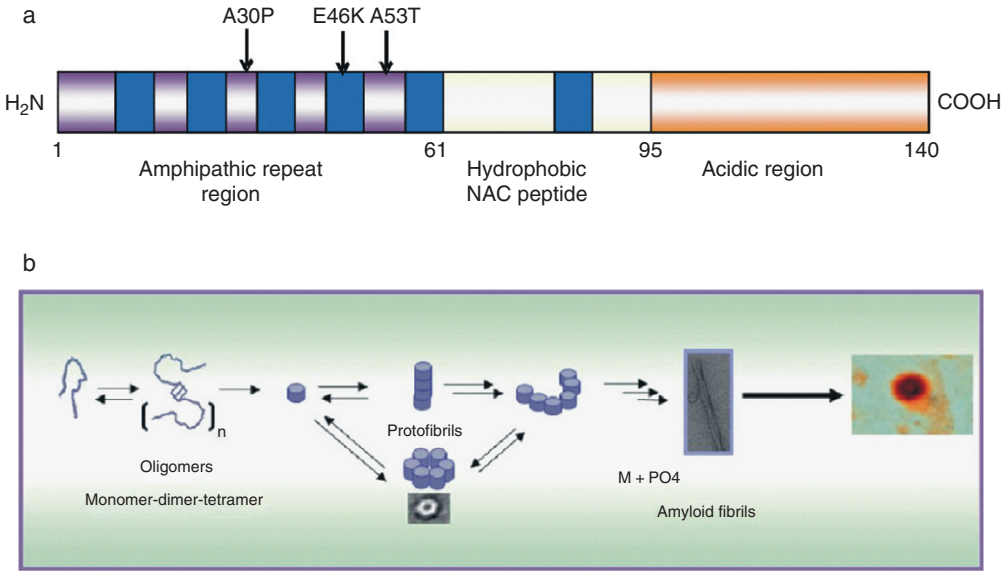

Fig. 1. α-syn structure and molecular mechanism of its oligomerization and fibrillogenesis. (a) Schematic representation of α-syn, where the N-terminal repeats (KTKEGV) are represented in blue; they partially overlap with the core domain (white). The pathogenic variants associated with familial forms of PD alter the N-terminus of α-syn and enhance its propensity to form protofibrils (A30P) or mature fibrils (E46K and A53T). (b) Illustration of the molecular steps involved in α-syn oligomerization and fibrillogenesis leading to Lewy body formation (adapted from Lashuel and Lansbury, 2006).

N-terminal region, comprising amino acids 1–60, contains four 11 residue imperfect repeats with a highly conserved hexameric motif (KTKEGV). The central region, comprising residues 61–95, also known as the non-amyloid component region (NAC), is composed of predominantly hydrophobic residues and is essential for α-syn fibrillization and LB formation. The C-terminal region (residues 96–149) is highly enriched in acidic (glutamate and aspartate) and proline residues and exists in a disordered conformation in the monomeric as well as the oligomeric and fibrillar forms of α-syn (Bertini et al., 2007; Wu et al., 2008). The C-terminus of α-syn has been proposed to function as a solubilizing domain and contributes to α-syn's thermostability. The C-terminal region of α-syn has been implicated in the majority of α-syn interactions with proteins (Cherny et al., 2004; Fernandez et al., 2004; Giasson et al., 2003; Jensen et al., 1999), metal ions (Brown, 2007; Paik et al., 1999) and other ligands (e.g. dopamine and polyamines) (Hoyer et al., 2004) and contains the majority of post-translational modification sites. α-Synuclein interactions with the microtubule-associated protein tau, brain-specific p25α and FKBP-type peptidyl-prolyl *cis–trans* isomerases have all been mapped to the C-terminal region encompassing residues 110–140, suggesting that the C-terminus of α-syn plays a critical role in modulating the stability, structure, aggregation and function of the protein *in vivo* (Kim et al., 2002).

In solution, α-syn (wild type, WT, A30P, E46K and A53T) exists as an ensemble of disordered conformations (Weinreb et al., 1996). However, in the presence of lipids, the N-terminal region adopts an α-helical conformation. Several factors have been shown to contribute to triggering the oligomerization and fibril formation by α-syn, including high protein concentrations, mutations, post-translational modifications and interactions with specific metals (Brown, 2007; Paik et al., 1999) and small molecules (Hoyer et al., 2004). Fibril formation by α-syn proceeds through series of discrete oligomeric intermediates, known as protofibrils of different sizes and morphologies, including spherical, annular and chain-like structures (Fig. 1b).

The rate of α-synuclein fibrillization is affected by several factors that may be relevant to PD

Several post-translational covalent modifications have been described for α-syn, including serine and tyrosine phosphorylation, ubiquitination, nitration, enzymatic cross-linking (e.g. tissue transglutaminase) and C-terminal truncation, some of which correlate well with PD (Fig. 2). In this section we will review the most commonly observed post-translational disease-associated modifications. We will present an overview of the current understanding of their role in disease pathogenesis on the basis of published *in vitro* and *in vivo* studies aimed at identifying the sites of modifications, the enzymes involved and the consequences of modulating these modifications on the structure, aggregation, membrane binding and toxicity of α-syn.

Phosphorylation

Increasing evidence from pathologic, genetic, animal model, biochemical and biophysical studies suggests that phosphorylation of α-syn at one or multiple sites may play an important role in regulating its structure, membrane binding, oligomerization, fibril formation, LB formation and neurotoxicity *in vivo* (Anderson et al., 2006; Fujiwara et al., 2002). The first evidence demonstrating phosphorylation of α-syn emerged from immunohistochemical and biochemical studies by Iwatsubo and colleagues and was supported later by many studies. Using a phospho-specific antibody and mass spectrometry, they demonstrated that the majority of α-syn within LB and inclusions isolated from brains of patients who died of PD, multiple system atrophy (MSA), dementia with Lewy bodies (DLB) and other synucleinopathies is phosphorylated at serine 129 (S129-P) (Anderson et al., 2006;

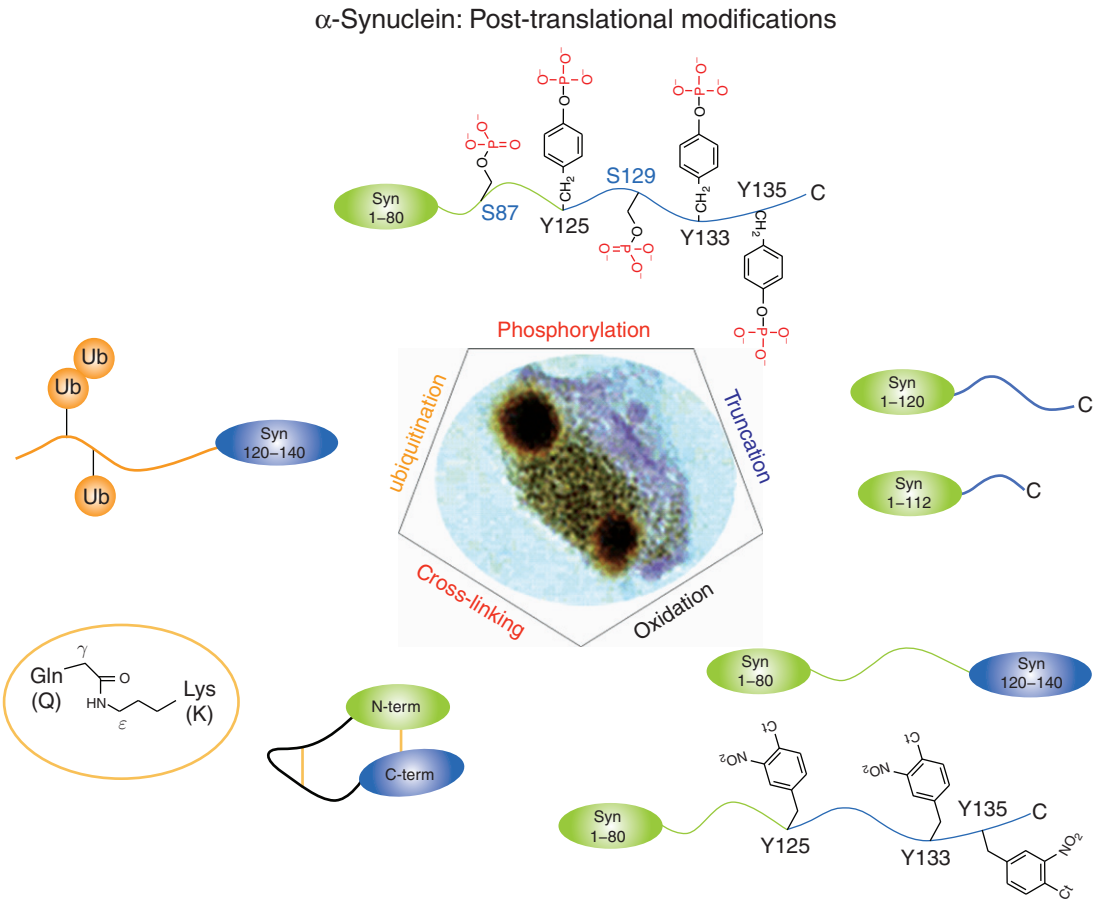

Fig. 2. Most studied α-syn post-translational modifications reported on the basis of their identification in LB. All the modifications identified thus far, including phosphorylation, truncation, nitration and covalent cross-linking by tissue transglutaminase, occur at and/or involve the C-terminal region of the protein. Only ubiquitination (mono-, di- and tri-) is restricted to the lysine residues in the N-terminal region of the protein comprising residues 1–36.

Fujiwara et al., 2002; Kahle et al., 2000; Okochi et al., 2000; Takahashi et al., 2003). Subsequent cell culture studies demonstrated that α-syn is constitutively phosphorylated at S87 and S129. Since then S129-P has emerged as a defining hallmark of PD and related synucleinopathies (Anderson et al., 2006; Fujiwara et al., 2002; Okochi et al., 2000; Takahashi et al., 2003) and is consistently observed in α-syn inclusion formed in cellular and animal models over-expressing WT or mutant α-syn (Chen and Feany, 2005; Lo Bianco et al., 2002; Neumann et al., 2002; Takahashi et al., 2003; Yamada et al., 2004). Recent studies from our group demonstrated that α-syn is phosphorylated at S87 *in vivo* and within LB. The level of S87-P is increased in brains of TG models of synucleinopathies and human brains from Alzheimer's disease (AD), DLB and MSA patients (Paleologou et al., 2010). A recent report by Feany and colleague demonstrated that α-syn is also phosphorylated at tyrosine 125 (Y125-P) and that phosphorylation at tyrosine residues Y125, Y133 and Y135 suppresses

S129-P-induced aggregation and toxicity. These studies demonstrate the presence of multiple phosphorylation sites within α-syn and suggest potential cross-talk between the different modified sites.

All potential phosphorylation sites in α-syn are highly conserved

A close examination and comparison of the amino acid sequences of all synucleins from humans and other species reveals that the majority of potential α-syn phosphorylation sites (4 serine, 10 threonine, 4 tyrosine residues, Fig. 3b) are highly conserved in all species, suggesting that phosphorylation at one or multiple sites may play important roles in regulating α-syn function in health and disease. Considering all α-syn phosphorylation sites identified *in vivo* [S129 (Chen and Feany, 2005; Fujiwara et al., 2002), S87 (Paleologou et al., 2010) and Y125 (Chen et al., 2009)] and *in vitro* [S87 (Kim et al., 2006; Okochi et al., 2000); S129 (Chen and Feany, 2005; Fujiwara et al., 2002; Kim et al., 2006; Okochi et al., 2000; Pronin et al., 2000; Takahashi et al., 2003) and Y125, Y133 and Y136 (Ahn et al., 2002; Ellis et al., 2001; Nakamura et al., 2001; Negro et al., 2002; Takahashi et al., 2003)], it is striking that all these sites cluster at the C-terminal region of α-syn spanning residues 120–140. Only S87 lies in the hydrophobic NAC region of α-syn, which is essential for α-syn aggregation and fibrillogenesis (El-Agnaf et al., 1998). These findings suggest important roles for these modifications in regulating protein–protein, protein–ligand and protein–

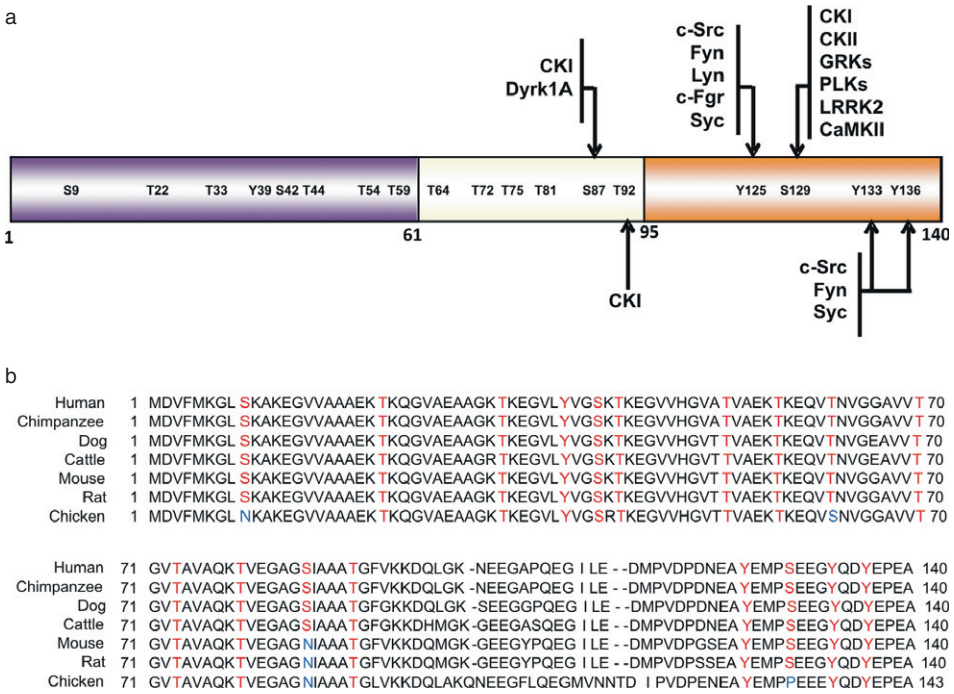

Fig. 3. α-syn phosphorylation in normal and pathological conditions. (a) Schematic depiction highlighting all the potential phosphorylation sites in α-syn: serine (S), threonine (T) and tyrosine (Y). When phosphorylation of the residue has been reported, the corresponding known kinases are indicated; the sites phosphorylated in human are listed in the upper part of the schema. (b) Multiple sequence alignment of α-syn generated by MUSCLE (version 3.6), in which serine, threonine and tyrosine residues are shown in color; note their high level of conservation trough mammals as only serine 87 is not preserved in rodents.

metal interactions, which have been mapped and shown to be mediated by the C-terminal domain of α-syn (see below).

Unravelling the role of phosphorylation in modulating the physiological and pathogenic activities of α-syn requires identification of the kinases and phosphatases involved in regulating its phosphorylation in vivo

Although the exact kinases and phosphatases responsible for regulating α-syn phosphorylation at S129 *in vivo* are still not known, a series of *in vitro* and cell culture-based studies have identified a number of kinases, which phosphorylate α-syn at S129 and/or S87, including casein kinase I (CKI) (*S87* and *S129*), casein kinase II (CKII) (*S129*) (Okochi et al., 2000) and the G protein-coupled receptor kinases (GRKs 1, 2, 5 and 6; *S129*) (Pronin et al., 2000), LRRK2 (leucine-rich repeat kinase 2) (*S129*) (Qing et al., 2009) and PLKs (polo-like kinases) (*S129*) (Inglis et al., 2009; Mbefo et al., 2010) (Fig. 3a). Tyrosine phosphorylation has also been reported at Y125 by Fyn (Nakamura et al., 2001), Syk (Negro et al., 2002), Lyn (Negro et al., 2002), c-Frg (Negro et al., 2002) and Src tyrosine kinases (Ellis et al., 2001), with Syk (Negro et al., 2002) also phosphorylating at Y133 and Y136.

Phosphorylation at S129 and/or S87 alters the conformation of monomeric α-syn and inhibits its fibrillization in vitro

Current cellular and *in vivo* studies aimed at dissecting the functional consequences of phosphorylation at serine, threonine or tyrosine residues mainly rely on genetic approaches to block (e.g. replacing serine/threonine with alanine, S/T → A, or tyrosine with phenylalanine, Y → F) or mimic constitutive phosphorylation (e.g. replacement of serine with glutamate or aspartate, S → E/D). The structural and electrostatic similarities between glutamate/aspartate (net charge of –1) and phosphoserine (net charge of –2) suggest that this type of substitution represents a reasonable approach to mimic constitutive phosphorylation at specific serine residues. However, detailed studies from our group have shown that S → D/E substitutions do not reproduce all aspects of phosphorylation. For example, structural comparisons of the phosphomimics S129D/E with S129-P using nuclear magnetic resonance (NMR) indicate that phosphorylation at S129 and S87 increases the hydrodynamic radius of α-syn to a value comparable to that observed for the WT protein in 8 M urea. In addition, paramagnetic relaxation enhancement of amide protons observed with the help of a spin label attached to residue 18 showed that long-range interactions between the N- and the C-terminal domains of α-syn are attenuated upon phosphorylation at S129 and S87. On the other hand, mutation of S129 and S87 to glutamate (S129E and S87E) or aspartate (S129D and S87D) does not lead to an extended conformation of monomeric α-syn. This result suggests that mutation of S → D/E cannot fully mimic the effect of phosphorylation on the structure and dynamics of α-syn. The extent of fibril formation by the phosphomimics S129E/S129D is similar to that of the WT α-syn, whereas phosphorylation at S129 (S129-P) consistently inhibits α-syn fibrillization. On the other hand, S → D/E substitution at S87 reproduces the effect of phosphorylation on α-syn aggregation at this residue, that is both substitutions inhibit the fibrillization of α-syn *in vitro*. However, phosphorylation at S87, but not the S87D mutation, increases the hydrodynamic radius of α-syn and influences its binding to membranes. Together, these studies demonstrate that phosphorylation of α-syn at S129 and/or S87 significantly inhibits its aggregation (Paleologou et al., 2008, 2010; Waxman and Giasson, 2008) and that the S → D/E substitutions do not reproduce all aspects of phosphorylation. Therefore, the results obtained with these mimics should be interpreted with caution.

Does phosphorylation promote or prevent α-synuclein aggregation in vivo?

To answer this question, one must be able to modulate the level of phosphorylation *in vivo*.

This modulation can be achieved by co-expression of α-syn and the relevant kinase or by comparing the effect of over-expressing the WT protein to the mutants designed to mimic (S129D) or abolish phosphorylation (S129A)(see Table 2). Although *in vitro* biophysical studies have clearly shown that replacement of serine by glutamate or aspartate does not reproduce all aspects of phosphorylation, the use of these mutants remains the only option for elucidating the role S129 phosphorylation in regulating α-syn aggregation and toxicity *in vivo*, especially given the lack of knowledge of efficient kinases that phosphorylate α-syn at this residue. In the next sections, we will present an overview of the findings from different *in vivo* studies aimed at elucidating the consequences of S129 phosphorylation.

GRK2-mediated phosphorylation at S129 or substitution of serine with aspartate (S129D) promotes α-syn oligomerization but does not influence inclusion formation in rodent and Drosophila models of PD

Initial studies aimed at investigating phosphorylation at S129 were carried out in *Drosophila*, a model in which α-syn expression induces a loss of dopaminergic neurons or retinal degeneration, depending on the promoter used (Feany and Bender, 2000). The co-expression of the *Drosophila* homologue of the kinase GRK2 with α-syn did not prompt or prevent the appearance of proteinase K-resistant inclusions (Chen and Feany, 2005), but it did enhance the formation of α-syn oligomers (Chen et al., 2009) compared to situations where only α-syn was over-expressed. These results suggest that increasing S129 phosphorylation does not promote α-syn fibrillization *in vivo* and argue in favour of the hypothesis that S129 phosphorylation results in the kinetic stabilization and/or accumulation of toxic α-syn oligomers. What remains unclear is whether the over-expression of GRK2 contributes to α-syn toxicity via mechanisms that are independent of α-syn phosphorylation. Further, the efficiency with which GRK2 phosphorylates α-syn and the turn-over of S129-P was not investigated in these studies.

To overcome the drawbacks associated with kinase over-expression, a complementary approach was used, based on the expression of the S129D or S129A variant. In *Drosophila*, over-expression of the phosphomimic variant (S129D) yielded results that are comparable to those obtained with GRK2 over-expression: (1) inclusion formation by S129D was similar to that observed for the WT protein and (2) S129D formed more oligomeric forms of α-syn than the WT protein. Similarly, *in vitro* studies using recombinant proteins demonstrated that fibril formation by the phosphomimics S129D/S129E is similar to that of the WT protein. Together, these studies demonstrate that S → D mutation at S129 alters the oligomerization, but not fibrillization, of α-syn.

Substitution of serine 129 with alanine (S129A) promotes α-syn inclusion formation and fibrillization in vivo and in vitro, respectively

Interestingly, in both the fly and the rodent models, the S129A mutants forms five times more insoluble, proteinase K-resistant inclusions (but fewer oligomers) than WT and S129D α-syn (Chen and Feany, 2005; Chen et al., 2009). *In vitro*, the S129A mutant forms fibrils more readily and to a greater extent than WT or the S129D mutant. Neither the S → A nor the S → E mutation alters the structure or morphology of α-syn fibrils *in vitro*. Initially, these findings appear to support the toxic hypothesis model, where accelerated fibrillization by this mutant ensures depletion of the toxic oligomers, thus explaining the reduced toxicity observed for S129A in the fly model. However, this correlation between accelerated fibrillization and reduced toxicity did not hold consistently in various rodent models.

In rodent models, the effects of S → D and S → A substitutions on α-syn aggregation are variable

In rodent, over-expression of S129D and S129A α-syn, using AAV-mediated gene transfer in the *substantia nigra*, led to inconsistent observations, partially due to the different approaches used to characterize and quantify α-syn aggregation. At first, Gorbatyuk et al. (2008) reported that both S129D and S129A form punctate α-syn aggregates *in vivo*. The authors described α-syn S129D immunostaining in neuronal cytoplasm as heterogeneous, whereas WT or α-syn S129A immunolabelling was more evenly distributed throughout the cell, without providing quantitative evaluation of the level of aggregation. Furthermore, neither the biochemical and structural properties of these aggregates nor the α-syn oligomerization in this model was thoroughly investigated, which precludes comparison of the extent and type of α-syn aggregation in other models. On the contrary, a detailed study by Azeredo da Silveira et al. (2009) provided experimental observations of aggregation that agreed with data obtained in *Drosophila*: α-syn S129A forms more β-sheet-rich (thioflavin S-positive), proteinase K-resistant aggregates than WT and S129D α-syn. Finally, a recent study (McFarland et al., 2009) described α-syn-positive deposits in dopaminergic neurons, but revealed similar aggregation patterns for all three proteins (WT, S129D and S129A α-syn). The discrepancy with the previous two models might be due to the fact that McFarland et al. (2009) used a bi-cistronic mRNA to co-express α-syn and green fluorescent protein (GFP), which may lead to lower expression of α-syn compared to classical monocistronic RNA. Recent reports suggest neuronal toxicity associated with AAV-mediated over-expression of GFP in the *substantia nigra*, which could mask the differences in terms of neuronal loss caused by α-syn and the two S129 mutants (Baens et al., 2006; Sawada et al., 2010; Ulusoy et al., 2009). An additional contributing factor might be the fact the immunohistological analysis was carried out 6 weeks after virus injection, compared to 8 weeks in the studies by Azeredo da Silveira et al. (2009) and Gorbatyuk et al. (2008).

Does phosphorylation enhance or protect against α-syn toxicity?

The accumulation of S129-P α-syn has been associated repeatedly with disease states both in post-mortem human brains (Fujiwara et al., 2002) and in various models of synucleinopathy (Lo Bianco et al., 2002; Neumann et al., 2002). However, the extent to which does S129 phosphorylation and/or the accumulation of α-syn S129-P represent toxic events in the pathogenesis of PD is not yet fully understood. In *Drosophila*, the co-expression of α-syn with the GRK2 homologue, as well as over-expression of α-syn S129D, accelerates neuronal loss compared with WT α-syn or S129A alone (Chen and Feany, 2005), suggesting a toxic effect of α-syn S129-P. On the contrary, in a screening performed in yeast, aimed at identifying modulators of α-syn-induced toxicity, PLK2, which was recently shown to be an efficient kinase and a major contributor to S129 phosphorylation *in vitro* and *in vivo*, was shown to be protective (Mbefo et al., 2010). This finding was confirmed in two other models, *Caenorhabditis elegans* and primary cultures of rat mesencephalic neurons (Gitler et al., 2009). Studies from two independent groups based on the over-expression of the α-syn variants S129D and S129A in rat models support the hypothesis that α-syn S129-P is protective (Azeredo da Silveira et al., 2009; Gorbatyuk et al., 2008). Indeed, over-expression of α-syn S129A in rat *substantia nigra* was reported to be more toxic than when the WT protein or S129D mutants were over-expressed (Azeredo da Silveira et al., 2009; Gorbatyuk et al., 2008). The results from the two rat models support the hypothesis that phosphorylation of α-syn is protective. The following findings are consistent with this hypothesis: (1) PLK2 over-expression protects against α-syn-induced toxicity in yeast, *C. elegans* and primary rat neurons (Gitler et al., 2009) and

(2) disease-associated α-syn mutations (E46K and A30P) reduce the level of α-syn phosphorylation at S129 in cell culture and upon *in vitro* phosphorylation with CK1 and PLK2 (Paleologou and Lashuel, unpublished data). However, a recent study by Hyman and colleagues reported a similar toxicity for the three variants (WT, S129D and S129A) (McFarland et al., 2009). As mentioned above, the results from this study might be biased by co-expression of GFP and α-syn (Baens et al., 2006; Sawada et al., 2010; Ulusoy et al., 2009).

Different mechanisms may be involved in modulating α-syn toxicity in the Drosophila and rodent models of PD

Although the increased aggregation of S129A correlates well with its enhanced toxicity in the rat model (Azeredo da Silveira et al., 2009), the opposite was observed in the fly model, where the increased aggregation of S129A, relative to WT and S129D, correlates with protection against α-syn-induced toxicity (Chen and Feany, 2005) (see Table 2). These observations clearly indicate the involvement of different neuropathologic mechanisms between the two models and suggest that a direct correlation between aggregation and toxicity in the fly and rat models may not be possible, especially if different approaches are used to assess and quantify toxicity and aggregation in the different models. Furthermore, there is no evidence that the aggregation pattern and structure of the aggregates/inclusions formed in the two models are identical. Initial studies by Chen and Feany reported the absence of thioflavin S staining or proteinase K-resistant aggregates in conditions of neuronal loss, where the accumulation of such aggregates occurs and correlates with neuronal loss in the rat model. Furthermore, in the rat model, S129D, which tends to be neuroprotective, formed fewer but larger aggregates than α-syn S129A (Azeredo da Silveira et al., 2009). More recently, Feany and colleagues showed, by western blot, that α-syn-associated toxicity correlates with the accumulation of soluble high molecular weight oligomeric α-syn species. Therefore, a careful analysis of data obtained in *Drosophila* suggests that large aggregates are well handled and do not impair cell survival, while soluble oligomers could be the primary toxic species in flies. Unfortunately, similar biochemical studies have not been carried out in the rat model, so direct comparison of the degree of oligomer formation and accumulation between the two models is not possible. An alternative hypothesis, which could account for the differential toxicity of inclusions between rat and fruit flies, relies on the fact that *Drosophila* is naturally devoid of α-syn, whereas a rat homologue does exist and bears strong sequence similarity to the human α-syn (>95%). Previous studies have shown that mouse and rat α-syn form fibrils more readily than human α-syn and influence the oligomerization and fibrillogenesis of human α-syn. Whether the interactions between rat and human α-syn could explain the differences in toxicity between the rat and the fly models remains unknown. However, this hypothesis can be tested by over-expressing α-syn, S129D or S129A in the substantia nigra of one of the α-syn knockout mouse models.

Despite the differences, some consistent observations emerged from both models

The effect of phosphorylation using the phosphomimicking approach has yielded different results depending on the host organism (fly vs. rat) and the methods employed to assess and/or quantify aggregation and toxicity (see Table 2). Despite the discrepancy in terms of α-syn toxicity in the different models, comparing the results obtained in the *Drosophila* and rat models reveal some consistent observations. (1) In addition to blocking phosphorylation, substitution of serine with alanine enhances the aggregation of α-syn in both animal models and *in vitro*, based on quantitative analysis of α-syn solubility and thioflavin S labelling. (2) In both fly and rat models, S129D exhibits similar aggregation properties as the WT protein (Azeredo da

Silveira et al., 2009; Chen and Feany, 2005). Interestingly, both observations are in complete agreement with *in vitro* biophysical studies using purified recombinant α-syn. These studies showed that α-syn S129A aggregates more rapidly than the WT protein (Paleologou et al., 2008). Although the effects of these two mutations (S → A and S → D) on α-syn aggregation are reproducible in these different models (Table 1), subsequent studies raised some concern about their utility to model the phosphorylation state of the protein *in vivo*. A direct comparison of the structural and aggregation properties of monomeric S129D and S129-P, which was prepared by phosphorylating α-syn with CK1 or PLK2, demonstrated that phosphorylation at S129 increases the conformational flexibility of α-syn and strongly inhibits its fibrillization, whereas monomeric S129D exhibits structural and aggregation properties similar to those of the WT protein (Fig. 4c). Observations of intraneuronal deposits are often a good starting point for assessing aggregation, but further analyses to characterize the solubility of structural properties (using electron microscopy, thioflavin S staining and proteinase K digestion) and oligomerization state (immunoblotting using denaturing and native gel electrophoresis) of α-syn within these inclusions are crucial to determine accurately how phosphorylation affects α-syn aggregation and to elucidate the relationship between α-syn aggregation and toxicity *in vivo*.

What are the molecular mechanisms underlying phosphorylation-dependent α-syn toxicity?

A limited number of studies have attempted to dissect the cellular mechanism associated with phosphorylation-dependent α-syn neurotoxicity *in vivo*. Using electron microscopy, Azeredo da Silveira et al. (2009) sought to examine subcellular abnormalities induced by enhancing or blocking α-syn phosphorylation. These studies revealed the presence of lysosomal bodies and phagosome-like structures trapped within the aggregates, suggesting an attempt by the neurons to degrade the inclusions by macroautophagy (Azeredo da Silveira et al., 2009). In addition, they reported the association of α-syn S129A with membranous structures, endoplasmic reticulum (ER) and Golgi apparatus, mostly in the vicinity of aggregates; these observations suggested an impairment of ER–Golgi trafficking by non-phosphorylated α-syn. On the contrary, in SH-SY5Y cells, over-expression of α-syn S129A or inhibition of CKII decreases the activation of ER stress and caspase 3 cleavage. This study also reported that the increase of S129-P α-syn levels, following rotenone treatment, is associated with activation of these pathways (Sugeno et al., 2008). Therefore, whether ER stress is due to α-syn or related to its phosphorylation state remains to be determined. Azeredo da Silveira et al. (2009) suggested that Golgi–ER impairment results from a dysfunction of the

Table 1. Consequences of mutations at position 129 of α-syn on its aggregation and on cell survival

		AGGREGATION			SURVIVAL		
MODEL		α-synS129A	α-syn	α-synS129D	α-synS129A	α-syn	α-synS129D
Rat	Gorbatyuk *et al.*, 2008	nd	nd	nd	††	†	–
Rat	Azeredo da Silveira *et al.*, 2009	✱✱	✱	✱	††	†	†
Rat	McFarland *et al.*, 2008	✱	✱	✱	†	†	†
Drosophila	Chen and Feany, 2005	✱✱	✱	✱	–	†	††

nd: not determined; ✱: aggregation; †: cell death; –: no cell loss.

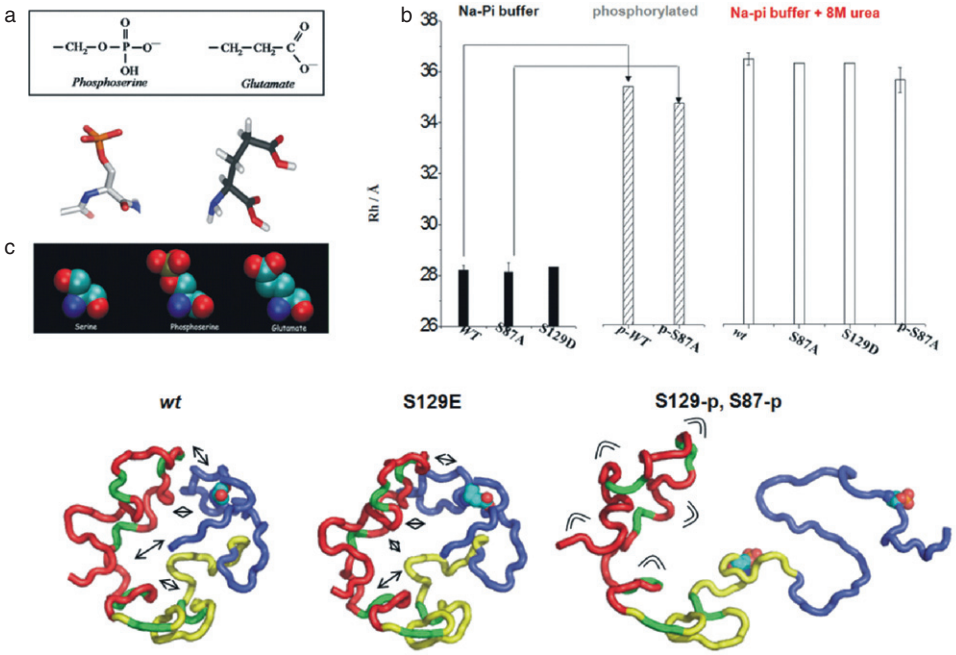

Fig. 4. The phosphomimics S129D/E or S87E do not reproduce the effect of phosphorylation on the structural properties of monomeric α-syn. (a) Structural comparison of the residue serine, phosphoserine and glutamate illustrating the similarities between the two last species. (b) The hydrodynamic radius of α-syn and α-syn S129D in a phosphate buffer is similar, around 28A (black bar), while the radius increased to 35A after phosphorylation of α-syn either at both S87 and S129 (p-WT) or at S129 only (pS87A) (dashed bars). In all cases, the radii rose to 36A in the presence of urea (white bars) corresponding to a true random-coiled state. (c) Illustration of the conformations populated by α-syn, α-syn S129E and α-syn S129-P (S129-p, S87-p) and of the long-range interactions involved (from Paleologou et al., 2008).

microtubule-mediated transport system, as indicated by the disarrayed neurofilament network when α-syn S129A is over-expressed. In cell lines, phosphorylation at S129 induces microtubule retraction (Kragh et al., 2009). Phosphorylated α-syn (S129-P) also co-localizes with activated caspase 9 in different models of synucleinopathy (Fournier et al., 2009; Yamada et al., 2004). It is worth notifying that activated caspase 9 immunostaining is preferentially found in cells positive for S129-P, although this later category of cells represented only a minor subpopulation among neurons accumulating a-syn (Fournier et al., 2009).Over-expression of α-syn S129A or α-syn S129D in rat *substantia nigra* leads to activation of caspase 9 (Azeredo da Silveira et al., 2009), indicating that caspase activation occurs independent of S129 phosphorylation and is likely to be a consequence of α-syn expression.

Novel phosphorylation sites

Recent studies from the Feany's laboratory and our group demonstrated that α-syn *in vivo* is phosphorylated at S87 and Y125. Studies by both groups suggest that modifications at this site correlate with disease and significantly influence the oligomerization and fibrillization of α-syn. The levels of S87-P were increased in brains of TG models of synucleinopathies and human brains from AD, Lewy body disease (LBD) and MSA patients. Using antibodies

against phosphorylated α-syn (S129-P and S87-P), significant levels of immunoreactivity were detected in the membrane in the LBD, MSA and AD cases but not in normal controls. Given that the remaining potential phosphorylation sites in α-syn are highly conserved, it would not be surprising that these residues also undergo phosphorylation and may play a role in modulating the physiologic and/or pathogenic properties of α-syn. Therefore, further studies, using phospho-specific antibodies targeting the different potential phosphorylation sites, are necessary to map all the phosphorylation sites in α-syn and elucidate their role in regulating its structure, aggregation and function(s).

Truncations

Truncated α-syn species are present in the normal brain and aggregate with the full-length α-syn in PD and related disorders

Biochemical characterization of aggregated α-syn from LBs revealed that it comprises predominantly full-length α-syn, in addition to small amounts of various truncated species with apparent molecular masses of 10–15 kDa (Anderson et al., 2006; Baba et al., 1998; Campbell et al., 2001; Crowther et al., 1998; Li et al., 2005; Liu et al., 2005; Okochi et al., 2000; Spillantini et al., 1998). At least five species were detected using mass spectrometry and represent C-terminal truncations (Fig. 5) (Anderson et al., 2006). A comparison between LB-derived and cytosolic α-syn forms showed that some truncated species were exclusively observed in the α-syn derived from LB. The cleavage sites were determined by tryptic digestion and sequencing using liquid chromatography followed by mass spectrometry (LC-MS/MS). Species terminating at D-115 (α-syn-D115), D-119 (α-syn-D119), N-122 (α-syn-N122), Y-133 (α-syn-Y133) and D135 (α-syn-D135) were identified (Fig. 5) (Anderson et al., 2006). Three additional truncated forms of α-syn were also identified in samples from PD, DLB and MSA brain tissues: two C-terminal truncated forms (ending approximately between amino acid residues 102–125 and 83–110, respectively) and a third N- and C-terminal truncated isoform, which were only detectable in aggregated forms of α-syn (Li et al., 2005; Liu et al., 2005; Tofaris et al., 2003). Interestingly, these isoforms are present in healthy and diseased brains, suggesting that α-syn truncation occurs under physiologic conditions. The most striking difference is that PD and DLB extracts contained appreciable amounts of truncated α-syn in SDS- and urea-soluble fractions and a significant level of the N- and C-terminal truncated forms (Li et al., 2005; Liu et al., 2005). All together, these observations show that α-syn truncation occurs under normal conditions and suggest that the truncated α-syn may have a normal physiologic role. These findings also suggest that the C-terminal truncated forms that accumulate selectively in LBs or insoluble fractions aggregate more readily and could act as effective seeds to accelerate the aggregation of the full-length protein.

C-terminal truncations promote the fibrillization of α-syn in vitro

Among the various post-translational modifications identified to date, the C-terminal deletion variants of α-syn consistently exhibit higher fibrillization propensity relative to the WT full-length protein. These findings, combined with the observation obtained for C-terminal deletion variants of α-syn in human brains and brains of TG animal models of PD (Li et al., 2005), led to the hypothesis that proteolytic processing of the C-terminus could be responsible for the initiation of α-syn fibrillogenesis in PD, possibly via a seeding mechanism. Lee and colleagues also reported several naturally occurring C-terminal deletion variants (including amino acids 1–119 or 1–122) in α-syn over-expressing TG mice and proposed that proteolytic processing of the C-terminus of α-syn may play a critical role in the initiation of α-syn aggregation and fibrillogenesis *in vivo*.

The effect of proteolytic cleavage on the aggregation of α-syn has been extensively investigated

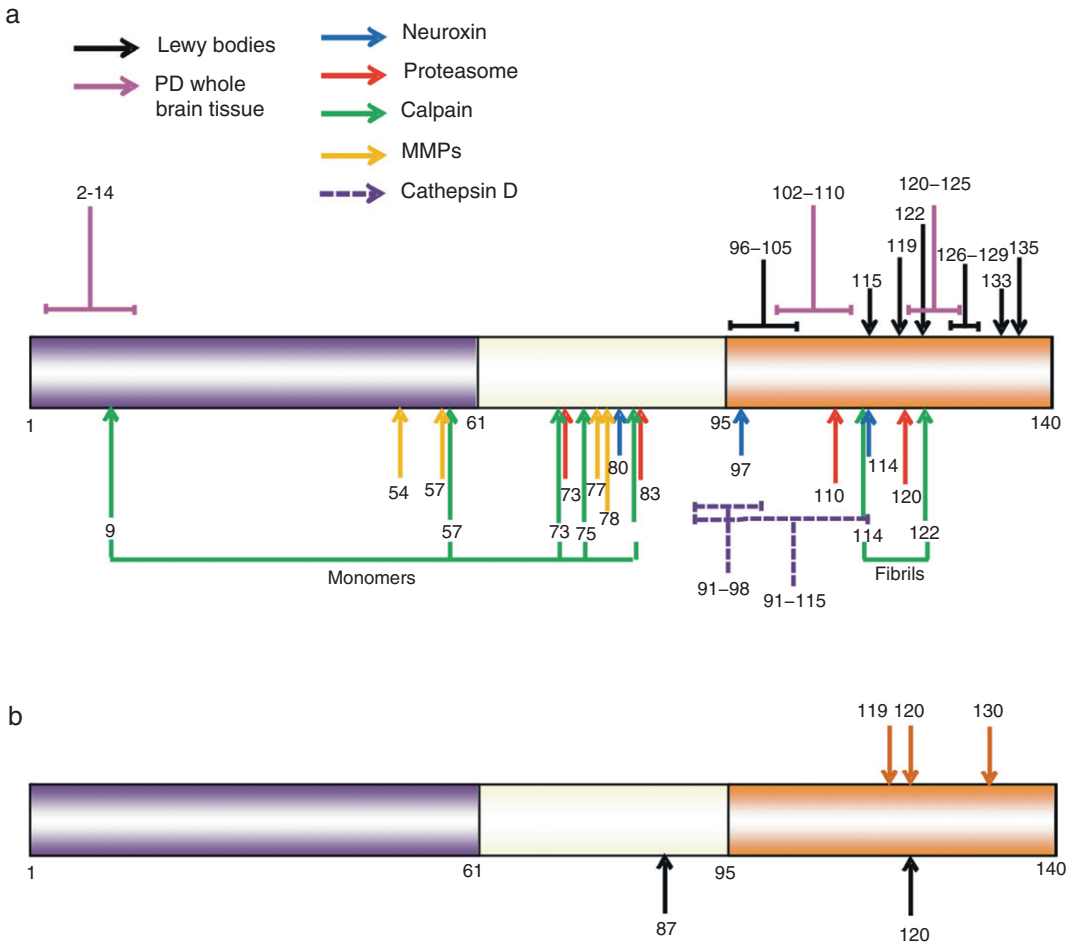

Fig. 5. Truncation of α-syn *in vivo* and *in vitro*. (a) In the upper part of the schema are represented the known sites of truncation of α-syn identified in extracts from LB (black arrows; Anderson et al., 2006) or from brain tissue of PD cases (pink arrows; Li et al., 2005, Liu et al., 2005). Last residue is indicated, or, when not defined, a range is given. Various enzymes able to cleave α-syn monomer or fibrils have been described; their site of digestion is indicated in the lower part of the schema. (b) Representation of the truncated forms studied in TG mice (upper part; Tofaris et al., 2006, Wakamatsu et al., 2008, Daher et al., 2009) and *Drosophila* (lower part; Periquet et al., 2007).

using recombinant proteins and by over-expressing C-terminal deletion variants in cell lines. *In vitro* fibrillization studies have consistently shown that truncation of various segments of the C-terminal residues 110–140 enhances the rate of α-syn aggregation and fibrillogenesis. Some fragments (1–110; 1–120) promote nucleation (Hoyer et al., 2004) and seed the aggregation of full-length α-syn (Li et al., 2005; Murray et al., 2003). Serpell et al. (2000) reported that 1–87 α-syn aggregates more rapidly than 1–120 α-syn and rat α-syn, which aggregates faster than human α-syn (Rochet et al., 2000). Similarly, studies by Murray et al. (2003) showed that the C-terminal

truncated α-syn variants 1–89, 1–102, 1–110, 1–120 and 1–30 aggregated more rapidly than the full-length protein with the 1–110 variant showing the most robust enhancement of α-syn fibril formation. Interestingly, the 1–102 and 1–110 variants, but not 1–120, seed the fibrillization of the full-length protein *in vitro*. Together, the results from these studies combined with data from solid-state NMR (Bertini et al., 2007; Wu et al., 2008), and immunogold labelling of α-syn fibrils (Murray et al., 2003) suggest that fibril formation by α-syn is mediated by its N-terminal domain (~1–90), whereas the C-terminal region remains flexible and may play a role in inhibiting the aggregation of monomeric α-syn (Murray et al., 2003). Subsequent NMR studies provided further evidence in support of this hypothesis and demonstrated that the C-terminal domain participates in long-range interactions with the N-terminal region of α-syn. These interactions shield the hydrophobic regions within the protein and prevents its self-assembly (Bertoncini et al., 2005; Pawar et al., 2005). Several groups have also shown that the C-terminal 20–30 amino acids, which are highly anionic, inhibit fibrillization (Crowther et al., 1998; Kim et al., 2002; Tofaris et al., 2006). This inhibition is mediated by transient interactions between the C-terminal region and the amyloidogenic NAC region. Truncations of residues in this region or a charge-neutralizing effect (e.g. by divalent metals such as Ca^{2+} and Cu^{2+} and polyamines) may account for the fibril acceleration by cations, including polyamines and C-terminal deletion mutants of α-syn (Antony et al., 2003; Cohlberg et al., 2002; Goers et al., 2003) and certain metals (Paik et al., 1999; Uversky et al., 2001; Yamin et al., 2003).

Several enzymes have been implicated in the proteolysis of α-syn

Although the inhibitory activity of the C-terminal sequence suggests that proteolytic cleavage of this region could promote the fibrillization of α-syn *in vivo*, a protease that selectively cleaves this sequence has not been identified. A number of enzymes have been implicated in α-syn cleavage and generation of truncated fragments. Neurosin, a trypsin-like serine protease, was detected in LBs (Iwata et al., 2003; Ogawa et al., 2000). The *in vitro* cleavage of α-syn by neurosin generates one major fragment 1–80, which does not aggregate *in vitro* (Iwata et al., 2003) and three minor ones: 1–97, 1–114 and 1–121 (Kasai et al., 2008) (Fig. 5). Interestingly, the phosphorylated form (S129-P) and the disease-associated mutants (A30P and A53T) are more resistant to proteolysis by neurosin (Kasai et al., 2008).

The intracellular calcium-dependent protease calpain cleaves monomeric WT or mutant (A30P and A53T) α-syn at several sites within the NAC region to yield fragments that inhibit the aggregation of the full-length protein (Mishizen-Eberz et al., 2003, 2005) (Fig. 5). The major generated fragments consist of two N-terminally truncated fragments, 58–140 and 84–140, and four C-terminally truncated forms, 1–57, 1–73, 1–75 and 1–83 (Mishizen-Eberz et al., 2003). Furthermore, using N-terminal sequencing and an antibody against the N-terminal truncated α-syn, Dufty et al. (2007) reported another calpain cleavage site between the residues 9 and 10. In the fibrillar state, calpain-mediated cleavage occurs exclusively within the C-terminal region (residues 114 and 122) (Mishizen-Eberz et al., 2005), probably due to this region remaining flexible and exposed to proteases. Calpain-cleaved α-syn species were found in LB and Lewy neurites in diseased brain and co-localize with activated calpain, suggesting a link between α-syn proteolysis by this enzyme and α-syn aggregation and pathology (Dufty et al., 2007).

Recently, a lysosomal enzyme, cathepsin D was reported to generate two forms of C-terminally truncated α-syn detectable at 12 and 10 kDa, respectively (Sevlever et al., 2008; Takahashi et al., 2007). Using antibodies to different α-syn epitopes, the authors demonstrated that these fragments end approximately between the

residues 91–98 and 91–115, respectively (Sevlever et al., 2008; Takahashi et al., 2007). Sevlever et al. (2008) also demonstrated that cathepsin-generated α-syn fragments are the major component of oligomeric a-syn species formed under oxidative stress. The formation of these correlated with an increase of CKII expression and α-syn phosphorylation in cell culture. These observations suggest a possible relationship among α-syn truncation, phosphorylation and oligomerization when cells are exposed to oxidative stress.

Although ubiquitin-mediated degradation of α-syn has been proposed by several studies, α-syn was reported to undergo proteolytic cleavage by the proteasome, in the absence of ubiquitination (Tofaris et al., 2001). The caspase-like activity of the 20S proteasome generates four α-syn fragments corresponding to 1–73, 1–83, 1–110 and 1–119 (Lewis et al., 2010; Liu et al., 2005). *In vitro* aggregation assay showed that the 1–110 and 1–120 α-syn fragments aggregate more rapidly than the full-length and can seed the aggregation and formation of hybrid protofibrils of truncated and non-truncated α-syn (Lewis et al., 2010; Liu et al., 2005). The PD-linked mutations do not significantly affect the cleavage of α-syn by the proteasome (Lewis et al., 2010); however, the fragments containing the mutation A53T aggregated more rapidly than the truncated WT and full-length A53T and were shown to seed and accelerate the aggregation of the full-length protein (Liu et al., 2005).

The matrix metalloproteases (MMPs, e.g. MMP-1 and MMP-3), a family of zinc-dependent endopeptidases, also cleave α-syn and generate several C-terminally truncated fragments *in vitro* (1–54, 1–57, 1–79 and 1–78) (Levin et al., 2009; Sung et al., 2005). Levin and collaborators showed that α-syn *in vitro* aggregation is increased after a limited proteolysis by MMPs. However, higher MMP concentrations resulted in an inhibition of α-syn aggregation (Levin et al., 2009), most likely due to increased MMP-mediated cleavage within the NAC region, which is essential for α-syn oligomerization and fibril formation.

***Truncation* (*in vivo studies*)**

To explore *in vivo* the effect of C-terminal truncations on the aggregation and toxicity of α-syn, TG flies and mice over-expressing various truncated forms of α-syn have been generated (see Table 2). In *Drosophila*, two different truncations have been investigated: α-syn 1–87, corresponding to a non-natural truncation with the entire acidic C-terminal domain was deleted and a pathological truncation, α-syn 1–120, with the last C-terminal amino acids removed (Periquet et al., 2007). The 1–87 α-syn variant did not aggregate or induce toxicity when expressed in *Drosophila*, although expression at levels similar to that of the WT α-syn could not be reached, which may account for these observations. Conversely, the pan neuronal expression of α-syn 1–120 resulted in the appearance in the *Drosophila* brain, of more abundant oligomers and α-syn-positive, proteinase K-resistant inclusions as compared to the full-length protein expressed at similar levels. The formation of inclusions by α-syn 1–120 was accompanied by a loss of dopaminergic neurons, which occurred slightly faster for the truncated than for the full-length α-syn.

Three different TG mice have been produced, expressing truncated variants of α-syn under the control of the TH or nestin promoter. The first TG mice were generated in a strain devoid of endogenous α-syn; the expression level of α-syn 1–120 was lower than what is expected for the rodent protein (Tofaris et al., 2006). Dopaminergic neurons from the olfactory bulbs and from the *substantia nigra* presented some α-syn-positive fibrillar inclusions (thioflavin S-positive). Despite the fact that expression of 1–120 resulted in the formation of both non-fibrillar and fibrillar α-syn aggregates, no neuronal loss was observed at 12 months of age, although DA and homovanilic acid (HVA) levels were decreased in 3-month-old animals. Wakamatsu et al. (2008) generated TG mice that over-express either the pathogenic mutant A53T or a truncated variant of this mutant in catecholaminergic neurons comprising residues 1–130 (α-syn A53T, 1–130). The expression of

Table 2. Animal models looking at consequences of truncation and phosphorylation on α-syn aggregation and toxicity

PTM studied	Model	Strategy	Aggregation method of assessment	Toxicity method of assessment	Publication(s)
α-syn 1-87	*Drosophila*	Pan neuronal Promoter	**n.d.**	– Number of TH neurons	Periquet et al. (2007)
α-syn 1-120	*Drosophila*	Pan neuronal Promoter	+ IHC (α-syn) PK digestion and HMW by WB	+ Number of TH neurons	Periquet et al. (2007)
	Mouse	TH promoter (α-syn KO background)	+ IHC (α-syn) e-microscopy Thio S	– Number of TH neurons DA decreased at 3 months	Tofaris et al. (2006) Michell et al. (2007)
α-syn 1-119	Mouse	Inducible expression (TH- or nestion-promoter)	– IHC (unpublished)	– Number of TH neurons DA decreased at 12 months with TH promoter	Daher et al. (2009)
α-syn A53T 1-130	Mouse	TH promoter	– IHC (α-syn)	+ number of TH neurons (developmental defect)	Wakamatsu et al. (2008a, 2008b)
α-syn S129-P	Yeast	**α-syn +PLK_2**	**n.d.**	– PKK_2 suppressed α-syn-induced cell death	Gitler et al., (2009)
	C. elegans	**α-syn + PLK_2**	**n.d.**	– PLK_2 suppressed α-syn-induced cell death	Gitler et al. (2009)
	Drosophila	**α-syn+GRK_2** pan neuronal or eye targeting promoter	– PK digestion but HMW in WB	+ Number of TH neurons Ommatidia disruption	Chen and Feany (2005) Chen et al. (2009)
		α-syn S129D pan neuronal or eye targeting promoter	– IHC (α-syn) PK digestion Solubility but HMW in WB	+ number TH neurons ommatidia disruption	
		α-syn S129A pan neuronal or eye targeting	+ IHC (inclusions) PK digestion Solubility	– Number of TH neurons Ommatidia disruption	
	Rat	**α-syn S129D** CBA promoter	+ IHC (α-syn)	– Number of TH neurons	Gorbatyuk et al. (2008)
		α-syn S129A CBA promoter	– IHC (α-syn)	+ Number of TH neurons DA decrease	
	Rat	**α-syn S129D** CMV promoter	– ThioS staining PK digestion e-microscopy	– Number of TH neurons	Azeredo da Silveira et al. (2008)
		α-syn S129A CMV promoter	+ ThioS staining PK digestion e-microscopy	+ Number of TH neurons	

(*Continued*)

Table 2. (*Continued*)

PTM studied	Model	Strategy	Aggregation method of assessment	Toxicity method of assessment	Publication(s)
	Rat	**α-syn S129D** GFP co-expressed CBA promoter	– IHC (α-syn, ubi)	– Number of TH neurons DA level	McFarland et al. (2009)
		α-syn S129A GFP co-expressed CBA promoter	– IHC (α-syn, ubi)	– Number of TH neurons DA level	
α-syn Y125-P, Y133-P, Y136-P	*Drosophila*	**α-syn Y125F, Y133F, Y136F** pan neuronal or eye targeting promoter	**n.d.** but HMW by WB	+ Number of TH neurons ommatidia disruption	Chen et al. (2009)
		α-syn + shark pan neuronal promoter	**n.d.** but reduced HMV by WB	– Number of TH neurons Shark reduced α-syn-induced cell death	

In various model over-expressing α-syn, the appearance of α-syn S129-P deposits correlates with the development of ubiquitin immunoreactivity, proteinase K-resistant and thioflavine S-positive inclusions. n.d.: no data; +: present; -: absent; CBA: CMV beta-actin; CMV: cytomegalovirus; DA: dopamine; Dopac: 3,4-dihydroxyphenylacetic acid; e- microscopy: electron microscopy; GFP: green fluorescent protein; HVA: homovanilic acid; HMW: high molecular weight species; IHC: immunohistochemistry; KO: knockout; PK: proteinase K; TH: tyrosine hydroxylase positive; Thio S: thioflavine S staining; ubi: ubiquitin; WB: western blot.

exogenous α-syn ranged from 1.3- to 1.6-fold the level of the endogenous protein. When the A53T 1–130 variant was over-expressed, the animals displayed a developmental decrease in the number of dopaminergic neurons from the *substantia nigra*, while animals expressing the full-length α-syn A53 did not present any defect. No abnormal proteinaceous accumulations were detected in the affected brain regions, despite the fact that the absence of endogenous α-syn was expected to promote aggregation (Wakamatsu et al., 2008). Finally, Daher et al. (2009) described the consequences of Cre-dependent expression of the C-terminal truncation α-syn 1–119. The authors did not observe any protein deposition by immunohistochemistry or neuronal loss, although dopamine, 3,4-dihydroxyphenylacetic acid and HVA were decreased in the striata of 10-month-old mice; the low protein level likely explains this mild phenotype, as the expression of α-syn 1–119 was 10-fold lower than that of the endogenous protein.

The toxicity of the C-terminal truncated forms of α-syn was also assessed using *in vitro* cell death assays. The addition of hybrid protofibrils, comprising truncated (1–110 or 1–120) and full-length α-syn, significantly increased α-syn-induced toxicity in SH-SY5Y neuroblastoma compared to the addition of monomeric truncated or full-length α-syn separately (Li et al., 2005). These data suggest that truncated α-syn may enhance cell death by promoting the formation of toxic protein aggregates (Liu et al., 2005). Co-over-expression of the C-terminal fragments (1–110 and 1–120) and the full-length protein also enhanced α-syn-induced cell death by increasing the cell vulnerability to oxidative stress (Li et al., 2005; Liu et al., 2005).

The emerging results from the various studies discussed above demonstrate that C-terminal truncated α-syn aggregates more than the full-length protein in *Drosophila*, but the results from TG mice are variable. Indeed, while Tofaris et al. (2006) reported the accumulation of fibril and non-fibrillar 1–120 α-syn inclusions, Wakamatsu et al. (2008) did not report the formation of any deposits of the A53T 1–130 variant, despite the fact that this mutant aggregates more than the full-length protein

in vitro. The discrepancies might be due to the lack of endogenous α-syn in the experiment by Tofaris et al. (2006). *In vitro* studies suggest that the presence of endogenous α-syn is expected to interfere with the fibrillization of human α-syn in the rat and mouse models. Consistent with this hypothesis, fibrillar aggregates were observed in mouse and fly models that do not express human α-syn (Chen and Feany, 2005; Tofaris et al., 2006). C-terminal truncations slightly increase neuronal loss compared to the normal α-syn in *Drosophila*, but not in two out of three TG mouse models. For comparison, previously published studies in mouse, targeting expression of full-length WT or PD-associated α-syn mutants (A30P & A53T) into dopaminergic neurons with the TH-promoter, report no neuronal loss (Matsuoka et al., 2001), or a age-associated neurodegeneration at 19 months in the cases of A30P and A53T (Richfield et al., 2002; Thiruchelvam et al., 2004). Only expression of α-syn 1–130 provoked a strong phenotype, with impaired development of neurons from the *substantia nigra pars compacta*. However this model does not reproduce the progressive cell loss or the associated neuropathology characteristic of synucleinopathies.

Ubiquitination

Ubiquitin-positive inclusions are a neuropathologic hallmark of PD and related disorders

Several neuropathologic studies have shown that a large proportion of LBs present in the *substantia nigra*, the *locus coeruleus*, the hippocampus and the cortex of brain with PD and related disorders, are positive for α-syn (Baba et al., 1998; Spillantini et al., 1997, 1998) and ubiquitin (Gomez-Tortosa et al., 2000; Hasegawa et al., 2002; Kuzuhara et al., 1988; Lowe et al., 1988; Manetto et al., 1988; Sampathu et al., 2003; Tofaris et al., 2003) (Fig. 6). Double-immunostaining using anti-α-syn and anti-ubiquitin antibodies revealed that the core of LBs is immunoreactive for both proteins and is surrounded by a rim of α-syn (Gomez-Tortosa et al., 2000; Mezey et al., 1998) (Fig. 6c). Other intra-cytoplasmic inclusions, larger than LB and without halo, called pale bodies, are positive for α-syn but only occasionally for ubiquitin (Gomez-Tortosa et al., 2000; Tofaris et al., 2003). α-Synuclein and ubiquitin co-staining is also frequent in Lewy neurites (e.g. in the hippocampus) (Gomez-Tortosa et al., 2000). Moreover, α-syn and ubiquitin show extensive co-localization in the glial cytoplasmic inclusions in MSA.

Biochemical analysis of purified LBs and/or LB-derived preparations, using western blotting and mass spectrometry techniques, confirmed that some of the α-syn within LBs is ubiquitinated (Anderson et al., 2006; Hasegawa et al., 2002; Sampathu et al., 2003; Tofaris et al., 2003). Interestingly, the majority of α-syn species found in LB are mono- or di-ubiquitinated. Some tri-ubiquitinated α-syn species have been detected by western blots, but no polyubiquitin chains were detected on α-syn isolated from LB or other brain tissues (Anderson et al., 2006; Hasegawa et al., 2002; Nonaka et al., 2005; Sampathu et al., 2003). These findings suggest that ubiquitination of α-syn may be involved in regulating some of its pathophysiologic properties and imply that ubiquitin-mediated degradation is not likely to be the major physiological mechanism for degrading α-syn. Consistent with this hypothesis, Tofaris et al. (2001) demonstrated that non-ubiqutinated α-syn can be degraded by the proteasome. Furthermore, studies from several groups have implicated other protein clearance pathways in the degradation and turnover of α-syn, including lysosomal and autophagic pathways (Cuervo et al., 2004; Vogiatzi et al., 2008; Webb et al., 2003; Xilouri et al., 2008).

E3 ubiquitin-protein ligases implicated in the ubiquitination of α-syn

Three E3 ubiquitin-protein ligases have been identified to play an important role in the ubiquitination of α-syn *in vitro* and *in vivo*: Parkin, ubiquitin

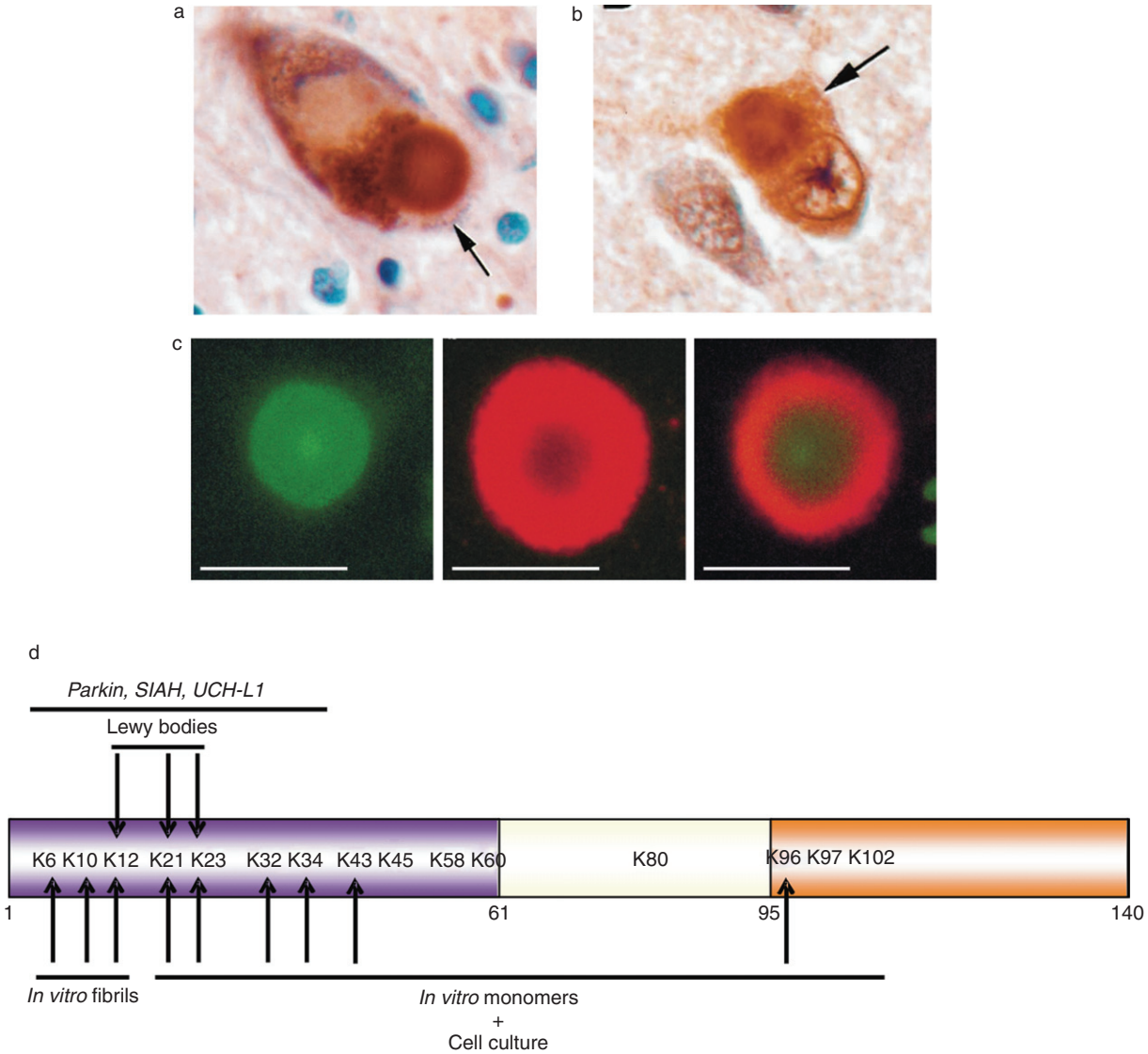

Fig. 6. Ubiquitin, a hallmark of LB, covalently modifies α-syn. Ubiquitin immunostaining in a nigral (a) and a cortical (b) LB (arrow) from synucleopathies diseased brains. (adapted from Chu et al., 2000). (c) Co-immunofluorescent labelling of α-syn and ubiquitin showing their co-localization in a LB from the *substantia nigra* of a PD patient: the staining shows ubiquitin and α-syn in the core of this inclusion and the α-syn alone in the periphery. Scale bar = 10 μm (adpated from Mezey et al., 1998). (d) Schematic representation of α-syn showing the lysine which can be ubiquitynated and the major sites of ubiquitination identified in LB (upper part; Anderson et al., 2006) or in cell culture and *in vitro* studies (lower part; Nonaka et al., 2005, Rott et al., 2008).

carboxy-terminal hydrolase L1 (UCH-L1) and seven in absentia homologue (SIAH) (Lee et al., 2008; Liani et al., 2004; Rott et al., 2008). Interestingly, the genes coding for Parkin and UCH-L1 are linked to familial forms of PD and to PD susceptibility, respectively (Liu et al., 2002; Shimura et al., 2000). Both Parkin and SIAH have been detected in LBs in PD brains (Bandopadhyay et al., 2005; Liani et al., 2004)

Ubiquitination sites

Ubiquitination of α-syn occurs at multiple lysine residues and the sites of ubiqutination depend on the conformational and/or aggregation state of the protein. There are 15 lysine (K) residues in α-syn, the majority of which are distributed within the N-terminal repeat sequences. The remaining residues are K80, K96, K97 and K103 (Fig. 6). Using single-site mutagenesis of lysine residues (K → R) and enzyme (lysyl endopeptidase AP1) digestion followed by peptide mapping using mass spectrometry, different groups have identified the possible lysine residues in α-syn that undergo ubiquitination *in vitro* and *in vivo*. *In vitro* ubiquitination of recombinant α-syn revealed that the monomeric and fibrillar forms of α-syn undergo ubiquitination at distinct lysine residues. *In vitro*, monomeric α-syn undergoes ubiqutination at several lysine residues including K10, K21, K23, K32, K34, K43 and K96, with the major ubiquitin-conjugated sites represented by K21, K23, K32 and K34 (Nonaka et al., 2005; Rott et al., 2008). Mutation of these residues to argnine (R) results in a >90% reduction of ubiquitinated α-syn (Nonaka et al., 2005). Similar results were obtained when these mutants were expressed in cell lines. Ubiquitination of recombinant α-syn using rabbit reticulocytes fraction II or rat brain extracts revealed similar ubiquitination patterns with K21 and K23 being the major ubiquitination sites (Nonaka et al., 2005). Interestingly, ubiquitination of α-syn fibrils prepared from recombinant α-syn, under identical conditions, occurs predominantly at K6, K10 and K12 (Nonaka et al., 2005). This observation may be explained by the fact that in α-syn fibrils, the N-terminal region remains accessible for interaction with ubiquitin-protein ligases. The ubiquitination sites linked to PD and related disorders have been identified form LB-purified α-syn. Using trypsin digestion followed by LC-MS/MS analysis, Anderson et al. (2006) identified residues K12, K21 and K23 as a major sites of ubiquitination in α-syn.

Does ubiquitination of α-syn enhance or prevent its aggregation and toxicity?

While the role of ubiquitination in modulating α-syn aggregation *in vivo* remains poorly understood, it is not essential for inclusion formation *in vivo*, as evidenced by the fact that not all α-syn inclusions in TG mouse models are ubiquitinated (Sampathu et al., 2003; van der Putten et al., 2000). The role of ubiquitination in modulating α-syn aggregation and toxicity was addressed by investigating the *in vitro* ubiquitination of recombinant α-syn, in cell culture and TG animal model. In the animal models, attempts to modulate the level of α-syn ubiquitnation, and thereby attenuate α-syn-induced neurotoxicity, were based on the regulation of Parkin expression or the over-expression of ubiquitin. In *Drosophila* (Haywood and Staveley, 2004; Yang et al., 2003), Parkin over-expression protects against α-syn-induced toxicity without modifying α-syn levels. Recently, Lee and collaborators demonstrated that the over-expression of ubiquitin has no effect *per se* on overall adult retinal or dopaminergic neuronal structure or viability (Lee et al., 2009), but co-expression of ubiquitin and α-syn suppresses α-syn-induced motor impairment (negative geotactic locomotor response) and cell degeneration in *Drosophila* eyes and in the DM1 cluster of dopaminergic neurons. Furthermore, the authors demonstrated that expression of the K48R, and not K63R ubiquitin mutants, suppresses the protective effect of ubiquitin

(Lee et al., 2009), suggesting that the ubiquitin-mediated neuroprotective effect is potentially dependent on the K48 polyubiquitin linkage, a signature targeting proteins for proteasomal degradation (Lim et al., 2005). Together, these data suggest that α-syn ubiquitination could protect against α-syn toxicity in *Drosophila* PD models, by targeting α-syn for proteasomal degradation and enhancing the function of the ubiquitin proteasome system.

In a rat model, over-expressing Parkin protects against α-syn toxicity without affecting α-syn levels (Lo Bianco et al., 2002). Unfortunately, none of the reported studies in fly and rat models characterized the amount of ubiquitinated α-syn or attempted to map the site of modification and/or characterize the ubiquitination pattern of α-syn. In TG mice over-expressing α-syn, the knockout of the *parkin* gene does not modify α-syn quantity (Fournier et al., 2009; Stichel et al., 2007; von Coelln et al., 2006) or levels of ubiquitinated α-syn (von Coelln et al., 2006). However in one of these models, the levels of S129-P α-syn deposits that are ubiquitinated decreased in the absence of Parkin (Fournier et al., 2009). But the same study showed, by *in vitro* ubiquitination, that neither α-syn nor S129-P α-syn is a Parkin substrate, suggesting that the fibrils rather than the monomeric protein might be ubiquitinated by Parkin. The decreased ubiquitination of S129-P α-syn inclusions is associated with a delayed appearance of the neurodegenerative phenotype, indicating a possible toxicity of Parkin-mediated ubiquitination. On the one hand, this report agrees with data describing a lack of synergy between Parkin deficiency and α-syn over-expression (von Coelln et al., 2006) and the previously described protective effect of Parkin expression against α-syn-induced toxicity (Haywood and Staveley, 2004; Lo Bianco et al., 2002; Petrucelli et al., 2002; Yang et al., 2003). On the other hand, these results are in agreement with data showing that ubiquitination of α-syn by SIAH promotes the formation of cytotoxic inclusions (Rott et al., 2008). To conclude, the limited studies reported in the literature indicate that Parkin does not modulate α-syn levels, but it might be involved in its deposition *in vivo*. Whether the ubiquitinated aggregates are toxic or protective is still controversial and requires further investigations.

In cell culture, endogenous SIAH co-localizes with α-syn and is, in part, responsible for its mono and di-ubiquitination in mammalian cell lines and human neuroblastoma, since the suppression of SIAH expression using shRNA completely abolishes α-syn ubiquitination (Lee et al., 2008; Rott et al., 2008). Moreover, the co-expression of α-syn and SIAH enhances α-syn mono and di-ubiquitination (Lee et al., 2008; Rott et al., 2008). Co-expression of α-syn, an E3 ubiquitin-protein ligase (Siah-1) and ubiquitin results in the formation of predominantly mono and di-ubiquitinated α-syn species (Lee et al., 2008). Siah-1- or Siah-2-mediated ubiquitination enhances the aggregation of α-syn and formation of α-syn-positive inclusion in PC12 cells and SH-SY5Y human neuroblastoma (Lee et al., 2008; Rott et al., 2008). *In vitro* ubiquitination of α-syn by SIAH promotes the formation of higher molecular weight α-syn aggregates as determined by western blot analysis (Rott et al., 2008). This observation suggests that ubiquitination by SIAH may enhance α-syn aggregation *in vitro*. These findings were confirmed by electron microscopy studies showing that SIAH-ubiquitinated α-syn forms more aggregates than the non-ubiquitinated form (Rott et al., 2008). Interstingly, the SIAH-ubiquitinated α-syn was reported to enhance the aggregation of non-ubiquitinated α-syn (Rott et al., 2008), suggesting that ubiquitinated α-syn may promote aggregation by seeding the non-ubiquitinated forms. The impact of the phosphorylation and the disease-linked mutations on α-syn ubiquitination *in vitro* is minor (Nonaka et al., 2005; Rott et al., 2008). A30P and 453T mutations reduce the ability of α-syn to bind to SIAH (Lee et al., 2008) but they do not disrupt the efficiency of the ubiquitination (Rott et al., 2008).

The formation of ubiquitinated inclusions associated with cell death after the inhibition of the

proteasome system in neuronal cell culture and *in vivo* has been reported by several groups (McNaught et al., 2002, 2004; Rideout and Stefanis, 2002). Inhibition of the proteasome or impairment of UCH-L1 activity induced neuronal degeneration and an increased intracellular expression of α-syn and ubiquitin (McNaught et al., 2002; Rideout and Stefanis, 2002). After 12 h incubation with proteasome inhibitors, intracellular inclusions immunoreactive for α-syn, ubiquitin and the chaperone Hsp70 were detected in the apoptotic cells (McNaught et al., 2002; Rideout and Stefanis, 2002). Interestingly, McNaught and collaborators highlighted the vulnerability of the dopaminergic versus the GABAergic neurons to proteasome inhibition (McNaught et al., 2002), which they suggested could explain the specific degeneration of the nigral dopaminergic neurons in PD. Similar observations were reported in differentiated and undifferentiated PC12 neuroblastoma after the inhibition of the proteasome (Rideout et al., 2001). Lactacystin-exposed cells showed diffuse ubiquitin immunoreactivity, and in some other cells, focal cytoplasmic accumulation of ubiquitin immunoreactivity was detected (Rideout et al., 2001). It is noteworthy that not all the ubiquitin inclusions were positive for α-syn. This observation suggests that the accumulation of α-syn within the intracellular inclusion depends on the cell type. In a rat model, McNaught showed that the impairment of the proteasome by systemic exposure to proteasome inhibitors induced the development of progressive parkinsonism, neuronal degeneration in different brain regions including the substantia nigra and the formation of LB-like inclusions positive for α-syn and ubiquitin (McNaught et al., 2004). Together, the data generated from the proteasome inhibition models show that the formation of the ubiquitin and α-syn-positive inclusions could be associated with neuronal toxicity *in vivo* and *in vitro*. However, whether the α-syn in these inclusions is ubiquitinated and which residues are implicated remain to be elucidated.

Conclusions

Significant advances have been made towards the identification of post-translational modifications of α-syn (Fig. 7). To date, several post-translational modifications have been identified, some of which appear to be strongly linked to the pathology in PD and related synucleinopathies (e.g. *phosphorylation*). Although the results from the studies discussed above demonstrate that introducing these modifications into α-syn or mutating the site of modification influences the structure and/or aggregation properties of the protein, reconciling the effects of these modifications in the different model systems is complicated by several factors: (1) different mechanisms of toxicity may be involved in modulating α-syn aggregation and toxicity in different model organisms (e.g. *Drosophila* vs. *rodents*); (2) differences in the methods used to assess aggregation and toxicity and lack of standardized rigorous approaches for the analysis of soluble and aggregated forms of α-syn, particularly in cellular and animal models, which have made it difficult to make direct comparisons between the various studies; (3) mutations that are generally used to mimic post-translational modifications do not truly reproduce all aspect of these modifications (e.g. *use of phosphomimics*; see below); (4) the majority of the *in vivo* studies focused primary on the effect of modifications on α-syn aggregation and toxicity (Table 2) and very little on elucidating the molecular mechanisms and cellular pathways altered by these modifications and (5) the cross-talk between different modifications is currently difficult to reproduce and study *in vitro* or *in vivo*. Recent studies suggest that α-syn is phosphorylated at multiple sites, and phosphorylation at tyrosine residues modulates α-syn aggregation and toxicity induced by S129-P, highlighting the importance of investigating the interplay between the different post-translational modifications. These challenges, combined with the lack of good cellular and animal models that reproduce the pathology and neuronal loss in humans, have

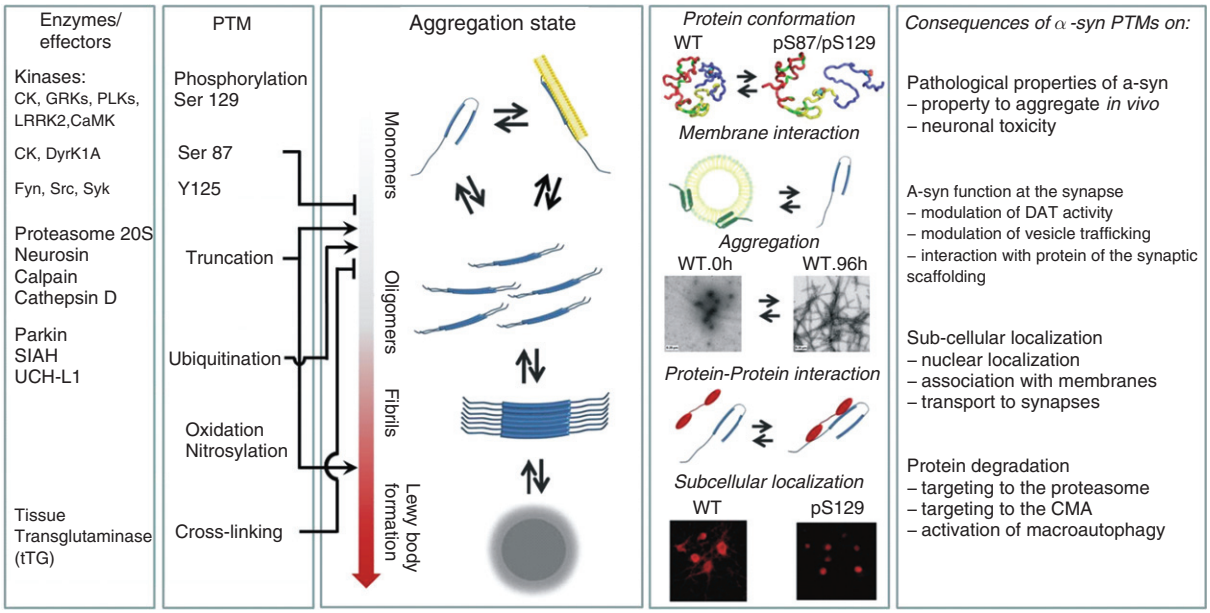

Fig. 7. Effects of α-syn post-translational modifications on its properties.

limited our ability to translate the knowledge gained from the identification of these modifications to an improved understanding of the molecular mechanisms underlying α-syn toxicity and developing new therapies for PD and related disorders. Therefore, several key outstanding questions concerning the role of post-translational modifications in PD remain unanswered (see Box 1).

Looking beyond aggregation

The majority of α-syn modifications were initially identified and implicated in disease pathogenesis solely on the basis of isolation and/or co-localization of modified α-syn within LBs or inclusions from diseased brains or TG animal models. Therefore, studies on these modifications have focused primarily on their role in modulating α-syn aggregation and toxicity rather than their effect on the functional and physiologic properties of the protein, including its stability, subcellular localization, membrane interactions and clearance mechanisms (Fig. 7). Post-translational modifications of proteins represent important molecular switches for regulating protein–protein and protein–ligand interactions and thus protein function in health and disease. The C-terminal region of α-syn has been implicated in the majority of α-syn interactions with proteins (Fernandez et al., 2004; Giasson et al., 2003; Jensen et al., 1999) and metal ions (Brown, 2007; Paik et al., 1999). Therefore, truncation and/or phosphorylation at single or multiple sites is likely to influence α-syn affinity to proteins, metals and other ligands (e.g. dopamine and polyamines) and alter the biochemical and biological processes regulated by its interaction with these molecules. For example, α-syn interacts with the microtubule-associated protein tau and stimulates tau phosphorylation (Jensen et al., 1999) and fibrillogenesis (Frasier et al., 2005) both *in vitro* and *in vivo*. The tau binding site was mapped to the C-terminal region (AA 87–140) of α-syn. Phosphorylation at S129, but not Y125, was also reported to reduce the rate of

Box 1

1. ***What percentage of α-syn is modified in vivo and is there a correlation between the level of modified α-syn and disease progression?*** With the exception of phosphorylation of α-syn at S129, quantitative assessments of the levels of modified α-syn in the soluble and aggregated states of the proteins, relative to total α-syn, are lacking. Studies by Iwatsubo and colleagues suggest that >90% of α-syn within LBs is phosphorylated at S129.

2. ***Do these modifications occur before and/or after α-syn aggregation and LB formation?*** For all reported modifications, it remains unclear whether the presence of modified α-syn in LB and other inclusions reflects their active role in the initiation of α-syn aggregation and development of pathology or a cellular response aimed at clearing unmodified forms of α-syn within LB. Interestingly, all disease-associated modifications, with the exception of phosphorylation at S87, occur at flexible regions that remain accessible in the monomeric, oligomeric and fibrillar states of α-syn. Recent findings that phosphorylation of α-syn at serines (S87 and S129) and tyrosines (Y125, Y133 and Y136), covalent cross-linking by tissue transglutaminase and nitration all inhibit α-syn fibril formation *in vitro* support the notion of these post-translational modifications being a late event rather than a prerequisite for α-syn aggregation.

3. ***What is the effect of each modification on the structure of monomeric α-syn and its binding to membranes, oligomerization and fibrillogenesis?*** The answer to this question lies in our ability to introduce site-specific modifications in α-syn and produce the desired protein in sufficient quantities to perform structural and biophysical studies. These site-specific modifications may not be possible at this stage *in vivo* but are achievable at the protein and single-cell level. Recent advances in chemistry have made it possible to introduce site specifically single or multiple post-translational modifications into proteins. A detailed structural understanding of how these modifications alter the structural properties and dynamics of monomeric α-syn will provide important insight into their role in triggering or inhibiting α-syn aggregation and/or interactions with other proteins and cellular compartments.

4. ***What is the effect of each modification on the stability, degradation and functional properties of α-syn?*** Only an integrative interdisciplinary approach with standardized methods and measures for assessing changes in α-syn properties would bring us closer to addressing the key outstanding mechanistic questions on the role of α-syn post-translational modifications in PD and translate this knowledge into novel therapies.

5. ***Is there cross-talk between the different post-translational modifications in α-syn?*** Thus far, all the modifications of α-syn have been investigated separately, and studies aimed at elucidating the interdependence and relationship between the different modifications are lacking. Different forms of α-syn are modified at multiple sites. Phosphorylation at Y125, ubiquitination or C-terminal truncations co-exist with S129-P, although the sequence of modification remains unknown. More importantly, the residues involved in these modifications are in close proximity to each other and result in a dramatic change in the structure and aggregation of monomeric α-syn. Therefore, it is clear that modifications at these residues will likely have a dramatic effect on α-syn interactions with other enzymes and susceptibility to modifications by these enzymes.

6. ***What are the natural enzymes involved in regulating each of these modification?*** Selective and efficient site-specific modification of α-syn at single or multiple sites *in vivo* are currently not possible because the identity of the natural enzymes (e.g. kinases, phosphatases, E3 ubiquitin ligases, hydrolyases and proteases) responsible for regulating the dynamics of these modifications remain unknown. The existing tools and methods (e.g. the use of phosphomimics or expression of truncated α-syn variants) do not allow for investigating the effect of post-translational modification with spatial and temporal resolution. Several candidate enzymes have been implicated in the phosphorylation and proteolysis of α-syn and are currently being tested and validated as potential therapeutic targets. The identification and validation of enzymes that regulate specific α-syn post-translational modifications will provide more effective means for modulating the level of these modifications and determining their role in disease pathogenesis *in vivo*, using genetic manipulations and/or small molecule inhibitors of these enzymes. Furthermore, this knowledge will allow us to identify the cellular pathways regulating these modifications, which may yield more effective therapeutic targets.
7. ***Can we prevent α-syn aggregation and toxicity in vivo by modulating the type and extent of post-translational modification at specific residue(s)?*** To be able to answer this question, we must use approaches and tools that allow site-specific modulation of post-translational modifications *in vivo*. Although the identification of candidate enzymes involved in regulating these modifications represents a first important step, it is crucial to demonstrate that the effect of modulating the activity of these enzymes is mediated specifically by α-syn, more effective means for modulating the level of these modifications and determining their role in disease pathogenesis *in vivo* using genetic manipulations and/or small molecule inhibitors of these enzymes. Furthermore, this knowledge will allow us to elucidate the cellular pathways in regulating these modifications, which may yield more tractable therapeutic targets and pathways.

α-syn transport (Saha et al., 2004). Together, these findings suggest that reversible phosphorylation or truncations within the C-terminal region (Y125 or S129) may be involved in regulating the association/dissociation with tau and other neuronal proteins (e.g. tau, synphilin (Lee et al., 2004), phospholipase D (PLD) (Payton et al., 2004; Pronin et al., 2000), 14-3-3 (Ostrerova et al., 1999), metals (Liu et al., 2005) and lipids. Phosphorylation within the C-terminal region (S129 or Y125) or the incorporation of phosphorylation mimicking mutations at these residues also reportedly reduces membrane binding and blocks α-syn-mediated inhibition of PLD2 (Okochi et al., 2000; Payton et al., 2004; Pronin et al., 2000), an enzyme involved in the hydrolysis of phosphatidylcholine and vesicular trafficking.

Recent studies by McFarland and colleagues demonstrated that phosphorylation at S129 and Y125 constitute an important switch for regulating α-syn interaction with other proteins. They explored the role of phosphorylation at S129 or Y125 in protein–protein interactions involving α-syn by comparing the protein–protein interactions of phosphorylated and non-phosphorylated C-terminal peptides encompassing these residues (residues 101–140) using pull down assays and mass spectrometry (McFarland et al., 2008). Their studies showed great differences in the set of proteins pulled down by phosphorylated forms of α-syn. The phosphorylated peptides showed preferential interactions with pre-synaptic cytoskeletal proteins and proteins involved in synaptic vesicle endocytosis, subunits of serine/threonine

kinases and phosphatases, whereas the non-phosphorylated peptide interacted preferentially with mitochondrial electron transport chain complexes. Some of the proteins reported to interact with α-syn [e.g. 14-3-3 and microtubule-associated protein 1B (MAP1B)] show a preference to the phosphorylated state (S129-P) of α-syn.

The NAC region in α-syn plays an important role in mediating α-syn fibrillization (Paleologou et al., 2010; Waxman and Giasson, 2008), membrane binding (Lotharius and Brundin, 2002) and interactions with other proteins, such as the enzyme PLD2 (Payton et al., 2004). Recent studies from our laboratory show that S87 phosphorylation alters the conformation of membrane-bound α-syn and decreases its affinity to lipid vesicles, but does not abrogate binding, probably by destabilizing the helical conformation and decreasing the lipid-binding affinity of the protein around the phosphorylation site. Together, these findings underscore the fact that elucidating the role of post-translational modifications in the pathogenesis of α-syn will require a better understanding how these modifications alter the physiologic and functional properties of the protein and how potential cross-talk between the different modifications influences the function of α-syn in health and disease. Only an integrative interdisciplinary approach with standardized methods and measures for assessing changes in α-syn properties would bring us closer to answering the key outstanding mechanistic questions regarding the role of α-syn post-translational modification in PD and translate this knowledge into novel therapies.

Abbreviations

α-syn	α-synuclein
AD	Alzheimer's disease
CKI/II	casein kinase I/II
DLB	dementia with Lewy bodies
ER	endoplasmic reticulum
GFP	green fluorescent protein
GRKs	G protein-coupled receptor kinases
LBD	Lewy body disease
LRRK2	leucine-rich repeat kinase 2
MSA	multiple system atrophy
MSA	multiple system ztrophy
NAC region	non-amyloid component region
PD	Parkinson's disease
PLKs	Polo-like kinases
S129-P	phosphorylated at serine 129
TG	transgenic
WT	wild type

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