Parkin ubiquitinates the α-synuclein–interacting protein, synphilin-1: implications for Lewy-body formation in Parkinson disease

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Parkinson disease is a common neurodegenerative disorder characterized by the loss of dopaminergic neurons and the presence of intracytoplasmic-ubiquitinated inclusions (Lewy bodies). Mutations in α-synuclein (A53T, A30P) and parkin cause familial Parkinson disease. Both these proteins are found in Lewy bodies. The absence of Lewy bodies in patients with parkin mutations suggests that parkin might be required for the formation of Lewy bodies. Here we show that parkin interacts with and ubiquitinates the α-synuclein–interacting protein, synphilin-1. Co-expression of α-synuclein, synphilin-1 and parkin result in the formation of Lewy-body–like ubiquitin-positive cytosolic inclusions. We further show that familial-linked mutations in parkin disrupt the ubiquitination of synphilin-1 and the formation of the ubiquitin-positive inclusions. These results provide a molecular basis for the ubiquitination of Lewy-body–associated proteins and link parkin and α-synuclein in a common pathogenic mechanism through their interaction with synphilin-1.

Parkinson disease (PD) is a relatively common neurodegenerative disorder, which is characterized by the loss of midbrain dopamine (DA) neurons[1,2] and the presence of Lewy bodies, proteinaceous cytoplasmic inclusions that are abundantly enriched in ubiquitin[3,4]. The identification of genes linked to familial PD holds tremendous promise for understanding the molecular pathogenesis of PD. Mutations in α-synuclein (A53T, A30P) cause autosomal dominant PD in isolated families[5,6]. Although familial-associated mutations in α-synuclein are a rare cause of PD, the observation that wild-type α-synuclein is a major component of Lewy bodies indicates that derangements in the function and/or disposition of α-synuclein may have prominent roles in the pathogenesis of sporadic PD (refs. 7–12). Targeted disruption of α-synuclein in mice does not yield a parkinsonian phenotype, but instead leads to abnormalities in striatal dopaminergic neurotransmission indicating that α-synuclein has important physiological roles in regulating dopamine activity[13]. Supporting the importance of α-synuclein in the pathogenesis of PD is the observation that over-expression of α-synuclein causes dopaminergic dysfunction and replicates many of the features of PD in transgenic flies and mice[14–16].

Although Lewy bodies are a prominent pathologic feature of PD, the underlying molecular pathogenic mechanisms accounting for Lewy-body formation, including the ubiquitination of proteins contained within, are poorly understood[3,4]. Short-lived and 'abnormal' proteins are targeted by ubiquitination for removal by the 26S proteasome[17,18]. The first step in ubiquitination is the ATP-dependent activation of ubiquitin by the ubiquitin-activating enzyme E1, followed by the transfer of ubiquitin to an ubiquitin-conjugating enzyme, E2. The final step requires an E3 ubiquitin-protein ligase, which facilitates the transfer of ubiquitin to the target protein. Derangements in the ubiquitin-proteasomal degradation system may have prominent roles in the pathogenesis of PD (refs. 14,19). A mutation (I93M) in ubiquitin carboxy-hydrolase L1 (UCHL1) may cause autosomal dominant PD in a small German kindred[20] and mutations in parkin cause autosomal-recessive juvenile-onset PD (AR-PD)[21]. Parkin appears to be in Lewy bodies[22] and affinity-purified antibodies against parkin recognize subsets of brainstem and cortical Lewy bodies in PD and dementia with Lewy-body brains[23]. The absence of Lewy bodies in patients with parkin mutations indicates that parkin may be required for the formation of Lewy bodies[14,19,24]. Recent studies indicate that parkin functions as an E3 ubiquitin-protein ligase and familial-linked mutations in parkin disrupt the E3 ubiquitin-protein ligase activity[24–26]. Part of parkin's function in the ubiquitination pathway may be to target misfolded proteins derived from the endoplasmic reticulum for degradation, as parkin protects against neurotoxicity induced by unfolded protein stress[26,27]. Here we report that parkin interacts with and ubiquitinates the α-synuclein–interacting protein, synphilin-1 (refs. 28,29), which is enriched in Lewy bodies in PD (ref. 30). Ubiquitin-positive Lewy-body–like cytosolic inclusions were formed when α-synuclein, synphilin-1 and wild-type parkin are co-expressed, whereas familial-linked mutations in parkin disrupted the ubiquitination of synphilin-1 and the formation of the ubiquitin-positive inclusions. Thus, parkin may have a prominent role in the ubiquitination of Lewy-body–associated proteins which are implicated in the pathogenesis of PD.

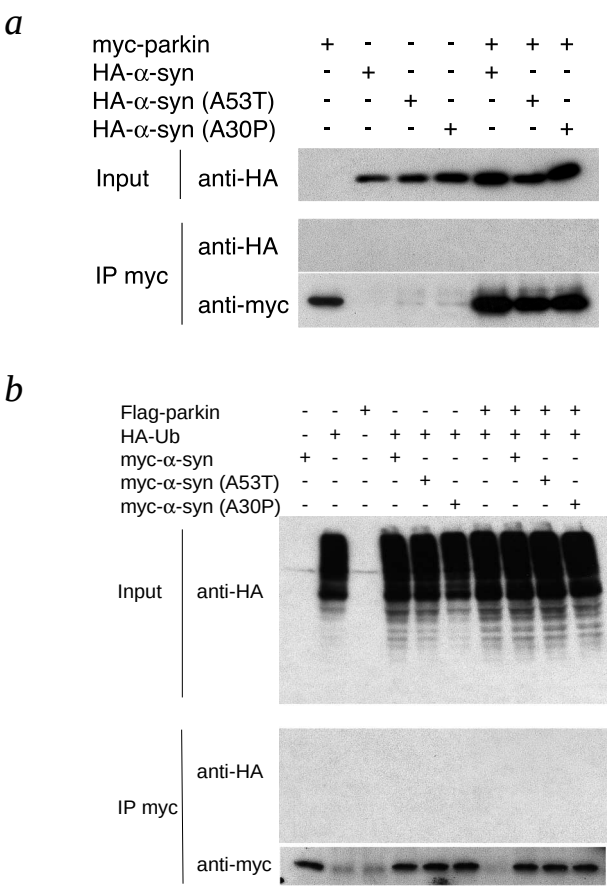


Fig. 1 Parkin fails to interact with and ubiquitinate non-glycosylated α-synuclein. ***a***, Lysates prepared from HEK293 cells transfected with myc-tagged parkin and wild-type HA-tagged α-synuclein or HA-tagged A53T α-synuclein or HA-tagged A30P α-synuclein, were subjected to IP with anti-myc followed by anti-HA immunoblotting. The blot was also stripped and reprobed with the anti-myc (lower panel) to illustrate that relatively equivalent amounts of parkin were expressed. ***b***, Lysates prepared from HEK293 cells transfected with Flag-tagged parkin, HA-tagged ubiquitin, and wild-type myc-tagged α-synuclein, myc-tagged A53T α-synuclein or myc-tagged A30P α-synuclein were subjected to IP with anti-myc followed by anti-HA immunoblotting to detect the possible ubiquitination of α-synuclein by parkin. The blot was also stripped and reprobed with the anti-myc (lower panel) to illustrate that relatively equivalent amounts of α-synuclein were expressed. Both experiments were replicated 3 times with similar results.

Parkin does not interact with non-glycosylated α-synuclein

To determine whether parkin could interact with the major component of the Lewy body, α-synuclein[7–12] and facilitate its ubiquitination, we did co-transfection experiments with wild-type α-synuclein, A53T α-synuclein or A30P α-synuclein, and parkin constructs followed by co-immunoprecipitation using both N-terminal HA-tagged α-synuclein and myc-tagged parkin constructs (tagged parkin constructs are fully functional[24–26]). Parkin failed to interact with α-synuclein (Fig. 1*a*). Parkin was recently shown to interact with a novel glycosylated form of α-synuclein (αSp22) in human brain[23]. We were also unable to detect an interaction with glycosylated higher-molecular-weight species of α-synuclein (Fig. 1*a*). Because glycosylation of α-synuclein may be unique to the nervous system, we did identical experiments in SH-SY5Y neuroblastoma cells and also failed to detect an interaction with α-synuclein and αSp22 (data not shown).

Previous studies suggest that α-synuclein is damaged by oxidative stress[31–35], which might target α-synuclein for degradation. Thus, we investigated whether oxidative damage of α-synuclein could predispose it to ubiquitination. We treated cultures with the peroxynitrite donor (5 mM SIN-1) and monitored potential interactions of α-synuclein with parkin. Oxidatively stressed α-synuclein also did not interact with parkin (data not shown). We next monitored whether parkin could ubiquitinate α-synuclein. In this assay, we transfected cells with Flag-tagged parkin, myc-tagged wild-type α-synuclein, A53T α-synuclein or A30P α-synuclein, and HA-tagged ubiquitin. Two days later, we immunoprecipitated cell lysates and probed the precipitates with an antibody against HA to monitor any possible ubiquitination of α-synuclein. Immunoprecipitated wild-type α-synuclein, A53T α-synuclein or A30P α-synuclein were not ubiquitinated (Fig. 1*b*).

Because α-synuclein is a small, highly conserved protein in which single amino-acid substitutions (A53T and A30P) can lead to different conformations, we investigated whether the N-terminal–tagged α-synuclein could interfere with its potential glycosylation and its interaction and ubiquitination by parkin by performing identical experiments with non-tagged α-synuclein. We found that parkin did not interact with or ubiquitinate α-synuclein in this setting, nor did we detect an interaction or modification of αSp22 (data not shown). Furthermore, parkin failed to ubiquitinate or interact with α-

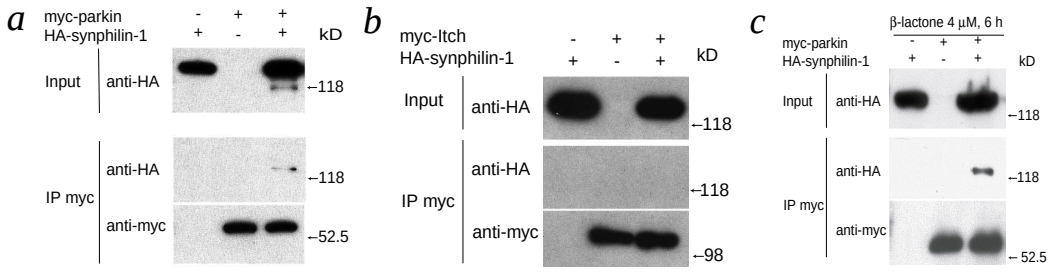


Fig. 2 Parkin interacts with the α-synuclein–interacting protein, synphilin-1. ***a***, Lysates prepared from HEK293 cells transfected with myc-tagged parkin and HA-tagged synphillin-1 were subjected to IP with anti-myc followed by anti-HA immunoblotting. The blot was also stripped and reprobed with anti-myc (lower panel) to illustrate that relatively equivalent amounts of parkin were expressed. ***b***, Lysates prepared from HEK293 cells transfected with myc-tagged Itch and HA-tagged synphilin-1 were subjected to IP with anti-myc followed by anti-HA immunoblotting. The blot was also stripped and reprobed with the anti-myc (lower panel) to illustrate that relatively equivalent amounts of parkin were expressed. ***c***, Same experiment as in *a*, but with the addition of the proteasome inhibitor β-lactone (4 μM) 6 h before the collection of cells for immunoprecipitation. The blot was also stripped and reprobed with the anti-myc antibody (lower panel) to illustrate that relatively equivalent amounts of parkin were expressed. All 3 experiments were replicated 3 times with similar results.

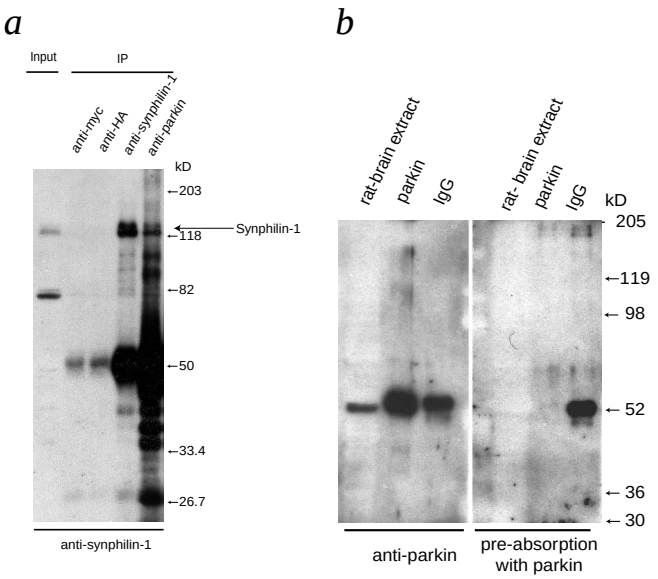


Fig. 3 Co-immunoprecipitation of synphilin-1 and parkin in rat brain extract. ***a***, Rat brain homogenate was subjected to IP with anti-myc, anti-HA, anti–synphilin-1 or anti-parkin followed by anti–synphilin-1 immunoblotting. Synphilin-1 migrates near the 118 kd marker. This experiment was replicated 3 times with similar results. ***b***, Preabsorption of anti-parkin with purified parkin protein. Anti-parkin was pre-absorbed with purified parkin protein and then tested for its specificity to parkin in rat brain extract and purified parkin protein. The last lane was loaded with IgG as a control for western blotting and shows the same migration level of parkin and IgG in the SDS–PAGE.

synuclein and we did not detect αSp22 in both HEK293 cells and SH-SY5Y neuroblastoma cells (data not shown). We may have failed to detect ubiquitination of α-synuclein and αSp22 by parkin because it might be rapidly degraded. However, even in the presence of the proteasome inhibitor, β-lactone, parkin failed to interact with or ubiquitinate α-synuclein, and we were unable to detect αSp22 (data not shown). Together these results indicate that parkin does not interact with, nor does it ubiquitinate, non-glycosylated α-synuclein, consistent with the observations of Shimura *et al*.[23] Furthermore, parkin interaction with αSp22 seems to be unique to human brain[23], as we were unable to detect αSp22 in both HEK293 cells and the neuronal cell line SH-SY5Y.

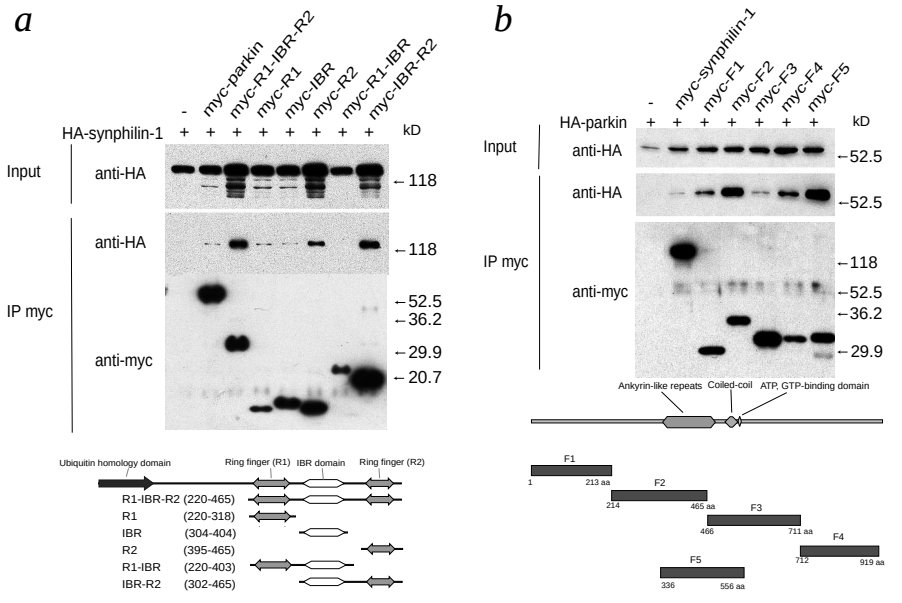
Parkin interacts with synphilin-1

Because parkin did not interact with or ubiquitinate non-glycosylated α-synuclein, the major α-synuclein species in brain[23], we investigated whether other proteins enriched in the Lewy body could interact with and be ubiquitinated by parkin. We chose to study the α-synuclein interacting protein, synphilin-1 (refs. 28,29). Although α-synuclein has over a dozen potential interactors whose function in PD is not known[36], we decided to focus our attention on synphilin-1 as it is enriched in Lewy bodies[30] and because it promotes the formation of Lewy-body–like inclusions[29]. We conducted co-transfection experiments with myc-tagged parkin and HA-tagged synphilin-1 followed by co-immunoprecipitation. Synphilin-1 co-immunoprecipitates with parkin (Fig. 2*a*), but did not interact with Itch, a newly identified E3 ligase (J.D. Wood and C.A. Ross, unpublished data) (Fig. 2*b*), confirming that the interaction between parkin and synphilin-1 is specific. To explore the possible role of the proteasomal pathway on the interaction of parkin and synphilin-1, we performed the same experiment following administration of the proteasome inhibitor, β-lactone, before the immunoprecipitation. β-lactone significantly increased the amount of synphilin-1 co-immunoprecipitated with parkin (Fig. 2*c*), but did not increase the steady-state level of synphilin-1, suggesting that the increase in synphilin-1 is due to the increase in parkin (compare Fig. 2*c* with *a*).

To determine whether synphilin-1 and parkin interact *in vivo*, we performed co-immunoprecipitation using an antibody against parkin in rat-brain homogenate followed by western-blot analysis with an antibody against synphilin-1 (Fig. 3). Synphilin-1 co-immunoprecipitated with parkin *in vivo* (Fig. 3), thus supporting the physiologic relevance of the interaction.

Parkin contains two ring fingers separated by an in-between ring finger (IBR) domain, which facilitates its interaction with

Fig. 4 Synphilin-1 interacts preferentially with the C-terminal R2 ring-finger domain of parkin and parkin interacts with the ankyrin domain of synphilin-1. ***a***, Lysates prepared from HEK293 cells transfected with HA-tagged synphilin-1 and various myc-tagged parkin domain constructs were subjected to IP with anti-myc followed by anti-HA immunoblotting. The blot was also stripped and reprobed with the anti-myc (lower panel) to illustrate the relative amounts of the parkin constructs that were expressed. Putative functional domains of parkin used in the mapping experiments are shown. ***b***, Lysates prepared from HEK293 cells transfected with HA-tagged parkin and various myc-tagged fragments of synphilin-1 were subjected to IP with anti-myc antibody followed by anti-HA immunoblotting. The blot was also stripped and reprobed with the anti-myc antibody (lower panel) to illustrate the relative amounts of the synphilin-1 constructs that were expressed. A schematic representation of the different fragments of synphilin-1 used in the mapping experiments is shown at the bottom of the figure. Both experiments were replicated 3 times with similar results.



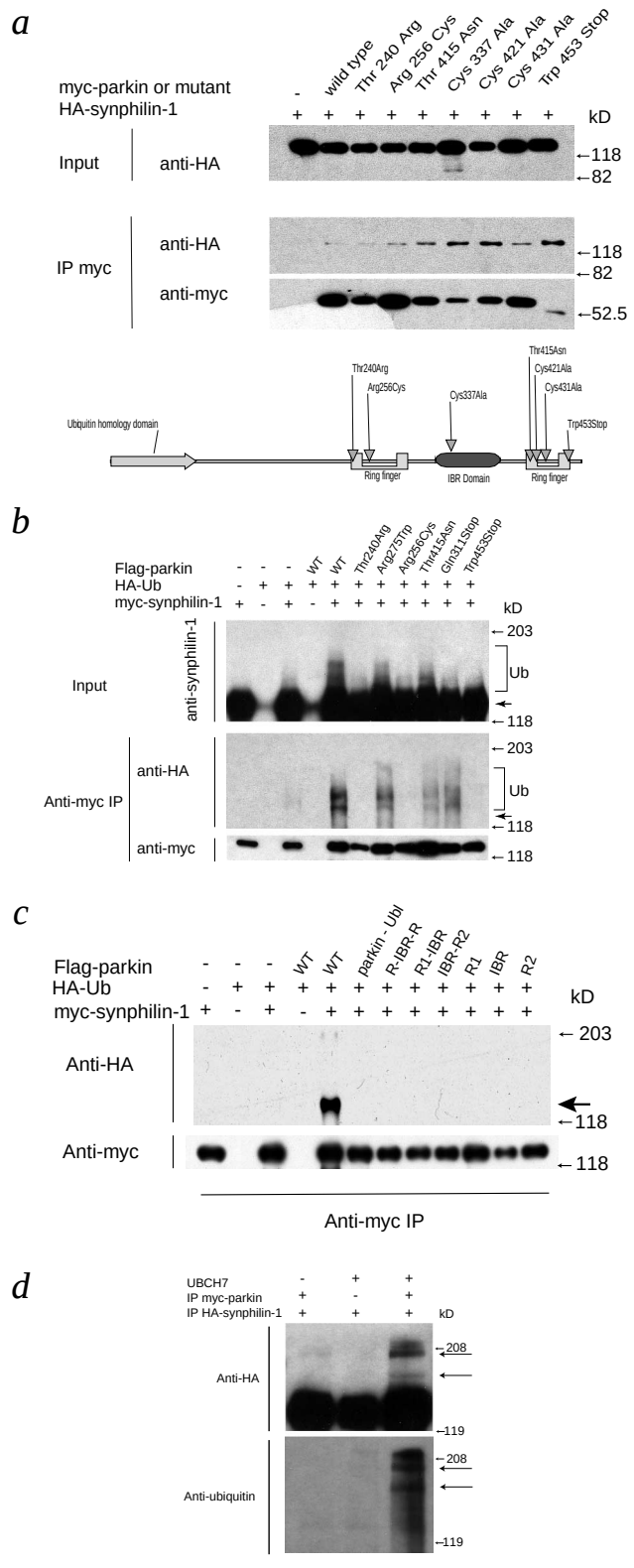


Fig. 5 Familial-associated mutations in parkin alter the interaction and ubiquitination of synphilin-1. ***a***, Lysates prepared from HEK293 cells transfected with HA-tagged synphilin-1 and various myc-tagged parkin mutant constructs were subjected to IP with anti-myc followed by anti-HA immunoblotting. The blot was also stripped and reprobed with anti-myc (lower panel) to illustrate the relative amounts of parkin that were expressed. A schematic representation of the location of different mutations in parkin used in the experiments is shown at the bottom of the panel. ***b***, Lysates prepared from HEK293 cells transfected with Flag-tagged parkin or various parkin mutants, HA-tagged ubiquitin and myc-tagged synphilin-1 were subjected to IP with anti-myc followed by anti-HA immunoblotting. Lysates were also probed with an anti–synphilin-1 antibody to demonstrate synphilin-1 laddering. The blot was stripped and reprobed with the anti-myc antibody to illustrate the relative levels of synphilin-1 constructs that were present in the extracts. Arrow indicates synphilin-1 and brackets indicate ubiquitinated synphilin-1. ***c***, Full-length parkin is required for the ubiquitination of synphilin-1. Lysates prepared from HEK293 cells transfected with Flag-tagged parkin or various putative functional domains of parkin, HA-tagged ubiquitin and myc-tagged synphilin-1 were subjected to IP with anti-myc followed by anti-HA immunoblotting. The blot was stripped and reprobed with anti-myc to illustrate that equivalent levels of synphilin-1 constructs were present in the extracts. Arrow indicates synphilin-1. ***d***, *In vitro* ubiquitination of synphilin-1 by parkin. The ubiquitination of synphilin-1 by parkin was reconstituted by using immunoprecipitated (IP) parkin and synphilin-1 with the addition of purified UBCH7 and the corresponding components in the ubiquitin system. The reaction products were analyzed by western-blot analysis with both anti-ubiquitin and anti-HA antibodies. Arrows and molecular marker at 208 kD indicate both synphilin-1 and ubiquitin-positive high molecular species. All four experiments were replicated 3 times with similar results.

substrate proteins[24,25]. To determine the site of interaction of parkin with synphilin-1, we monitored the interaction of synphilin-1 with ring-finger–containing constructs of parkin (Fig. 4*a*). A construct containing the first ring finger (R1), the IBR domain and the second ring finger (R2) potently interacted with synphilin-1, which contrasts with the weak interaction with full-length parkin. Additional mapping studies indicate that synphilin-1 mainly interacted with the R2 domain of parkin (Fig. 4*a*). We constructed a series of truncated synphilin-1 constructs to determine the domain of synphilin-1 that interacted with parkin (Fig. 4*b*). There was a strong interaction between the ankyrin domain of synphilin-1 and parkin (Fig. 4*b*).

Mutations in parkin alter the interaction with synphilin-1

We next monitored whether the interaction of parkin and synphilin-1 is disrupted or altered by familial-PD–associated mutations in parkin (Fig. 5*a*). The E3 ubiquitin protein ligase activity of these mutants was limited or absent[24,25] (Y.Z., *et al.*). Familial-associated mutations in the R1 domain have minimal effects on the interaction between synphilin-1 and parkin (Fig. 5*a*). Familial-associated mutations in the R2 domain and IBR domain seemed to increase the interaction of parkin with synphilin-1 (Fig. 5*a*). We further explored the role of the R2 domain of parkin in regulating the interaction with synphilin-1 by mutating the cysteines within the R2 domain. Mutations of either the first or second cysteine of R2 (Cys421Ala and Cys431Ala) also seemed to increase the interaction of parkin with synphilin-1 (Fig. 5*a*). The familial-linked mutation (Trp453Stop) that resulted in a truncated protein lacking the last 12 amino acids also increased the interaction of parkin with synphilin-1 (Fig. 5*a*). Together these results suggest that synphilin-1 interacts primarily with the R2 domain and that familial-linked mutations in the R2 domain appear to promote the interaction of synphilin-1 with parkin.

Parkin mutations interfere with synphilin-1 ubiquitination

To ascertain whether parkin ubiquitinates synphilin-1, we transfected cells with Flag-tagged parkin, myc-tagged syn-

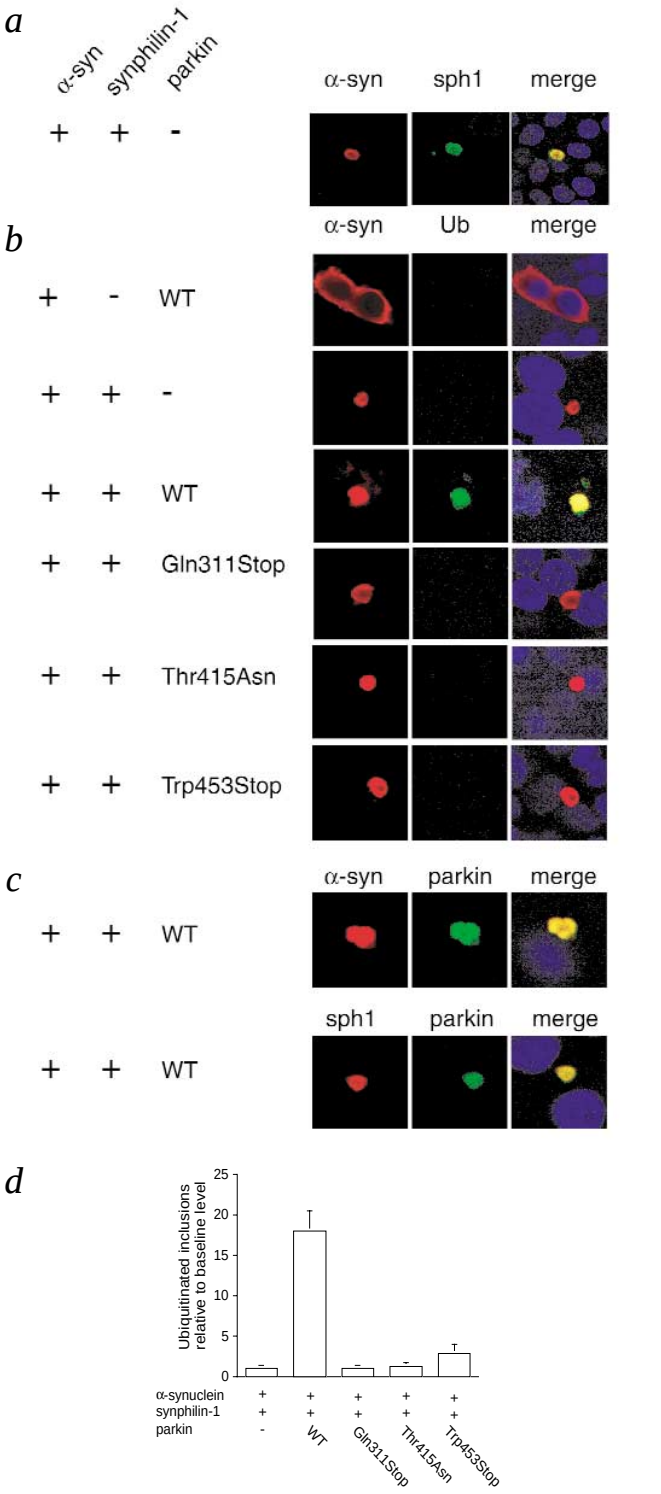


Fig. 6 Parkin ubiquitinates Lewy-body–like inclusions that are formed when synphilin-1 and α-synuclein are co-expressed. ***a***, Lewy-body–like inclusions are formed when synphilin-1 and α-synuclein are co-expressed in HEK293 cells. Both proteins stain positive in the inclusions (red, α-synuclein; green, synphilin-1; yellow, merged). Nuclei are shown in blue. ***b***, Inclusions formed by synphilin-1 and α-synuclein are not ubiquitin-positive. Co-expression of synphilin-1, α-synuclein and parkin results in the formation of ubiquitin-positive Lewy-body–like inclusions (red, α-synuclein; green, ubiquitin (Ub); yellow, merged). Familial-linked mutations of parkin fail to ubiquitinate these Lewy-body–like cytosolic inclusions. Nuclei are shown in blue. ***c***, The α-synuclein and synphilin-1 inclusions are parkin positive (red, α-synuclein or synphilin-1; green, parkin; yellow, merged). Nuclei are shown in blue. ***d***, Quantification of ubiquitin-positive Lewy-body–like inclusions in HEK 293 cells co-transfected with the various constructs. The number of ubiquitin-positive inclusions is relative to the cells transfected with α-synuclein and synphilin-1. Co-transfection of parkin with α-synuclein and synphilin-1 results in a 18-fold increase in ubiquitin-positive inclusions. Familial-linked parkin mutations substantially decrease the formation of ubiquitin-positive inclusions. All experiments in *a–d* were replicated 3 times with similar results.

philin-1 and HA-tagged ubiquitin (Fig. 5*b*). Two days later, we immunoprecipitated cells with an antibody against myc and probed with an antibody against HA to monitor ubiquitination. Immunoprecipitated synphilin-1 showed significant anti-HA immunoreactivity consistent with parkin-mediated ubiquitination of synphilin-1 (Fig. 5*b*). We also tested whether familial-linked mutations in parkin affect the parkin-mediated ubiquitination of synphilin-1 (Fig. 5*b*). All the familial-linked mutations in parkin impaired the ability of parkin to ubiquitinate synphilin-1; in particular, the mutants Thr240Arg, Arg256Cys and Trp453Stop were almost devoid of ubiquitinating activity. We probed the cell lysates with an antibody against synphilin-1 and found a ladder of synphilin-1, which indicates that synphilin-1 is being directly ubiquitinated. All the familial-linked mutations had reduced ladder formation comparable with cells lacking transfected Flag-tagged parkin (Fig. 5*b*). We further explored the possible functional domains of parkin that can ubiquitinate synphilin-1 by comparing wild-type parkin-mediated ubiquitination with ubiquitination mediated by different domains of parkin (Fig. 5*c*). We found that full-length parkin was required for the ubiquitination of synphilin-1 (Fig. 5*c*). The specificity of the interaction and ubiquitination of synphilin-1 by parkin was demonstrated by the lack of an interaction of parkin with α-synuclein (Fig. 1). To further investigate the interaction between parkin and synphilin-1, we reconstituted the ubiquitination reaction of synphilin-1 by parkin *in vitro*. In this experiment, we used immunoprecipitated parkin and synphilin-1 with the addition of the required ubiquitin system components. Immunoprecipitated parkin ubiquitinated synphilin-1 (Fig. 5*d*), demonstrating that synphilin-1 is a direct target of parkin.

Parkin ubiquitinates Lewy-body–like inclusions

To determine whether parkin might be involved in the ubiquitination of proteins contained within Lewy-body inclusions, we monitored whether parkin could ubiquitinate the cytosolic, Lewy-body–like inclusions that are formed when synphilin-1 and α-synuclein are co-expressed[28,29]. When we co-transfected cells with synphilin-1 and α-synuclein, we saw the formation of cytosolic inclusions (Fig. 6*a*). Inclusions were only formed when both synphilin-1 and α-synuclein are co-expressed (data not shown). Immunofluorescence of cells co-transfected with synphilin-1 and α-synuclein showed that both proteins are found in the inclusions (Fig. 6*a*). These inclusions were rarely (≤ 1%) ubiquitinated (Fig. 6*b* and *d*). However, when wild-type parkin was co-expressed with synphilin-1 and α-synuclein, there was an 18-fold increase in the number of ubiquitin-positive inclusions (Fig. 6*b* and *d*). We obtained similar results with the PD-related mutations in α-synuclein (data not shown). We also tested whether familial-linked mutations in parkin affect the parkin-mediated ubiquitination of these inclusions (Fig. 6*b*

and *d*). Gln311Stop, Thr415Asn and Trp453Stop mutations in the R2 domain of parkin impaired the ability of parkin to ubiquitinate these cytosolic inclusions. We further found that the α-synuclein and synphilin-1 inclusions are also parkin positive (Fig. 6*c*). α-synuclein only formed inclusions in the presence of synphilin-1. When parkin and α-synuclein were co-transfected, we did not observe inclusions and α-synuclein was not ubiquitinated (Fig. 6*b*). When parkin and the PD-related mutations in α-synuclein were co-transfected, we did not observe inclusions (data not shown). Together these results indicate that parkin ubiquitinates protein(s) contained within cytosolic Lewy-body–like inclusions that contain both synphilin-1 and α-synuclein, and that familial-linked mutations in parkin impair the ubiquitination of protein(s) within these cytosolic inclusions.

Discussion

The major findings of this report are that parkin interacts with and ubiquitinates the α-synuclein interacting protein, synphilin-1, and that parkin ubiquitinates the Lewy-body–like inclusions that are formed when α-synuclein is co-expressed with synphilin-1. Furthermore, ubiquitination of synphilin-1 and the ubiquitination of the Lewy-body–like inclusions are impaired by familial-associated parkin mutants. These findings have important implications for understanding the pathogenesis of PD as they provide the molecular mechanism by which Lewy-body–associated proteins are ubiquitinated and potentially link the two familial associated PD genes, α-synuclein and parkin, through the α-synuclein-interacting protein, synphilin-1, in the ubiquitination of Lewy-body–like inclusions.

The Lewy body is morphologically composed of two major components, the dense core and the surrounding halo[3,4]. α-synuclein is mainly concentrated in the surrounding halo whereas synphilin-1 is mainly concentrated within the dense core[12,30,37]. Parkin is also expressed in Lewy bodies[22,23]. Consistent with the notion that synphilin-1 may be a major target of parkin-mediated ubiquitination, ubiquitin staining is mainly concentrated in the dense core of Lewy bodies where synphilin-1 expression predominates[30,37]. Parkin has three other potential substrates including CDCrel-1 (ref. 24), Pael-R (ref. 27) and αSp22 (ref. 23). Levels of Pael-R and αSp22 are increased in AR-PD brains, suggesting that the accumulation of both Pael-R and αSp22 may have a role in the pathogenesis of AR-PD (refs. 23,27). Whether CDCrel-1, αSp22 or Pael-R are contained within Lewy bodies is not known. Although αSp22 is a target of parkin-mediated ubiquitination *in vitro*[23], α-synuclein may not be the major ubiquitinated protein in Lewy bodies since α-synuclein intracytoplasmic deposits in dementia with Lewy bodies are often ubiquitin-negative[38]. In addition, similar to our observations, other investigators have also found that α-synuclein is not ubiquitinated[39].

Patients with mutations in parkin do not have Lewy bodies[40–42]. This observation has led to the speculation that pathogenic mechanisms caused by mutations in parkin are different from those that occur in sporadic PD, and in PD due to mutations in α-synuclein. Our data on the other hand, suggest a common link between the different causes of PD. The ability of synphilin-1 to interact with parkin and α-synuclein provides a link in the pathogenesis of PD caused by mutations in parkin, and alterations in the major non-glycosylated form of α-synuclein. We propose that parkin is intimately involved in the ubiquitination of Lewy-body–associated proteins, such as synphilin-1. In addition, parkin may be directly linked to α-synuclein through αSp22 (ref. 23). In the absence of parkin, Lewy-body–associated proteins would not be ubiquitinated and the formation of Lewy bodies would be impaired. Consistent with this notion is our observation that familial-associated mutations of parkin fail to ubiquitinate synphilin-1. In an analogous manner, absence of a related E3 ubiquitin-protein ligase, E6-AP, leads to a relative paucity of ubiquitinated polyglutamine-associated nuclear inclusions[43] and dramatically worsens the phenotype of transgenic mice overexpressing the polyglutamine-expanded protein ataxin-1 (ref. 43). Inhibition of the ubiquitin system in cellular models of Huntington disease suppresses the formation of inclusions and results in increased mutant-huntingtin–induced neuronal death[44]. Further understanding of the molecular mechanisms of Lewy-body formation, ubiquitination and the interaction of parkin, α-synuclein and synphilin-1 holds promise for better understanding the pathophysiology of PD and the development of therapeutic agents.

Methods

Antibody generation. To generate a peptide antigen of parkin, a peptide containing the last 18 amino acids of the C-terminal of parkin was synthesized and cross-linked to keyhole limpet hemocyanin (KLH). The conjugated peptide was then used to immunize a New Zealand white rabbit (JH540) (Cocalico Biologicals, Reamstown, Pennsylvania). Antibody specificity was confirmed by the ability to preadsorb the immunostaining with excess purified parkin protein.

Cell culture and transfection. HEK293 cells were grown in DMEM containing 10% FBS in a 5% CO_2 atmosphere. Cells were transiently transfected with the target vector by the lipofectamine method according to the manufacturers instructions (Gibco Life Technologies, Gaithersburg, Maryland).

Co-immunoprecipitation. For co-immunoprecipitation from cell cultures, HEK293 cells were transfected with 2 μg of each plasmid. After 36 h, cells were washed with cold PBS and harvested in immunoprecipitation buffer (1% Triton X-100, 2 μg/ml aprotinin, 100 μg/ml PMSF in PBS). The lysate was then rotated at 4 °C for 1 h followed by centrifugation at 14,000 r.p.m. for 15 min. The supernatants were then combined with 50 μl Protein G Sepharose (Amersham) preincubated with antibodies against HA or myc (Roche) followed by rotating at 4 °C for 2 h. The protein G sepharose was pelleted and washed 3 times using immunoprecipitation buffer or buffer with additional 500 mM NaCl followed by 3 washes with PBS. The precipitates were resolved on SDS–PAGE gel and subjected to western-blot analysis. Bands were visualized with chemiluminescence (Pierce, Rockford, Illinois).

For co-immunoprecipitation of the endogenous proteins from rat brain, adult rat brain was homogenized in 4 volumes of ice-cold PBS containing 320 mM sucrose and 0.1% Triton X-100 with protease inhibitor cocktail (Sigma). The tissue homogenate was centrifuged at 37,000*g* at 4 °C for 20 min. The supernatant was used for immunoprecipitation with one of the following antibodies: anti-HA, ant-myc, anti–synphilin-1 (ref. 29) or anti-parkin. The precipitates were subjected to western-blot analysis with an anti–synphilin-1 antibody[29].

Ubiquitination assay. HEK293 cells were transfected with 2 μg of pCMV-Flag-parkin or Flag-tagged parkin mutants, pRK5-myc-synphilin-1 or pRK5-myc-α-synuclein, and 2 μg of pMT123-HA-ubiquitin plasmids. After 36 h, immunoprecipitation was performed with an antibody against myc. The precipitates were submitted to western blotting with an antibody against HA.

***In vitro* ubiquitination assay.** Reactions were performed in 50 μl mixture containing 50 mM TrisHCl, pH7.5, 2.5 mM MgCl, 2 mM DTT, 2mM ATP, 10 μg ubiquitin (Sigma), 100 ng of E1 (Calbiochem, San Diego,

California), 200 ng of UbcH7 (Affinti-Research, Exeter, UK), immunoprecipitated myc-tagged parkin and HA-tagged synphilin-1. Reactions were carried out for 2 h at 37 °C before terminating with an equal volume of 2× SDS sample buffer. The reaction products were then subject to western-blot analysis with anti-ubiquitin (Dako, Carpinteria, California) or anti-HA antibodies.

Generation of plasmids. Full-length parkin cDNA was cloned into pRK5-myc vector between *Sal*I and *Not*I sites. The cDNA sequences encoding amino acids 220–465, 220–403, 32–465, 220–318, 304–404 and 395–465 of parkin were cloned into pRK5-myc vector between *Sal*I and *Not*I sites to generate pRK5-myc-R1-IBR-R2, pRK5-myc-R1-IBR, pRK5-myc-IBR-R2, pRK5-myc-R1, pRK5-myc-IBR, pRK5-myc-R2, respectively. The mutants pRK5-myc-parkinThr240Arg, pRK5-myc-parkinThr415Asn, pRK5-myc-parkinCys421Ala, pRK5-myc-parkinCys431Ala, pRK5-myc-parkinGln311Stop and pRK5-myc-parkinTrp453Stop were generated by PCR-mediated site-directed mutagenesis[45]. Full-length cDNAs of synphilin-1, α-synuclein and α-synuclein mutants (A53T or A30P) were cloned into pRK5-myc and HA vectors between *Sal*I and *Not*I sites. The cDNA sequences encoding amino acids 1–213, 214–465, 466–711, 712–919 and 336–556 of synphilin-1 were cloned into pRK5-myc vector between *Sal*I and *Not*I sites to generate pRK5-myc-F1, pRK5-myc-F2, pRK5-myc-F3, pRK5-myc-F4 and pRK5-myc-F5. The integrity of the constructs was confirmed by sequencing.

Immunocytochemistry. HEK293 cells were transfected with 0.5 μg of pRK5-α-synuclein, pRK5-HA-synphilin-1 and pRK5-myc-parkin per well in 2-well chamber slides. After 48 h, cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.2% Triton X-100 for 15 min at room temperature. They were first labeled with either of the following combination of the primary antibodies: anti-α-synuclein (BD Transduction Laboratories, Lexington, Kentucky) and anti-HA (Upstate Biotechnology, Lake Placid, New York), anti–α-synuclein and anti-ubiquitin (DAKO, Carpinteria, California), anti–α-synuclein and anti-myc (Developmental Studies Hybridoma Bank, Iowa City, Iowa), or anti-HA and anti-myc. After washing, the cells were labeled with Cy3-conjugated anti-mouse and FITC-conjugated anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania). The cells were then analyzed by confocal microscopy (Zeiss LSM 410, Thornwood, New York).

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