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# Visions & Reflections (Minireview)

# Synphilin-1 isoforms in Parkinson's disease: regulation by phosphorylation and ubiquitylation

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**Abstract.** Parkinson's disease (PD) is characterized by the death of dopaminergic neurons and the presence of Lewy bodies in the substantia nigra pars compacta. The mechanisms involved in the death of neurons as well as the role of Lewy bodies in the pathogenesis of the disease are still unclear. Lewy bodies are made of aggregated proteins, in which α-synuclein represents their major component. α-Synuclein interacts with synphilin-1, a protein that is also present in Lewy bodies. When expressed in cells, synphilin-1 forms inclusions together with α-synuclein that resemble Lewy bodies. Synphilin-1 is ubiquitylated by various E3 ubiquitin-ligases, such as SIAH, parkin and dorfin. Ubiquitylation of synphilin-1 by SIAH is essential for its aggregation into inclusions. We recently identified a new synphilin-1 isoform, synphilin-1A, that is toxic to neurons, aggregation-prone and accumulates in detergent-insoluble fractions of brains from α-synucleinopathy patients. Synphilin-1A inclusions recruit both α-synuclein and synphilin-1. Aggregation of synphilin-1 and synphilin-1A seems to be protective to cells. We now discuss several aspects of the neurobiology and pathology of synphilin-1 isoforms, focusing on possible implications for PD.

**Keywords.** Parkinson's disease, synphilin, α-synuclein, ubiquitylation, inclusion body, Lewy body.

## Synphilin-1 isoforms: interaction with several Parkinson's disease proteins

In the last decade, different gene mutations have been shown to cause familial Parkinson's disease (PD) [1]. α-Synuclein was the first gene found to be mutated in families with PD [2–5]. Although mutations in the α-synuclein gene represent a rare cause of PD [1], its robust presence in Lewy bodies [6] and ability to form fibrils [7] place α-synuclein as a major player in the pathogenesis of sporadic PD. Despite the central role of α-synuclein in the disease, the mechanisms by which α-synuclein promotes neurodegeneration and how its aggregation into Lewy bodies takes part in this process remain unclear.

We and others found that α-synuclein interacts *in vivo* with synphilin-1 [8–11]. Synphilin-1 is a protein of 919 amino acids, which contains different domains, such as ankyrin-like repeats, a coiled-coil domain and a putative ATP,GTP-binding domain [8] (Fig. 1a). Synphilin-1 localizes to the presynapse where it binds to synaptic vesicles [12] and may affect dopamine release [13].

Various synphilin-1 regions were found to contribute to the interaction with α-synuclein [9, 12, 14], with both the N-terminus and the central region involved in the interaction [12, 14]. The interaction of synphilin-1 with α-synuclein indicates that synphilin-1 may also play a central role in PD. Supporting this possibility is

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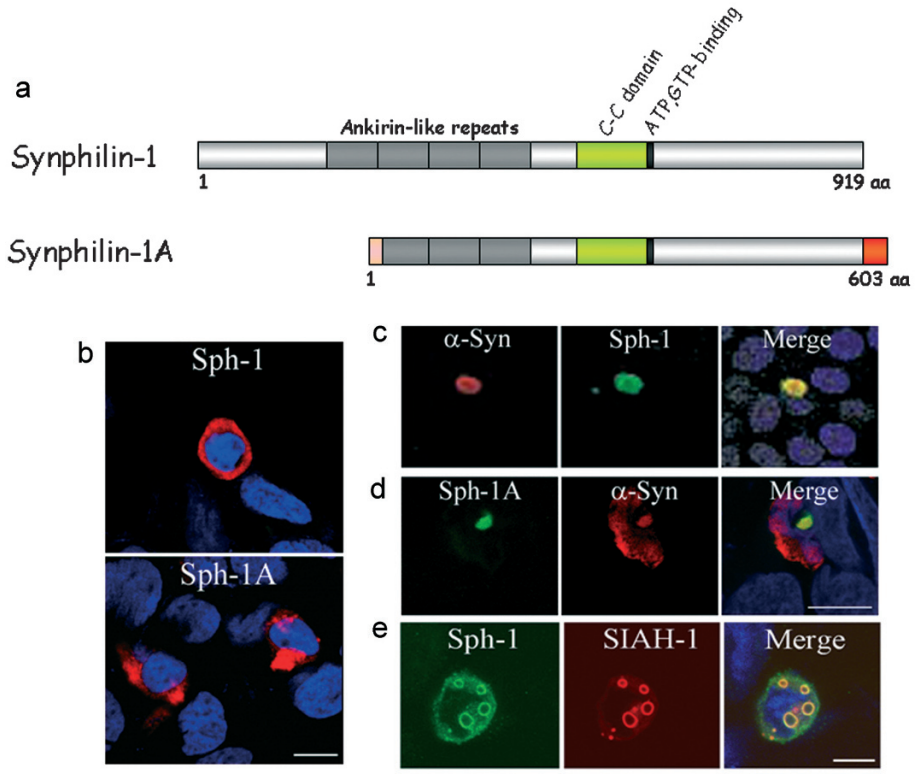


**Figure 1.** (*a*) Schematic representation of synphilin-1 and synphilin-1A open reading frames. The ankyrin-like repeats are shown in dark gray while the coiled-coil domain and ATP,GTP-binding domain are shown in green and black, respectively. Due to differential alternative splicing, synphilin-1A lacks part of the synphilin-1 N-terminus, including the first and part of the second ankyrin-like domains. In addition, it contains additional amino acid stretches at the N-terminus (28 amino acids) and C-terminus (51 amino acids), shown in light pink and red, respectively. (*b*) Immunofluorescence showing the formation of cytosolic inclusions in untreated HEK293 cells overexpressing synphilin-1A but not in cells overexpressing synphilin-1 [30]. (*c*) Immunofluorescence showing the formation of cytosolic inclusions in untreated HEK293 cells overexpressing synphilin-1 and α-synuclein. Reproduced with permission from Macmillan Publishers Ltd: Chung et al., Nat Med 7: 1144–50, 2001 [15]. (*d*) Immunofluorescence showing the formation of cytosolic inclusions in untreated HEK293 cells overexpressing synphilin-1A and α-synuclein [30]. (*e*) Immunofluorescence showing the formation of cytosolic inclusions in HEK293 cells overexpressing synphilin-1 and SIAH-1 in the presence of the proteasome inhibitor lactacystin [38].

the finding that co-expression of synphilin-1 and α-synuclein in cells in culture leads to the formation of Lewy body-like inclusions [8, 11, 15] (Fig. 1c).

Besides interacting with α-synuclein, synphilin-1 interacts with other proteins involved in the pathogenesis of PD (Fig. 2). Synphilin-1 interacts *in vivo* with and is ubiquitylated by parkin [15], an E3 ubiquitin-ligase responsible for the majority of juvenile PD cases [16–18]. Ross and co-workers recently reported that synphilin-1 also interacts with LRRK2 [19, 20], a protein kinase involved in both autosomal dominant and sporadic PD [21]. The interaction of synphilin-1 with different proteins involved in PD (α-synuclein, parkin and LRRK2) suggests that it may assemble these proteins into a multi-protein complex. Synphilin-1 was found to be present in the core of Lewy bodies from PD brains [22]. The observation that synphilin-1 is an integral component of Lewy bodies implies that it may play a role in their formation. Synphilin-1 was also found in Lewy bodies of Diffuse Lewy Body Disease and Multiple System Atrophy [23], suggesting that it may be connected with the aggregation of α-synuclein in different α-synucleinopathies. Synphilin-1 mRNA is increased in the cortex of Diffuse Lewy Body Disease patients [24], indicating a more widespread role of synphilin-1 in α-synucleinopathies.

The presence of synphilin-1 in Lewy bodies has been confirmed in different studies using antibodies generated against different synphilin-1 epitopes [22, 23, 25–27]. The percent of Lewy bodies positive for synphilin-1 varied from 5 to 96% depending on the antibodies used. As pointed out by Bandopadhyay et al. [27], the high variability in the percent of Lewy bodies positive for synphilin-1 in the various studies is probably due to differences in antibody sensitivity and suitability for immunohostochemistry assays. Nevertheless, the presence of synphilin-1 in a variety of α-synucleinopathy lesions [23] and its ability to form Lewy body-like inclusions with α-synuclein in cells [8, 15, 28] suggest that synphilin-1 may nucleate Lewy body formation.

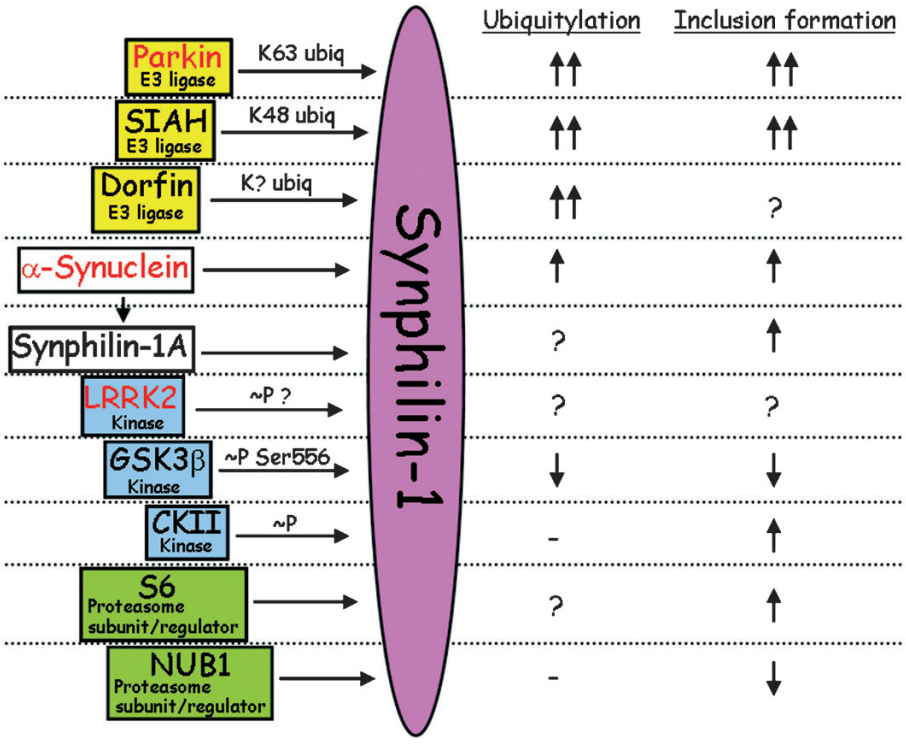


**Figure 2.** Interaction of synphilin-1 isoforms with PD-related proteins, protein kinases and ubiquitin proteasome system-related proteins. Synphilin-1 interacts with different proteins mutated in familial forms of PD, namely α-synuclein, parkin and LRRK2 (in red) [8, 15, 19, 20, 27]. Synphilin-1 co-localizes with α-synuclein into Lewy bodies and forms inclusions that resemble Lewy bodies [8, 22]. Parkin, SIAH and dorfin represent E3 ubiquitin-ligases (yellow boxes) that directly polyubiquitylate synphilin-1 [15, 38, 39]. While ubiquitylation by SIAH occurs via K48-linked polyubiquitin chains and leads to synphilin-1 degradation by the proteasome [38, 40], parkin ubiquitylates synphilin-1 in a nonclassical, proteasomal-independent manner that involves the formation of K63-linked polyubiquitin chains [40]. The type of synphilin-1 polyubiquitylation promoted by dorfin still needs to be determined [39]. Both SIAH and parkin increase the formation of ubiquitylated synphilin-1 inclusions [15, 38, 40, 47, 69]. Ubiquitylation of synphilin-1 is essential for its aggregation into inclusions since a synphilin-1 mutant that is unable to be ubiquitylated by SIAH does not form inclusions in the presence of proteasome inhibitors [38]. α-Synuclein increases synphilin-1 ubiquitylation and inclusion formation, an effect that depends on the levels of α-synuclein phosphorylation at serine 129 [28]. Synphilin-1A interacts with and recruits synphilin-1 to inclusion bodies [30]. The presence of synphilin-1A in Lewy bodies and interaction with both synphilin-1 and α-synuclein suggest that synphilin-1A may work as a core for the formation of Lewy bodies [30]. Synphilin-1 also interacts with various protein kinases (blue boxes). Synphilin-1 interacts with and is phosphorylated by the protein kinases GSK3β and casein kinase II (CKII) [11, 47]. While GSK3β decreases synphilin-1 ubiquitylation and inclusion formation [47], CKII increases the formation of synphilin-1 inclusion bodies by a mechanism independent of ubiquitylation [11, 47]. The ability of LRRK2 to phosphorylate synphilin-1 and modulate its ubiquitylation and inclusion body formation is still unknown. Synphilin-1 interacts with the proteasome subunit/regulators S6 and NUB1 (green boxes). The interaction of synphilin-1 with the proteasome subunit S6 decreases proteasome function and promotes the formation of inclusion bodies containing synphilin-1 [48]. The data are compatible with the possible accumulation of ubiquitylated synphilin-1 leading to inclusion formation. NUB1 seems to accelerate the degradation of synphilin-1 by the proteasome, leading to decreased synphilin-1 inclusion formation [49]. The interaction of synphilin-1 with several proteins involved in the disease suggests that it may assemble the PD proteins into a multi-protein complex.

In agreement with the possible role of synphilin-1 in the formation of Lewy bodies, synphilin-1 was recently shown to co-localize with accumulated α-synuclein in the brain of homozygous A53T α-synuclein mice [29]. A mutation in the synphilin-1 protein (R621C) was identified in two apparently unrelated German PD patients [10]. This further suggests that synphilin-1 may play a role in the disease. Screening different populations for the R621 mutation will be important to confirm the genetic association of synphilin-1 with PD.

We have recently identified a new isoform of synphilin-1, synphilin-1A [30]. Synphilin-1A is an unusual splice variant of synphilin-1. Strikingly, synphilin-1A has a different start codon that leads to a different initial reading frame. It lacks the first 394 amino acids of synphilin-1 and contains an additional 28 amino acids at the N-terminus as well as 51 additional ones at the C-terminus [30] (Fig. 1a). These two new amino acid stretches present in synphilin-1A display no homology to any other protein in the database. Synphilin-1A is an aggregation-prone and neurotoxic protein [30] (Fig. 1b). It interacts with α-synuclein and synphilin-1, and promotes their recruitment to inclusion bodies [30] (Figs 1d and 2). Moreover, synphilin-1A accumulates into detergent-insoluble protein

fractions from brains of α-synucleinopathy patients but not in controls [30].

The synphilin-1A isoform is also present in Lewy bodies of PD and Diffuse Lewy Body Disease patients [30]. Although the exact percent of Lewy bodies positive for synphilin-1A is still not known, the findings that synphilin-1A interacts with both α-synuclein and synphilin-1 and that it is insoluble in α-synucleinopathies brains suggest that synphilin-1A may play a role in the formation of Lewy bodies as well. Moreover, due to its intrinsic neurotoxicity, changes in the strength of interaction between synphilin-1A and α-synuclein may influence not only the formation of Lewy bodies but also the death of dopaminergic neurons.

## Synphilin-1 isoforms, ubiquitylation and inclusion formation

Several findings point to a dysfunction of the ubiquitin-proteasome system in PD. Proteasomal activity in the substantia nigra is decreased in sporadic PD patients [31]. Furthermore, parkin and UCH-L1 proteins, that belong to the ubiquitin-proteasome system, were found mutated in some familial forms of PD [16–18, 32]. Moreover, Lewy bodies can be easily detected by antibodies against ubiquitin [33], indicating that they are heavily ubiquitylated. Accordingly, α-synuclein isolated from Lewy bodies was shown to be monoubiquitylated [34–36].

Synphilin-1 has been shown to be robustly ubiquitylated and degraded by the ubiquitin-proteasome system [37, 38]. Synphilin-1 interacts with and is ubiquitylated by various E3 ubiquitin-ligases: parkin, dorfin, SIAH-1 and SIAH-2 [13, 15, 38, 39] (Fig. 2). While SIAH-1 and SIAH-2 promote the proteasomal degradation of synphilin-1, parkin does not cause its degradation [38, 40].

The inability of the proteasome to degrade SIAH-ubiquitylated synphilin-1 elicits the formation of synphilin-1 inclusions [38]. These inclusions are ubiquitin positive and recruit α-synuclein. Ubiquitylation of synphilin-1 is essential for its inclusion formation, since a synphilin-1 mutant that is unable to be ubiquitylated by SIAH does not undergo aggregation into inclusion bodies [38]. These data, together with the finding that α-synuclein isolated from Lewy bodies is monoubiquitylated, suggest that ubiquitylation might represent a primary event for inclusion formation in PD.

Further supporting the role of ubiquitylation in the formation of Lewy bodies is the finding that most Lewy bodies (79–96%) in Diffuse Lewy Body Disease are positive for ubiquitin [41]. On the other hand, the lack of ubiquitin staining in some Lewy bodies [41, 42] indicates that ubiquitylation is not the major determinant for protein aggregation. However, it is still possible that small amounts of ubiquitylated proteins, below the sensitivity threshold of anti-ubiquitin antibody, could suffice to trigger Lewy body formation.

The findings that SIAH monoubiquitylates α-synuclein and is present in Lewy bodies [38] raise the possibility that SIAH is a novel component of the ubiquitin-proteasome system involved in PD and that its dysregulation may play a role in the pathology. Although no SIAH-1 mutation was identified in screenings of 209 familial and sporadic PD patients [43], additional SIAH-1 and possibly SIAH-2 screenings will be important to ascertain the genetic association of SIAH with PD.

Degradation of ubiquitylated proteins by the proteasome occurs when polyubiquitin chains are linked via lysine 48 (K48) [44]. Some proteins are ubiquitylated with polyubiquitin chains linked via lysine 63 (K63), which are not targeted for degradation but, rather, regulate intracellular signaling [45]. In contrast to the ubiquitylation of synphilin-1 promoted by SIAH, which occurs via K48-linked polyubiquitylation [38, 40], parkin ubiquitylates synphilin-1 via K63-linked polyubiquitin chains [40] (Fig. 2). Dawson and co-workers have shown that synphilin-1/α-synuclein inclusions promoted by parkin contain predominantly polyubiquitin chains linked via K63 [40]. In addition, the formation of K63-linked polyubiquitin augments the number of synphilin-1/α-synuclein inclusions, suggesting that K63-linked ubiquitylation of synphilin-1 by parkin may also be involved in the formation of Lewy bodies. The fact that synphilin-1 is modified by two forms of polyubiquitylation, K48-linked and K63-linked chains, indicates that ubiquitylation may modulate distinct aspects of synphilin-1 function and aggregation. Further understanding of synphilin-1 physiological function will help determine the role of K63-linked polyubiquitylation. It is conceivable that K63-linked polyubiquitylation may represent an initial event that leads to the aggregation of synphilin-1 in the absence of proteasome impairment.

In addition to being ubiquitylated, synphilin-1 seems to interfere with proteasomal function. Overexpression of synphilin-1 in cells in culture decreases the degradation of a reporter protein (GFPu) that is highly degraded by the proteasome, indicating an inhibition of proteasomal function [46, 47]. More recently, Krüger and co-workers demonstrated that synphilin-1 interacts with the regulatory proteasomal protein S6 ATPase (Fig. 2), providing a molecular target for synphilin-1 effects on the proteasome [48]. Co-expression of synphilin-1 and S6 protein inhibits

the proteasomal activity and increases the number of aggresome-like inclusions containing synphilin-1 [48]. The interaction of synphilin-1 and S6 protein is the first evidence that synphilin-1 may directly interfere with the proteasomal function.

Synphilin-1 was recently shown to interact with NUB1 protein [49] (Fig. 2). NUB1 increases the proteasomal degradation of the ubiquitin-like protein NEDD8 [50]. NUB1 also decreases synphilin-1 steady-state levels, indicating that it may help target synphilin-1 to the proteasome [49]. The exact mechanism by which NUB1 accelerates the degradation of synphilin-1 and the E3 ubiquitin-ligases involved in this process have not been investigated.

Similar to synphilin-1, proteasomal inhibitors increase the number of synphilin-1A inclusion bodies in primary neuronal cultures, suggesting that synphilin-1A may be ubiquitylated *in vivo* [30]. These ubiquitylated synphilin-1A inclusions contain α-synuclein, implying that synphilin-1A may also recruit α-synuclein to Lewy bodies. Future studies will be important to establish both the identity of the E3 ubiquitin-ligase(s) that endogenously ubiquitylate synphilin-1A and the cellular implications caused by synphilin-1A ubiquitylation.

Although the extent of α-synuclein degradation by the ubiquitin-proteasome system is still controversial [51–54], ubiquitylation may also directly influence α-synuclein aggregation, as α-synuclein is monoubiquitylated in Lewy bodies. We have previously shown that SIAH monoubiquitylates α-synuclein [38]. Monoubiquitylation of α-synuclein by SIAH increases its aggregation and inclusion formation [our unpublished observation], indicating a role for ubiquitylation *per se* in Lewy body formation. Parkin, UCH-L1 and PINK1 also form intracellular inclusions when ubiquitylated [55–57]. Thus, ubiquitylation of different proteins involved in PD may also contribute to the formation of Lewy bodies.

Proteasomal dysfunction and accumulation of ubiquitylated proteins also occur in other neurodegenerative diseases [58, 59]. In Huntington's disease (HD), mutant huntingtin forms inclusions when ubiquitylated [60]. Likewise, tau accumulates into aggregates caused by proteasome inhibitors in cellular models of Alzheimer's disease (AD) [61]. Moreover, similar to Lewy bodies, both HD intranuclear inclusions and AD tangles are heavily ubiquitylated [62, 63]. Together, these data suggest that ubiquitylation may be involved in the formation of protein aggregates in several neurodegenerative diseases.

## Modulation of synphilin-1 ubiquitylation

Phosphorylation is a post-translational modification known to modulate the ubiquitylation of a variety of proteins [44]. α-Synuclein purified from Lewy bodies is both phosphorylated and monoubiquitylated [35, 36], suggesting a connection between these two events in PD. In support, most proteins linked to familial PD belong to the ubiquitin-proteasome system or work as protein kinases [1].

α-Synuclein is phosphorylated *in vivo* at serine 129 [64]. This phosphorylated form of α-synuclein displays an increased ability to form fibrils *in vitro* and is enriched in α-synucleinopathy lesions [65]. Ross and co-workers established that a phosphorylation-deficient α-synuclein mutant (S129A) decreases the ubiquitylation of synphilin-1 and the formation of intracellular inclusions [28], suggesting that phosphorylation of α-synuclein at serine 129 modulates synphilin-1 ubiquitylation and aggregation by a still unclear mechanism.

Several kinases phosphorylate synphilin-1 *in vivo* and alter its aggregatory activity. Mouradian and co-workers found that casein kinase II phosphorylates synphilin-1 *in vivo* [11]. Phosphorylation of synphilin-1 by casein kinase II decreases its interaction with α-synuclein and inclusion formation [11], but does not change synphilin-1 ubiquitylation [47] (Fig. 2). These results imply that the formation of synphilin-1 inclusions may depend on other factors in addition to ubiquitylation, such as phosphorylation and strength of interaction with α-synuclein.

There are several reports showing that protein kinases, such as GSK3β and Cdk5, modulate the death of dopaminergic neurons in pharmacological models of PD [66, 67]. We have recently demonstrated that endogenous synphilin-1 is phosphorylated by GSK3β [47] and that this phosphorylation decreases synphilin-1 ubiquitylation and inclusion formation [47] (Fig. 2). When the phosphorylation of synphilin-1 by GSK3β at serine 556 was prevented by the use of siRNA to GSK3β, a significant increase in synphilin-1 inclusion formation was observed [47]. These results further strengthen the notion that ubiquitylation of synphilin-1 is important for its aggregation into inclusions in a phosphorylation-dependent manner.

Parkin decreases synphilin-1 and α-synuclein toxicities and promotes the formation of inclusions containing both proteins [28, 68], supporting the idea that synphilin-1 inclusions are not toxic to cells. The ability of parkin to increase the formation of synphilin-1/α-synuclein inclusions has been ascribed to its ability to ubiquitylate synphilin-1, since parkin ubiquitylates synphilin-1 but not α-synuclein [15, 28].

We recently found that parkin is phosphorylated *in vivo* by Cdk5 at serine 131, located in its linker region [69]. Phosphorylation of parkin by Cdk5 decreases its autoubiquitylation and ability to ubiquitylate synphilin-1 [69]. Thus, a phosphorylation-deficient parkin mutant (S131A) was more efficient in ubiquitylating synphilin-1 and promoted higher inclusion body formation [69]. These data confirm the role of parkin in the formation of synphilin-1 inclusions.

Taken together, these findings imply that different protein kinases modulate directly and indirectly the levels of synphilin-1 ubiquitylation and may contribute to Lewy body formation.

## Cellular effects of synphilin-containing inclusions

Concentrated efforts have been spent to determine the role of Lewy bodies in the viability of neurons. Since a pharmacological or transgenic mouse model that recapitulates all the pathological events of PD is still not available, it has not been possible to ascertain definitively the role of Lewy bodies. Different cellular models of PD suggest a protective role for intracellular inclusion bodies against cell death [38, 70, 71]. The possibility that PD inclusions are neuroprotective is in accordance with cell models of other neurodegenerative diseases, such as HD, where inclusion bodies were not correlated with toxicity promoted by mutated soluble huntingtin [72].

In PD, the toxicity of α-synuclein has been attributed to its soluble rather than fibrillar forms [73, 74]. For example, α-synuclein protofibrils were shown to cause membrane permeabilization [75]. In addition, dopamine prevents the conversion of protofibrils to fibrils, implying that the death of dopaminergic neurons in the disease may be triggered by accumulation of intracellular α-synuclein protofibrils [76].

Synphilin-1 strongly increases α-synuclein toxicity in cultured cells [68, 77]. Interestingly, cells that exhibit synphilin-1/α-synuclein inclusion bodies are relatively spared, implying that the inclusions are cytoprotective [70]. Likewise, the neurotoxicity of synphilin-1A is alleviated by its aggregation into inclusions, indicating an inverse correlation between inclusion formation and cell toxicity in PD [30]. Conceivably, inclusions may prevent α-synuclein and synphilin-1 isoform toxicity by sequestering them in an insoluble structure. Electron microscopy analysis revealed that synphilin-1/α-synuclein inclusions contain fibrils as well as amorphous and granular aggregates, resembling Lewy bodies [28]. The amount of fibrils present in the synphilin-1/α-synuclein inclusions is less than that observed in Lewy bodies *in vivo* [28], suggesting that α-synuclein fibrils may take longer to accumulate.

A synphilin-1/α-synuclein cell model may be useful to screen for therapeutic compounds for PD that affect inclusion formation [78]. Recently, Kazantsev and coworkers demonstrated that the drugs B2 (5-[4-(4-chlorobenzoyl)-1-piperazinyl]-8-nitroquinoline) and a sirtuin 2 inhibitor decrease α-synuclein-mediated toxicity while increasing the number of synphilin-1/α-synuclein inclusions [79, 80].

The investigation as to how additional PD-related proteins affect synphilin-1 isoform toxicity and inclusions will be key to understanding the pathological role of synphilin-1 proteins. Understanding how different cellular stresses, such as oxidative and endoplasmic reticulum stress, influence the formation of synphilin-1 isoforms inclusions will be valuable for understanding the formation of Lewy bodies as well.

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