Human Molecular Genetics, 2005, Vol. 14, No. 17 ***2571–2586***
doi:10.1093/hmg/ddi292
Advance Access published on July 27, 2005

Familial-associated mutations differentially disrupt the solubility, localization, binding and ubiquitination properties of parkin

Sathya R. Sriram[1,2], Xiaojie Li[1,2,3], Han Seok Ko[1,3], Kenny K.K. Chung[1,3], Esther Wong[6], Kah Leong Lim[6], Valina L. Dawson[1,2,3,4,5] and Ted M. Dawson[1,2,3,4,*]

[1]Institute for Cell Engineering, [2]Cellular and Molecular Medicine Graduate Program, [3]Department of Neurology and [4]Department of Neuroscience and [5]Department of Physiology Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA and [6]Neurodegeneration Laboratory, National Neurosciences Institute, Singapore

Received May 12, 2005; Revised and Accepted July 19, 2005

Mutations in *parkin* are largely associated with autosomal recessive juvenile parkinsonism. The underlying mechanism of pathogenesis in parkin-associated Parkinson's disease (PD) is thought to be due to the loss of parkin's E3 ubiquitin ligase activity. A subset of missense and nonsense point mutations in parkin that span the entire gene and represent the numerous inheritance patterns that are associated with parkin-linked PD were investigated for their E3 ligase activity, localization and their ability to bind, ubiquitinate and effect the degradation of two substrates, synphilin-1 and aminoacyl-tRNA synthetase complex cofactor, p38. Parkin mutants vary in their intracellular localization, binding to substrates and enzymatic activity, yet they are ultimately deficient in their ability to degrade substrate. These results suggest that not all parkin mutations result in loss of parkin's E3 ligase activity, but they all appear to manifest as loss-of-function mutants due to defects in solubility, aggregation, enzymatic activity or targeting proteins to the proteasome for degradation.

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disease affecting over 1 million people in North America alone (1,2). This debilitating movement disorder has an estimated prevalence of >0.1% of the population >40 years, making age the most consistent risk factor for PD (3). Primarily a sporadic disease with an unknown etiology, PD has long been attributed to abnormal environmental factors (4–6). However, recent insights into the genetics of PD and the subsequent identification of several genes for monogenically inherited forms has revolutionized the field of PD research (7–10). Although the familial forms account for only ~5–10% of all PD cases, there are sufficient overlapping clinical and pathological features to justify studying the contribution of these rare genes to the pathogenesis of PD.

Several distinct loci have been linked with rare Mendelian forms of PD and subsequent causal mutations have been identified in *alpha-synuclein, parkin, DJ-1, PINK1* and *LRRK2* (11,12). The PARK2 locus on chromosome 6 encodes the parkin protein and is linked to autosomal recessive juvenile parkinsonism (ARJP) (13,14). This early-onset disease is characterized by the loss of nigral and locus coeruleus neurons with the absence of Lewy body formation and gliosis (15,16). Parkin-associated PD is pathologically unique because they lack Lewy bodies, suggesting a role for parkin in the formation of these proteinaceous inclusions.

Parkin functions as an E2-dependent E3 ubiquitin ligase in the ubiquitin–proteasome degradation pathway (17–19). Several substrates for parkin have been identified so far—the synaptic vesicle-associated CDCrel-1 and CDCrel-2; the α-synuclein-interacting protein, synphilin-1; parkin-associated endothelin-like receptor (Pael-R); synaptotagmin XI; α-/β-tubulin; cyclin E; the p38 subunit of aminoacyl-tRNA synthetase and the dopamine transporter (DAT)—but their relevance in the pathogenesis of parkin-linked PD is yet to be fully appreciated (17,20–33). However, the p38 subunit of aminoacyl-tRNA synthetase appears to be an authentic parkin

*To whom correspondence should be addressed at: Institute for Cell Engineering, Johns Hopkins University School of Medicine, 733 N Broadway Street, Suite 731, Baltimore, MD 21205, USA. Tel: +1 4106143359; Fax: +1 4106149568; Email: tdawson@jhmi.edu

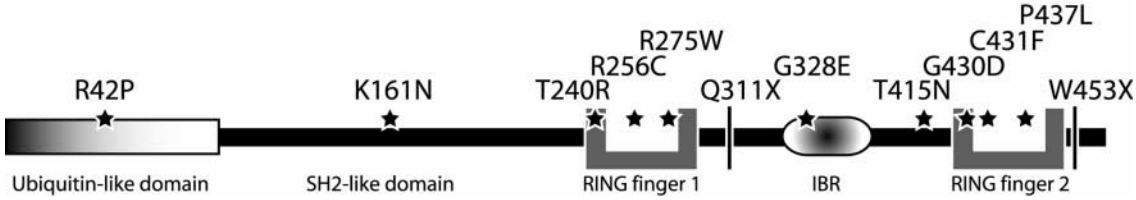

Figure 1. Schematic representation of the 12 missense (stars) and nonsense (rectangle) point mutations in *parkin*, linked to familial PD, that were selected for the study.

substrate as it is upregulated in the ventral midbrain/hindbrain of both young and old parkin null mice and patients with ARJP and idiopathic PD and diffuse Lewy body disease (34). Moreover, adenovirus-mediated overexpression of p38 in the substantia nigra in mice leads to loss of dopaminergic neurons (34). A protective role for parkin against toxicity induced by ceramide, kainate, Pael-R-induced ER stress, overexpression of α-synuclein alone and in the presence of synphilin-1 has also been described (22,25,35–38). Parkin may also function as part of larger complexes such as the Skp1-Cullin-F-box protein complex, with chaperones such as CHIP and Hsp70, the bcl-2-associated anthogene 5 (BAG5) complex and CASK in the post-synaptic complex (22,25,39,40). Association with BAG5 and two distinct post-translational modifications on parkin, S-nitrosylation and phosphorylation, have been shown to negatively regulate both its E3 ubiquitin ligase activity and its protective function in the cell (37,41,42).

Originally identified in consanguineous Japanese families, mutations in *parkin* have since been identified across varied ethnicities and constitute ∼50% of familial early-onset recessive PD cases (43). Although the majority of parkin-linked PD is early-onset and inherited in an autosomal recessive manner, there is marked variation in the age of onset and inheritance patterns, including a potential role for heterozygous *parkin* mutations as susceptibility alleles in late-onset PD (44). A diverse range of mutations in *parkin*, including missense mutations, truncations, exon deletions, exon multiplications and frameshifts, have been described (45). Although mutations are observed along the entire length of the *parkin* gene, there is some evidence for mutational hot spots and founder mutations for the more frequently observed mutations (46).

The association of *parkin* mutations with recessive forms of familial PD led to the hypothesis that these loss-of-function mutations result in an obliteration of parkin's enzymatic function, a significant accumulation of cytotoxic parkin substrate(s), and the subsequent onset of disease. However, parkin mutants have been shown to retain or have only partially disrupted ubiquitination activity, suggesting that these mutations may not result in a complete loss of enzymatic activity (19,20,21,47). This observation prompted us to further investigate the enzymatic activity of parkin mutants and also search for alternative mechanisms by which a subset of point mutations and truncations may contribute to parkin's loss-of-function. Here, we report that not all the selected mutants result in complete obliteration of enzymatic activity. Some of the familial-associated parkin mutants have increased ubiquitination activity compared with wild-type. In addition, some parkin mutants have altered distribution and solubility in the cell. Investigating the effect of these mutants on binding, ubiquitination and degradation of two characterized substrates, synphilin-1 and the p38 subunit of aminoacyl-tRNA synthetase, suggest that all the mutations, through alternative mechanisms including defective ubiquitination and altered solubility, ultimately results in the improper degradation of substrates by the proteasome and their subsequent accumulation in the cell. Thus, each mutant may be defective in one or more aspects of the ubiquitination process or is abnormally localized, resulting in an apparent loss-of-function of the parkin protein.

RESULTS

Twelve point mutations in parkin were analyzed to determine common and relevant abnormalities compared with wild-type protein (Fig. 1). The 12 selected mutations are distributed across the various domains of parkin and cover a wide spectrum of reported inheritance patterns, ensuring reasonable representation of the over 70 known point mutations (Table 1). The parkin protein has an N-terminal ubiquitin-like domain, an SH2-like linker and a C-terminal RING TRIAD domain, containing two RING domains separated by an in-between-RING (IBR) domain. One mutation each in the ubiquitin-like domain (R42P), SH2-like linker domain (K161 N), IBR domain (G328E) and the domain between IBR and RING 2 (T415 N) was selected for the analysis. The RING domains are critical for parkin's E3 ligase activity by mediating ubiquitin transfer between the E2 conjugating enzymes and the substrate (48). Three point mutations in each RING domain were chosen for the study: T240R, R256C and R275W in RING 1 and G430D, C431F and P437L in RING 2. We also included two truncations: W453X, which excludes the last 12 amino acids comprising a PDZ domain, and Q311X, which includes the first RING finger domain, but excludes the IBR domain, second RING finger domain and the PDZ domain.

Some PD-linked parkin mutations lead to an altered solubility in cells

Parkin mutants were compared with wild-type parkin for their ability to be sequentially extracted by two commonly used detergents, Triton X-100 and sodium dodecyl sulfate (SDS), respectively, based on previous studies showing parkin mutants' differential extractability due to altered localization (49). Wild-type parkin protein is observed in both the Triton X-soluble and Triton X-insoluble fractions (Fig. 2A). A number of mutant proteins have extraction profiles similar to wild-type, such as R42P, K161 N, T240R, G328E, T415 N

Table 1. Selected parkin point mutations

Mutation	Domain	Type	References
R42P	Ub-like	Homozygous Hetero (nonpathogenic)	(69,70)
K161N	SH2-like	Compound heterozygous	(61)
T240R	R1	Homozygous	(61,71)
R256C	R1	Heterozygous Homozygous Compound heterozygous	(60,61,72)
R275W (12 distinct families)	R1	Compound Heterozygous (P437L) (40 bp ex 3 del) (G430D; del3-6; del5-6) Homozygous Heterozygous Dominant negative effect?	(60,61,63,64,72–77)
Q311Stop	Truncate	Homozygous	(61,71)
G328E	IBR	Heterozygous	(60)
T415N	Before R2	Homozygous	(61)
G430D	R2	Compound heterozygous (with 40 bp del) (with R275W)	(72,74,75)
C431F	R2	Compound heterozygous Homozygous	(60,78)
P437L	R2	Heterozygous Hetero (and homo R275W)	(70,76,77,79)
W453Stop	After R2	Homozygous	(61)

The 12 pathogenic point mutations result in amino acid substitutions in the protein and span the various domains of parkin. The range of inheritance patterns that are associated with each of the selected mutants is indicated.

and P437L, whereas two mutants, G430D and C431F, have lower extractability in the Triton X-100 fraction when compared with wild-type protein (Fig. 2A). Two proteins with point mutations, R256C and R275W, and the two truncated proteins, Q311X and W453X, are absent in the Triton X-100 fraction but produce immunoreactive bands on a western blot of the Triton X-insoluble cell lysates (Fig. 2A). These observations suggest that some point mutations in parkin may result in altered localization in the cell, rendering them resistant to extraction by Triton X-100, and thus affect their ability to function normally. In light of this finding, all subsequent experiments, unless otherwise stated, were performed using cells lysed in 1% SDS buffer.

Parkin mutants with altered extraction properties tend to form intracellular aggregates

The differential extractability of the parkin mutants prompted us to study the intracellular distribution of these mutant parkin proteins. Myc-tagged wild-type and mutant parkin constructs were transfected into SH-SY5Y cells and prepared for examination under a confocal microscope. Wild-type parkin was found homogeneously expressed in the cytoplasm of SH-SY5Y neuroblastoma cells with occasional parkin-positive aggregates that were observed in at most an average of 10% of the total population of transfected cells (Fig. 2B and C). The majority of the mutants that were easily extracted by Triton X-100, i.e. K161 N, T240R, G328E, T415 N, G430D and P437L, showed a similar intracellular localization to wild-type parkin (Fig. 2B). In contrast, a significant number of cells transfected with mutants that were predominantly observed in the Triton X-insoluble fraction (R256C, R275W, Q311X, C431F, W453X) consistently had intracellular aggregates that were parkin-positive (Fig. 2B and C). However, one mutant, R42P, which is easily extracted by both Triton X-100 and SDS, showed statistically significant propensity to form parkin-positive aggregates (Fig. 2B and C). Thus, almost all the mutants that are largely extracted in the Triton X-insoluble fraction are observed to have an increased propensity to be confined in intracellular aggregates.

Parkin mutants show differential auto-ubiquitination

Parkin self-ubiquitination has been established as a reliable method to study parkin's enzymatic function as parkin is capable of auto-ubiquitination (18,25,37). To study whether familial-linked parkin mutations affect self-ubiquitination activity, immunoprecipitation experiments were performed in SH-SY5Y cells with myc-tagged wild-type and mutant parkin constructs co-transfected with HA-tagged ubiquitin. A myc IP of the parkin protein followed by immunoblotting with anti-HA shows the level of ubiquitin modification on each mutant compared withwild-type (Fig. 3). Some mutations show reduced ubiquitin modification as a function of their abolished activity (K161N, T240R, Q311X, T415N, P437L), whereas others retain their ability to auto-ubiquitinate (R42P, R256C, R275W, G328E, G430D, C431F, W453X). The reduced ubiquitination for some mutants could indicate either a very high turnover of the protein or an impaired enzymatic activity. Performing the experiment in the presence of a proteasome inhibitor did not alter the ubiquitination profile of these mutants (data not shown), thus supporting the latter theory. The abundance of high molecular weight ubiquitinated species observed with some mutants could indicate either a gain-of-function mutation, which results in an increased enzymatic activity, or an impaired targeting to the proteasome

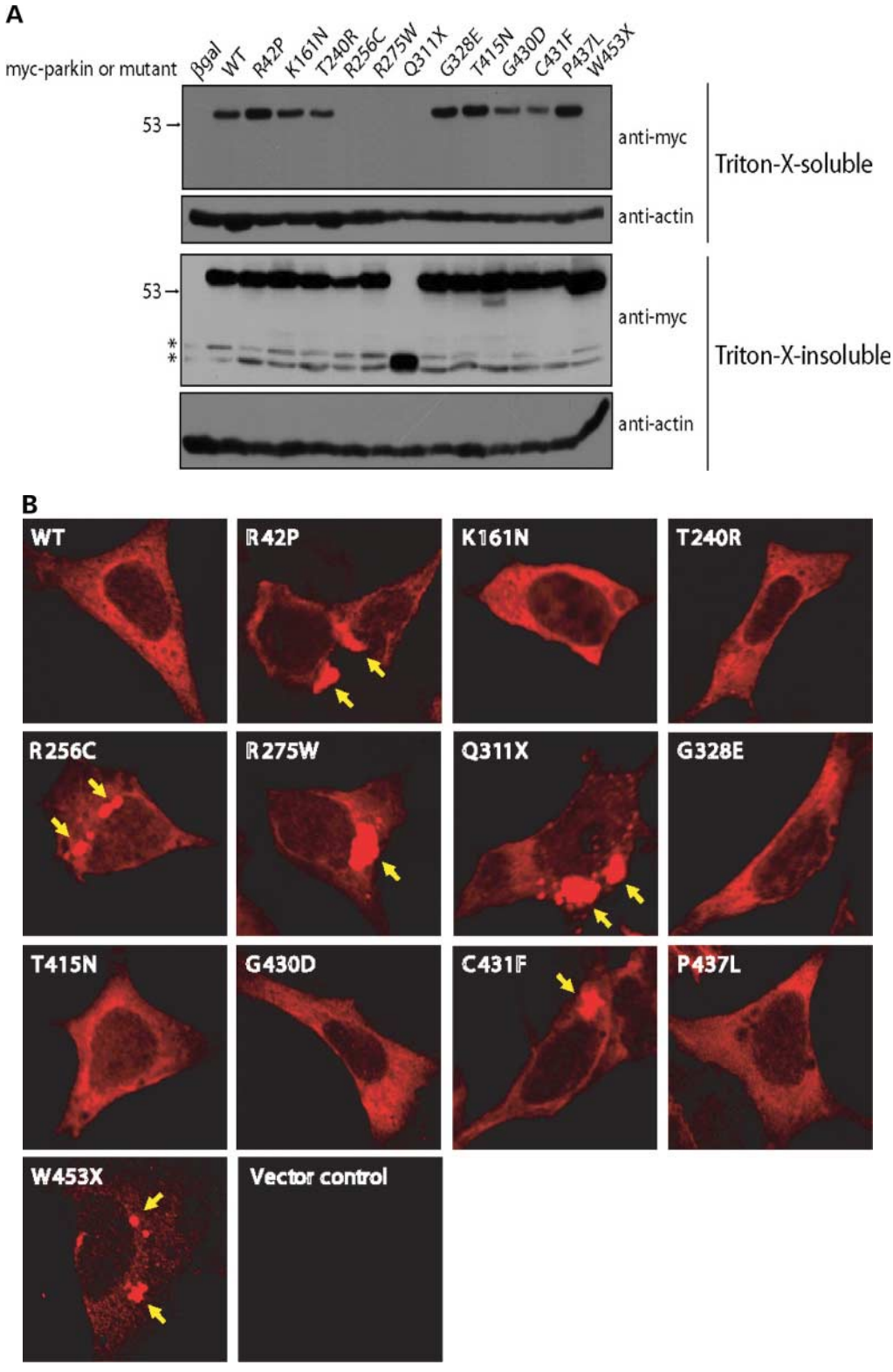

Figure 2. Mutations in *parkin* result in altered solubility and localization of the protein. (**A**) Transiently transfected SH-SY5Y cells were lysed sequentially with Triton X-100 and SDS, and the lysates were analyzed on a western blot for myc-tagged wild-type and mutant parkin, as indicated. The blots were stripped and reprobed for actin as a loading control. The experiment was repeated three times with similar results. Asterisk indicates non-specific bands observed on the blot. (**B**) Cellular localization of exogenously expressed wild-type and parkin mutants were studied using an anti-myc antibody (red). Cells transfected with the vector served as a negative control, whereas those transfected with wild-type and Triton-soluble mutant parkin showed uniform cellular distribution. The SDS-soluble parkin mutants formed aggresome-like perinuclear structures (arrows). (**C**) Statistical analysis showing the percentage of cells containing parkin-positive aggregates. Bars shown are the mean of at least three independent experiments, and error bars indicate $\pm$SEM. Statistical significance is indicated on the graph. ($^{*}P < 0.05$, $^{**}P < 0.01$, ANOVA test)

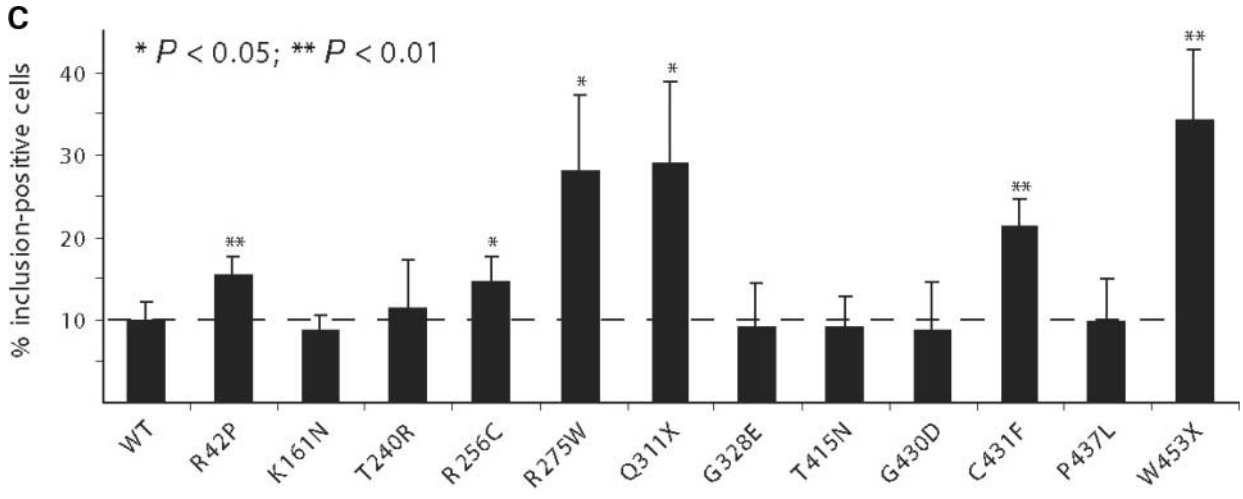

Figure 2. Continued.

and therefore accumulation of the ubiquitinated protein. To account for the potential mechanisms by which the parkin mutants with increased ubiquitination activity show a loss-of-function phenotype, we studied the relative ability of the selected mutants to bind and ubiquitinate substrate(s).

Parkin mutants have altered binding to substrates

Parkin catalyzes its own ubiquitination and that of several substrates, including synphilin-1 and the p38 subunit of aminoacyl-tRNA synthetase complex (20,21). Further, parkin has been shown to have dual function as a ubiquitin ligase, mediating both proteasome-dependent as well as proteasome-independent ubiquitination of substrates via lysine 48-linked and lysine 63-linked polyubiquitin chains, respectively (50,51). Although parkin-mediated ubiquitination of p38 promotes its degradation, parkin predominantly ubiquitinates synphilin-1 via lysine 63; however, proteasome-mediated degradation of synphilin-1 can be achieved by high parkin to synphilin-1 ratios *in vivo* (21,51). Because parkin may ubiquitinate these two substrates via two potentially different pathways, both the binding and the ubiquitination of p38 and synphilin-1 by the selected mutants were investigated.

Myc-tagged parkin wild-type and mutant constructs were transfected into SH-SY5Y cells along with HA-tagged p38 for co-immunoprecipitation experiments to study the relative binding abilities of the selected parkin mutants to p38. Because non-covalent interactions, as is typical for interactions between E3 ligases and their substrates, may be easily disrupted by harsh detergent conditions, the cells were lysed in 1% Triton buffer. To account for the differential solubilities, the lysates were immunoprecipitated with equivalent amounts of anti-myc antibody to ensure that similar amounts of mutant and wild-type parkin were assayed in the experiment. Probing the immunoprecipitated samples with anti-HA on a western blot revealed that the majority of the mutants have a reduced capacity to bind substrate relative to wild-type parkin (Fig. 4A). Point mutations in either RING finger domain almost completely abolish binding (T240R, R256C, R275W in RING finger 1 and G430D, C431F, P437L in RING finger 2), whereas mutations in non-RING domains maintain a reduced binding ability (R42P, K161N, G328E and T415N) (Fig. 4A). The two truncation mutants, Q311X and W453X, have an increased propensity to bind p38 when compared with wild-type parkin, suggesting a role for the PDZ domain, localized to the last 12 amino acids of parkin, in substrate binding and release (Fig. 4A).

Similar experiments with myc-tagged parkin wild-type and mutant constructs and HA-tagged synphilin-1 were done to study any variation in the binding pattern observed with p38. The second RING finger domain, and not the first RING domain, is crucial for synphilin-1 binding to parkin (Fig. 4B). The point mutations in RING finger 2 (G430D, C431F, P437L) have highly reduced abilities to bind synphilin-1, as do mutations in non-RING domains, such as K161N, G328E and T415N (Fig. 4B). Several point mutants (R42P, R256C, R275W) and the two truncations (Q311X, W453X) promote binding to synphilin-1 (Fig. 4B). These results suggest that while some mutations in *parkin* may be unable to release substrates effectively, perhaps due to disrupted binding to adapter proteins required in the ubiquitination process, other mutations may affect the ability of substrate to associate with parkin, thus resulting in a disrupted function.

To further analyze the results observed in the co-immunoprecipitation experiments, we studied the intracellular localization of p38 and synphilin-1 in the presence of wild-type and mutant parkin. Immunocytochemistry using a subset of the selected mutants and p38 revealed that the parkin mutants that show an increased tendency to form intracellular aggregates (R42P, R275W, Q311X, C431F, and W453X) sequester p38 into these aggresome-like structures (Fig. 4C). In contrast, synphilin-1 does not co-localize with the aggregates formed by parkin mutants (Fig. 4D).

Mutations in parkin differentially disrupt the ubiquitination of substrates

To study the ability of these selected parkin mutants to ubiquitinate substrate, SH-SY5Y cells were transfected with

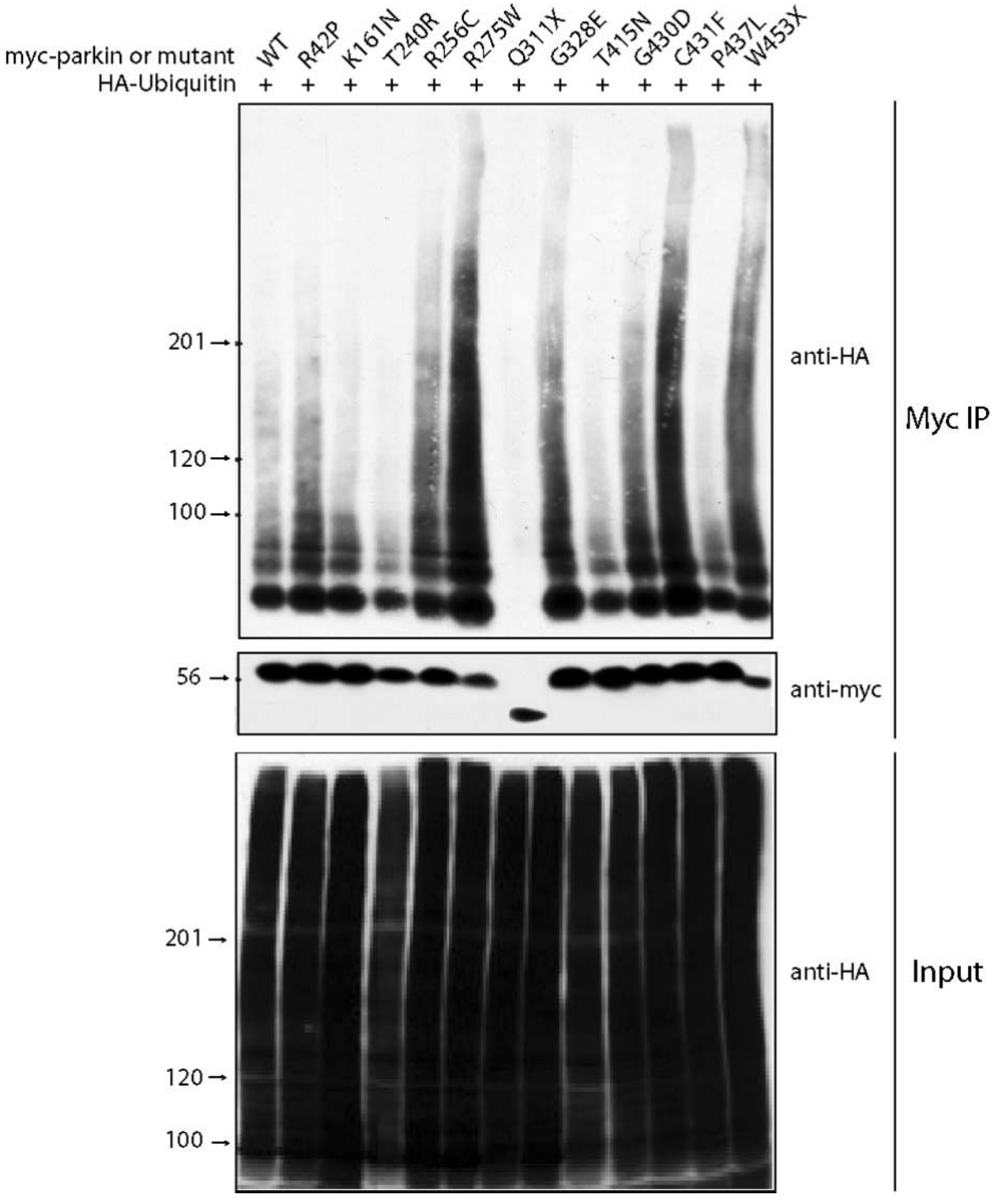

Figure 3. Parkin mutants have different ubiquitination activity. Co-immunoprecipitation (co-IP) experiments were set up for myc-tagged wild-type and mutant parkin with HA-tagged ubiquitin. Cell lysate was immunoprecipitated (IP) with anti-myc antibody and separated using SDS–PAGE on a gradient gel. Immunoblotting the IP samples on a western blot with anti-HA revealed a characteristic ubiquitin-positive smear in each lane corresponding to the amount of ubiquitination on each parkin mutant. The blot was stripped and reprobed with anti-myc antibody to show that equivalent amounts of wild-type and mutant parkin were pulled down by IP. Input samples were probed with anti-HA to show the levels of transfected HA-ubiquitin in each sample. The experiment was performed three times with similar results.

myc-tagged parkin wild-type and mutant constructs, along with FLAG-tagged p38 and HA-tagged ubiquitin. Cell lysates were immunoprecipitated with an antibody against FLAG and probed with an antibody to HA on a western blot to study the level of ubiquitination on the FLAG-tagged substrate in the presence of wild-type and mutant parkin. The presence of increased anti-HA immunoreactivity on the immunoprecipitated p38 in the presence of wild-type parkin confirmed that

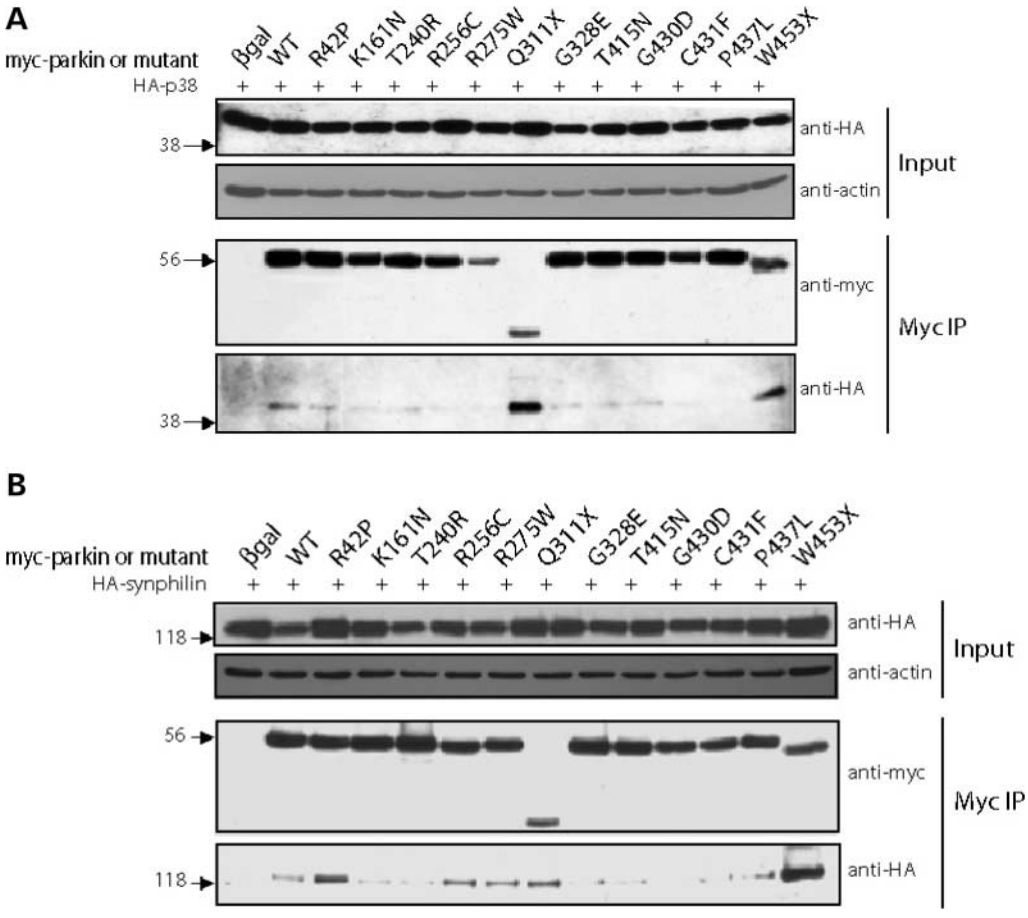

Figure 4. Parkin mutants differentially bind and co-localize with substrates. (**A**) Cells were transfected with myc-tagged wild-type and mutant parkin and HA-tagged p38 as indicated. Cell lysates were subjected to IP with anti-myc antibody and separated on a 12% polyacrylamide gel. Input and IP samples were probed with anti-HA antibody to observe the amount of exogenous p38 that was present. The IP blot was stripped and reprobed with anti-myc antibody to indicate the relative efficiency of IP. (**B**) Co-immunostaining of myc-tagged wild-type and mutant parkin and HA-tagged p38 was performed with anti-myc (red) and anti-HA (green). The merged pictures show uniform cellular distribution of wild-type and some mutants with p38 (yellow). Three mutants show parkin- and p38-positive inclusions (yellow). (**C** and **D**) Experiments similar to (A) and (B) were performed using flag-tagged synphilin-1 with parkin mutants. The experiment was repeated three times with similar results.

p38 is a substrate of parkin (Fig. 5A). Reduced ubiquitin modification on p38 is observed in the presence of mutants that also have reduced auto-ubiquitination, T240R, Q311X, T415N and P437L, confirming that these mutants have diminished enzymatic activity (Figs 3 and 5A). All other mutants show a spectrum of similar or increased ubiquitin modification on p38, with the highest immunoreactivity seen in the presence of R275W (Fig. 5A).

Analogous experiments were done to examine the effect of familial-linked parkin mutations on the ubiquitination of synphilin-1. FLAG-tagged synphilin-1 was co-transfected with HA-ubiquitin and myc-tagged wild-type and mutant parkin, and immunoprecipitated FLAG-synphilin-1 was analyzed for ubiquitin-positive smear on a western blot. Substantial ubiquitination of synphilin-1 observed in the presence of wild-type parkin confirms that synphilin-1 is a parkin substrate (Fig. 5B). Most mutations impaired parkin-mediated ubiquitination on synphilin-1 compared with wild-type, whereas R42P and R275W exhibit enhanced ubiquitination of synphilin-1 (Fig. 5B).

Parkin mutants are unable to degrade substrates

Parkin mutants differentially bind and ubiquitinate substrates, but are they still able to effectively target them for degradation via the 26S proteasome? The degradation of p38 and synphilin-1 in the presence of wild-type parkin is proteasome-dependent, as the reduction is blocked by co-incubation with proteasome inhibitors (data not shown). To determine whether substrates are degraded in the presence of the selected parkin mutants, stable SH-SY5Y cell lines expressing HA-tagged p38 or FLAG-tagged synphilin-1 were transfected with myc-tagged parkin wild-type and mutant constructs. Cell lysates were probed on a western blot to assess the steady state levels of p38 or synphilin-1. The bands were quantified using a densitometer, and substrate levels were normalized to actin loading controls and graphed to examine the relative ability of each mutant to degrade substrate.

In the presence of wild-type parkin, the steady state level of p38 is significantly decreased by ~50% compared with the control validating that p38 is indeed degraded by parkin

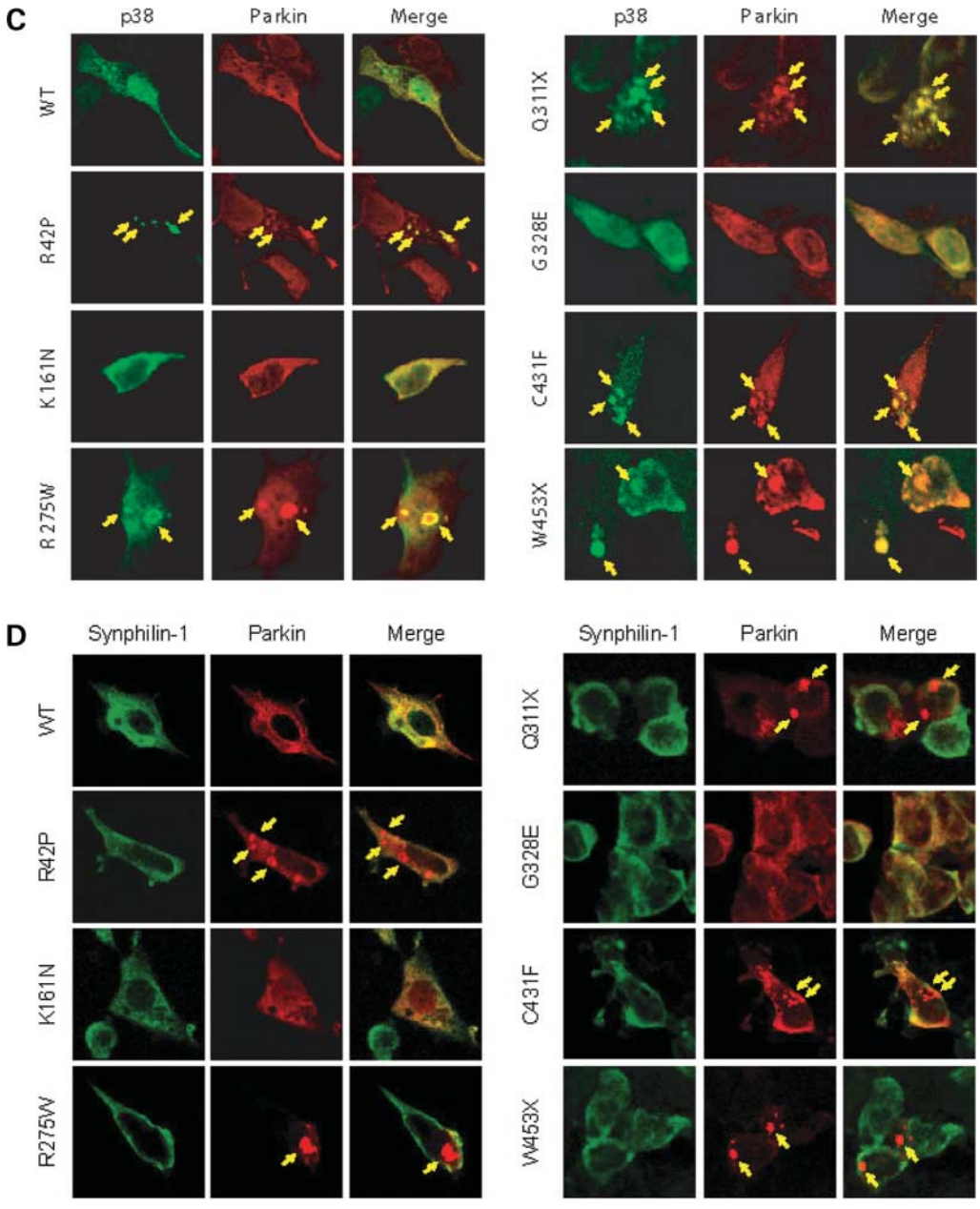

Figure 4. Continued.

(Fig. 6A and B). Interestingly, none of the parkin mutants was able to reduce the steady state levels of p38 with comparable efficiency to wild-type parkin (Fig. 6A and B). Although parkin does not primarily ubiquitinate synphilin-1 at lysine-48, an increased parkin to synphilin-1 ratio can induce ubiquitin-mediated degradation of synphilin-1 by parkin (51). Our results corroborate this observation because addition of wild-type parkin significantly reduces the amount of synphilin-1 in cell lysate by 50% compared with the βgal control (Fig. 6C and D). Further, similar to p38, efficient degradation of synphilin-1 was not observed in the presence of the parkin mutants, suggesting that these mutants are defective in their ability to degrade synphilin-1 as well (Fig. 6C and D). Cycloheximide-chase experiments performed in the stable cell lines show gradually decreasing levels of substrate with wild-type, but not mutant parkin, confirming that the observed steady state levels of p38 and synphilin-1 are due to degradation, and not altered protein synthesis (data not shown).

DISCUSSION

Pathogenic mutations in parkin occur as either homozygous or compound heterozygous mutations that mostly result in the onset of a recessive form of juvenile parkinsonism, although a wide variety of inheritance patterns have been reported (52).

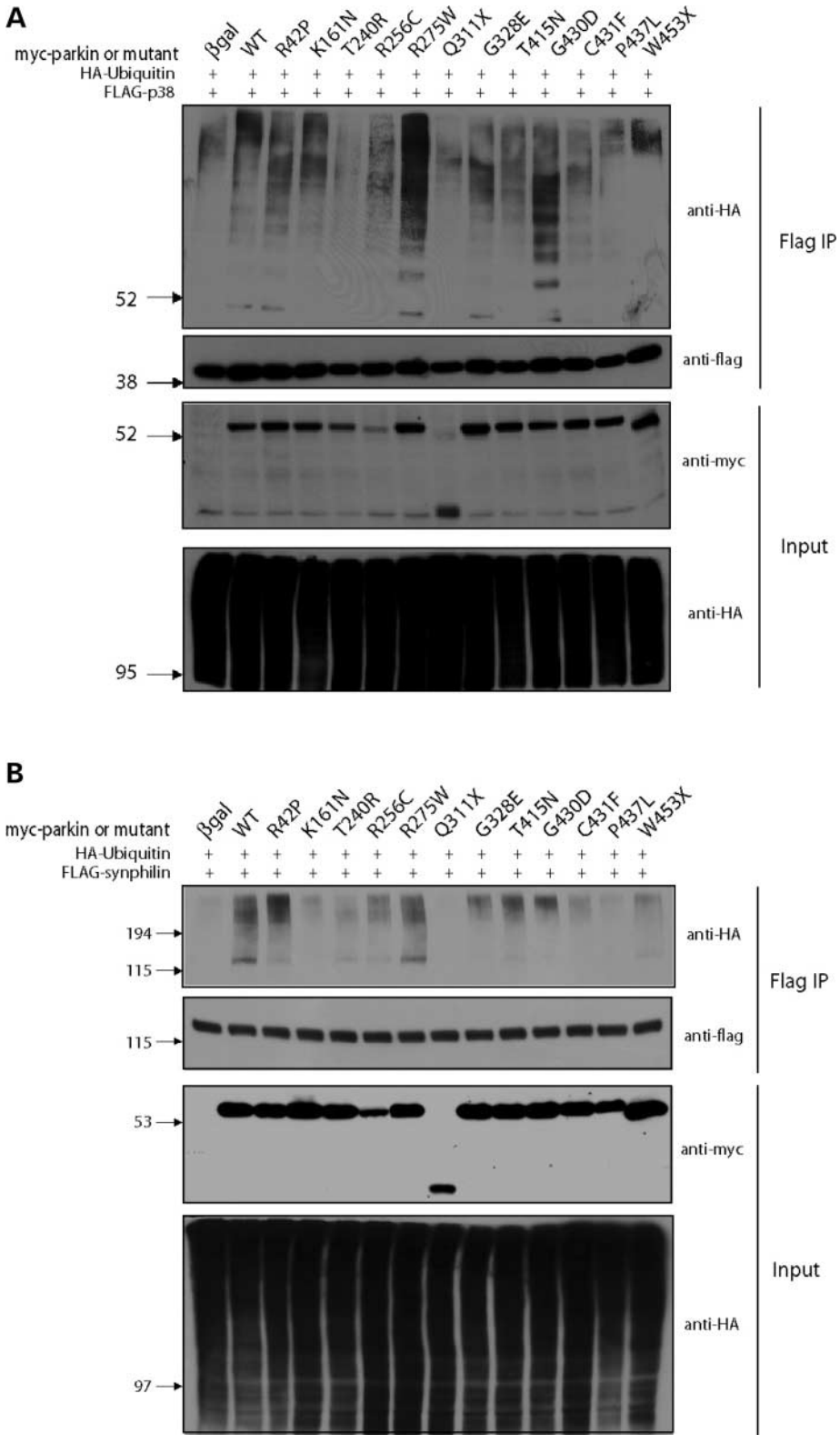

Figure 5. Parkin mutants differentially ubiquitinate substrates. Co-IP experiments were set up to study the relative ubiquitination of flag-tagged p38 (**A**) and flag-tagged synphilin-1 (**B**) in the presence of HA-tagged ubiquitin and myc-tagged wild-type and mutant parkin. The substrates were immunoprecipitated using anti-flag antibody and the samples were probed on a western blot with anti-HA to observe the amount of ubiquitin-positive species in the presence of the various mutants. The IP blots were stripped and reprobed with anti-flag to show the relative amount of p38 (A) and synphilin-1 (B). Input samples were analyzed for the amount of exogenously expressed parkin (anti-myc) and ubiquitin (anti-HA) to show the efficiency of transfection. The experiment was performed three times with similar results.

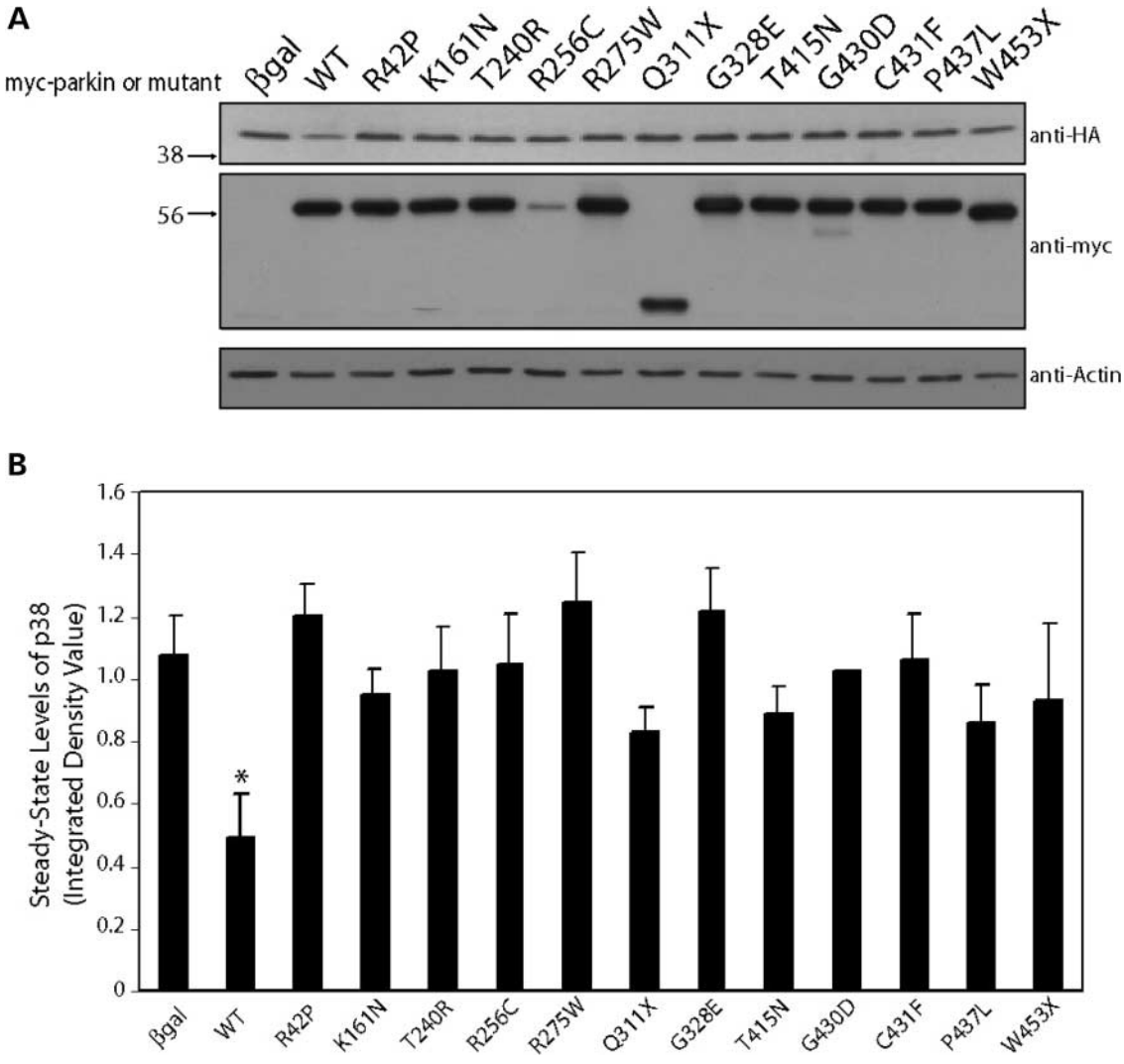

Figure 6. Degradation of p38 and synphilin by parkin mutants. Stable cell lines expressing HA-tagged p38 (**A**) or FLAG-tagged synphilin-1 (**C**) were transiently transfected with βgal, myc-tagged wild-type or mutant parkin. The relative amount of substrate in the cell lysate was assessed by western blotting using anti-HA (A) or anti-FLAG (C) antibody. The blot was stripped and reprobed for exogenously expressed parkin (anti-myc) and a loading control, actin (anti-actin). Densitometric analysis of three independent experiments is represented as a bar graph showing the amount of p38 (**B**) or synphilin-1 (**D**) as a function of integrated density value in the presence of each mutant. The error bars indicate $\pm$SEM and any statistical significance using ANOVA is indicated ($^{*}P < 0.05$, $^{**}P < 0.001$).

The remarkable diversity and number of mutation types that have been discovered in parkin-associated PD patients prompted the search for shared dysfunction of the mutant parkin proteins in the disease process. Most biochemical analyses of parkin have led to the identification of substrates and interactors; only a handful of studies have analyzed the functional consequences of mutations in parkin (47,49,51–55). This study is an extensive biochemical characterization of a representative subset of point mutations in parkin to gain further insight into the mechanism by which mutations in parkin result in disease.

Parkin mutants have differential solubility properties in transfected cells assayed by their extractability using two detergents of varying strengths. Our data confirm and extend recently published data on the altered solubility of parkin mutants (55). Wild-type parkin and some mutants become detergent-insoluble and form intracellular aggresome-like structures, primarily under stress conditions of proteasome inhibition and oxidative stress (40,53,56,57). Consistent with the findings of Wang *et al.* (55), under basal conditions, mutants that were extracted easily by SDS, but not by Triton X-100, had a higher propensity to be sequestered in aggresome-like structures. Thus, some point mutations may cause parkin to be compartmentalized away from its normal cytoplasmic distribution and site of enzymatic activity, leading to an apparent loss-of-function. This phenomenon can be explained for the two nonsense mutants, Q311X and W453X, because the truncations may adopt abnormal conformations that interfere with their normal functioning. In fact, unlike wild-type parkin, Q311X and W453X have been shown to spontaneously misfold and aggregate, which impairs their association with cellular membranes and high

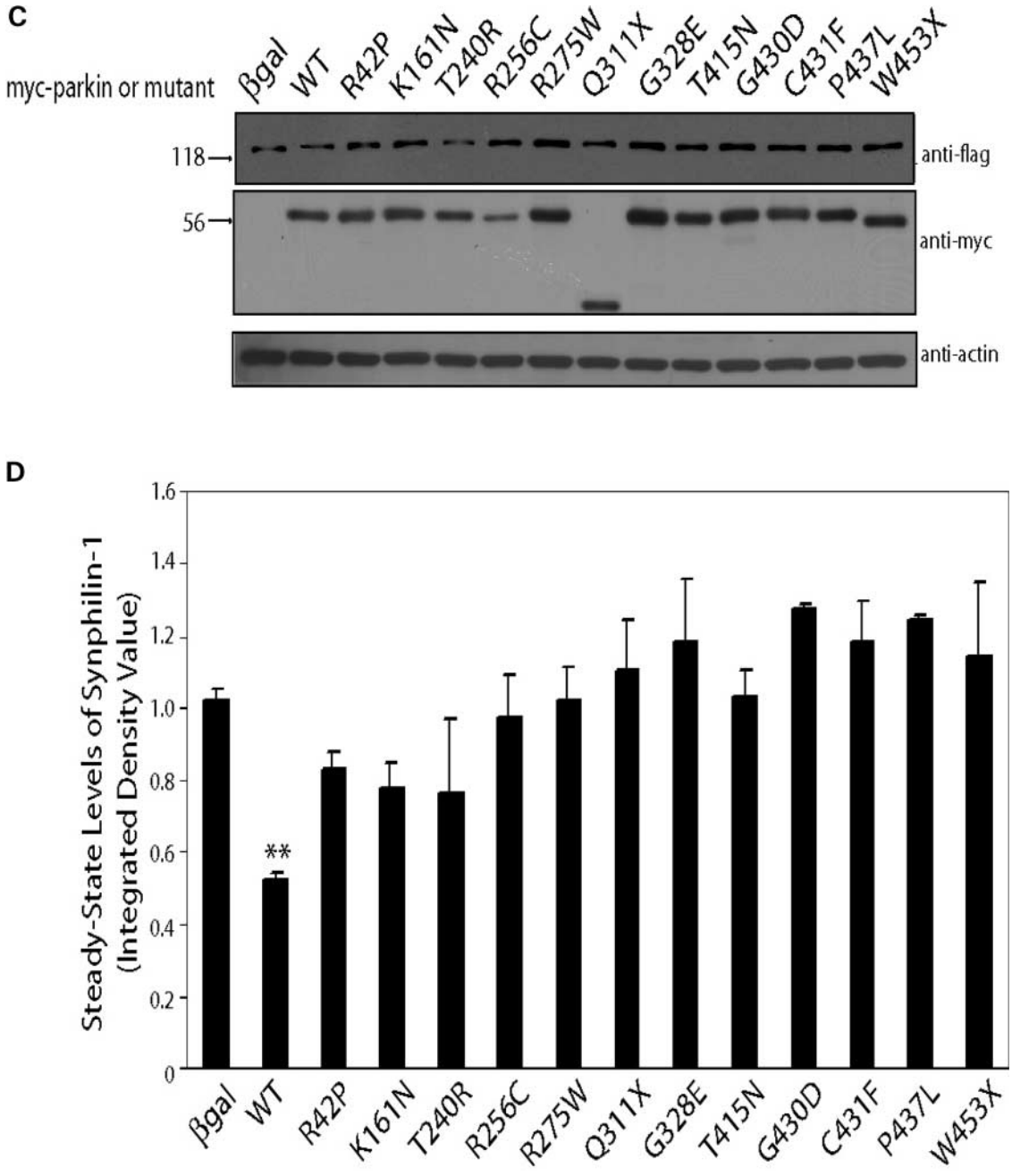

Figure 6. Continued.

molecular weight complexes (53,55). Both Q311X and W453X show an increased affinity for both substrates, p38 and synphilin-1, which could be attributed to disrupted conformations and binding to other, yet unknown, factors that may play a role in substrate release from the E3 ligase. Moreover, structural analysis of the C-terminal RING domain in the RING–IBR–RING motif of parkin suggests that deletion of the PDZ-containing C-terminal amino acid residues in W453X could potentially disrupt the integrity of the second RING finger domain (58). Thus, although the mutant could still be enzymatically active, it may be unable to bind factors that are required to successfully complete the ubiquitination process.

It is more challenging to account for the poor solubility and inclusion formation of the missense mutations, R42P, R256C, R275W and C431F; however, these point mutations occur in domains that are critical to parkin's enzymatic activity, the UBL domain and RING finger domains, and therefore may be more prone to misfolding and aggregation. Further, contrary to what is hypothesized, most of these mutants are observed to have increased auto-ubiquitination activity when compared with wild-type parkin, indicating that the point

mutations may not necessarily abolish (or diminish) parkin's ability to catalyze the addition of ubiquitin molecules. NMR studies suggest that the UBL domain of parkin binds the Rpn10 subunit of the proteasome (59). The R42P mutation has been shown to cause a conformational change in the Rpn10-binding site of the UBL domain in an NMR study, potentially disrupting binding to the proteasome (59). This impaired targeting to the proteasome could potentially explain the accumulation of high molecular weight ubiquitin-positive bands seen with the R42P mutant that are subsequently shuttled into aggresome-like compartmentalized structures in the cell. This finding is in contrast to published data suggesting decreased stability and enhanced degradation of N-terminal parkin mutants, including R42P, compared with wild-type parkin (53). It is conceivable that the use of an N-terminal myc-tag on the R42P mutant used in our study may mask the instability effect caused by this N-terminal mutation. The R256C and R275W mutations have been identified in families with homozygous and compound heterozygous inheritance, with a potential role as susceptibility mutations in late-onset PD (60–62). One patient, with a compound heterozygous mutation in the *parkin* gene (deletion of 40 bp in exon 3 and R275W), was observed to have Lewy body pathology, suggesting a unique effect caused by the R275W mutation that may be absent with other mutations (63). Patients carrying the missense R275W mutation tended to have an earlier age of onset and increased severity of diseases compared with patients with two truncating mutations, implicating a dominant negative effect (64). The identification of single heterozygous mutations in *parkin* may be attributed to the limitations of techniques available for screening genes as large and complex as *parkin.* However, if there is no second mutation in these patients, the single mutation may be dominant negative or may result in haploinsufficiency, which when coupled with nitrosative modifications that further repress activity, manifests disease comparable to loss-of-function mutations (37,52). A potential explanation for the dysfunction of aggregation-prone parkin missense mutants is based on the impairment of the ubiquitin–proteasome system by protein aggregation, either by exhausting one or more factors, such as chaperones, required for normal function or by 'clogging up' the proteasome (65). Thus, the observed mutant parkin-induced aggregation in cells with even a heterozygous mutation could eventually result in severe proteasomal dysfunction, which may be selectively toxic to dopaminergic neurons (35,66). A recent study suggests that BAG5 levels increase after dopaminergic injury, leading to loss of parkin's E3 ligase activity as well as Hsp70-mediated chaperone activity, while enhancing the sequestration of parkin into aggregates and susceptibility of dopaminergic neurons to death (40). It remains to be seen whether parkin's interaction with BAG5 and Hsp70 has a role in the increased propensity of some mutants to be included in protein aggregates and thus affect dopaminergic cell death.

The lack of correlation between the levels of auto-ubiquitination of the mutants and their abilities to ubiquitinate substrate may be attributed to the different linkages that parkin can catalyze. Although it has been established that parkin primarily catalyzes a non-classical lysine-63-linked polyubiquitin chain on synphilin-1, it remains to be seen what type of linkage parkin catalyzes on itself and on p38, although it is clear that parkin is involved in the degradation of p38. Because the various types of ubiquitin linkages result in distinctly different signals, it is not inconceivable to suppose that they require discrete machinery in the cell and therefore may be disrupted differentially by the mutants. A subset of the selected mutants (K161N, T240R, Q311X, T415N and P437L) can be confirmed 'ligase dead' because of their consistent inability to catalyze ubiquitination of self and the chosen substrates. In contrast, mutants that retain their ability to auto-ubiquitinate but not so much the ability to ubiquitinate substrate (R256C, G328E, G430D, C431F and W453X) are largely defective in binding to substrate. Ultimately, in spite of variable binding and ubiquitination properties, all the parkin mutants are unable to effect efficient degradation of substrate(s), leading to an abnormal accumulation that may be toxic to the cell. Overexpression of p38 in cells has been shown to be cytotoxic, and protection from this toxicity is mediated by parkin (21,34). Along with α-synuclein, synphilin-1 has been shown to exert significant cell toxicity, whereas aggresomes formed with both α-synuclein and synphilin-1 are cytoprotective (37,67). However, the absence of synphilin-1 in the mutant parkin-induced protein aggregates suggests that in the absence of α-synuclein, these do not represent the sites of synphilin-1 aggregation.

Some parkin mutations (K161N, T240R, T415N and P437L) are 'model' loss-of-function mutations in that they almost completely abolish binding and ubiquitination activity of parkin. These mutants are similar to wild-type in their extractability and inclusion-forming properties. Two mutants, T240R and T415N, have been shown to impair self-degradation (18). The accumulation of substrates in the presence of these mutants, similar to control, can be straightforwardly ascribed to their diminished E3 ubiquitin ligase activity coupled with the poor binding to substrate.

The mutations described in this study can be classified into one or more of three categories of mechanisms by which they result in loss-of-function mutations (Fig. 7). One class of mutations has modified physical properties, causing them to be sequestered into aggresome-like structures in the cell and away from their site of normal function. A second group of mutations show an abundance of high molecular weight ubiquitin-positive species, suggesting that they may have increased enzymatic activity than wild-type, or are inefficiently targeted to the proteasome. In the event of proteolytic stress, i.e. an overload of the ubiquitin–proteasome system, these mutants may be forced into aggregates (68). The final set of mutations causes significantly reduced or abolished enzymatic activity and binding that is typical of loss-of-function mutations. The three mechanisms converge into a common outcome, the accumulation of substrate due to impaired degradation by 26S proteasome.

In summary, we show that parkin mutants vary considerably in their biochemical properties, such as extractability, intracellular localization, binding to substrate and enzymatic activity, yet they are all associated with a similar end point (Table 2). All the mutants selected in this study were consistently unable to reduce the steady state levels of two substrates,

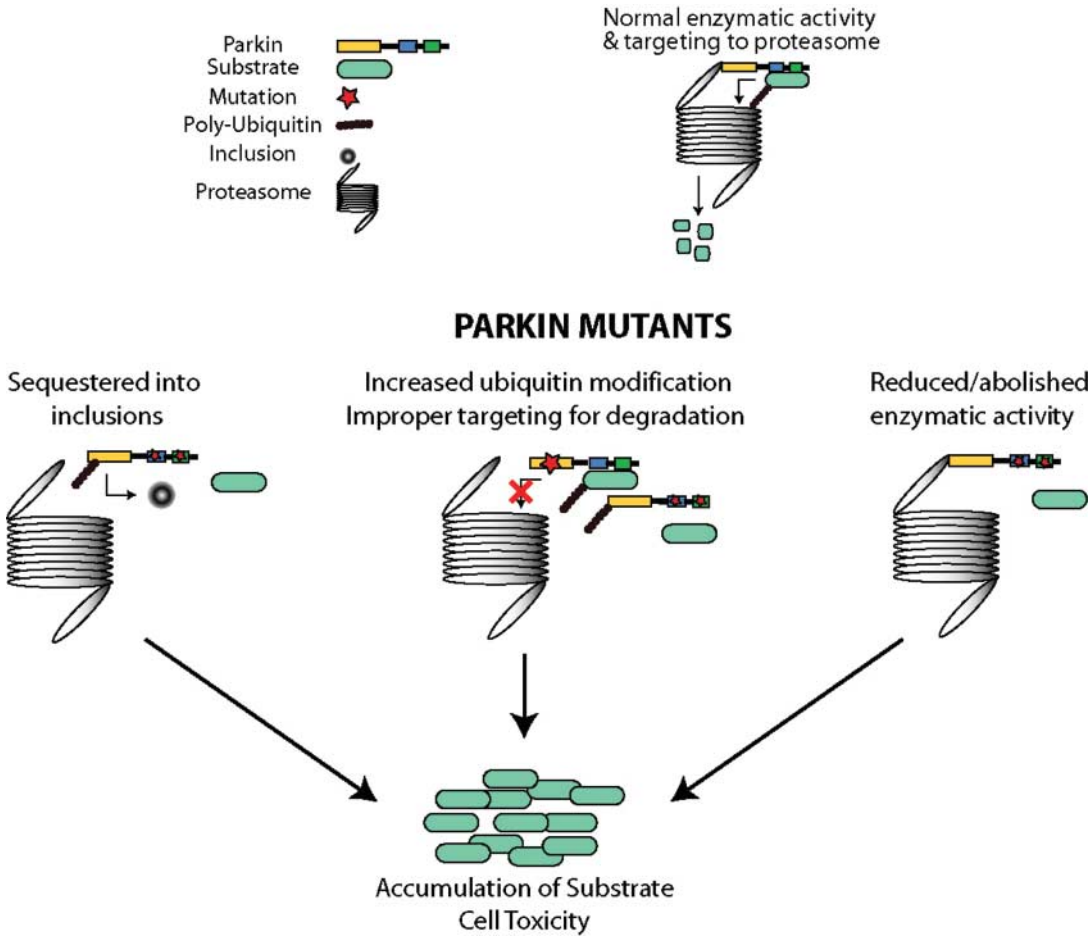

Figure 7. Schematic representing the classification of three main mechanisms by which mutations in *parkin* result in a loss-of-function of the parkin protein and potentially disrupt the cellular homeostasis, leading to cell toxicity. Some mutations result in an altered solubility and localization, sequestering them away from normal site of action, whereas other mutations could result in the improper targeting of self to the proteasome and be cleared from the cytosol into insoluble aggregates. Some mutations result in increased enzymatic activity, suggesting that the gain-of-function mutation results in the disruption of normal parkin function. Finally, the third category of mutations causes reduced or abolished E3 ligase activity. All the mutations in *parkin*, albeit through distinctly different mechanisms, are unable to efficiently degrade substrate.

p38 subunit of aminoacyl-tRNA synthetase and synphilin-1, suggesting that they are deficient in their ability to degrade substrates. These results indicate that not all mutations in parkin result in loss of enzymatic function, but they may manifest as loss-of-function mutations and cause recessive disease because of a defect in one or more steps of the ubiquitination–degradation process.

MATERIALS AND METHODS

Generation of plasmids

Full-length parkin cDNA was cloned into pRK5-myc vector between *Sal*I and *Not*I sites. The parkin mutants pRK5-myc-parkinArg42Pro, pRK5-myc-parkinLys161Asn, pRK5-myc-parkinThr240Arg, pRK5-myc-parkinArg256Cys, pRK5-myc-parkinArg275Trp, pRK5-myc-parkinGln311Stop, pRK5-myc-parkinGly328Glu, pRK5-myc-parkinThr415Asn, pRK5-myc-parkinGly430Asp, pRK5-myc-parkinCys431Phe, pRK5-myc-parkinPro437Leu and pRK5-myc-parkinTrp453-Stop were generated by PCR-mediated site-directed mutagenesis. Full-length cDNA of ubiquitin was cloned into pRK5-HA vector between *Sal*I and *Not*I sites, whereas the cDNAs of synphilin-1 and p38 subunit of aminoacyl-tRNA synthetase were cloned into pRK5-HA vectors as well as pCMV-FLAG vectors between *Sal*I and *Not*I sites. The integrity of the plasmids was confirmed by sequencing and expression on western blots.

Cell culture and transfection

SH-SY5Y cells were grown in DMEM containing 10% FBS in a 5% CO_2 atmosphere at 37°C. Cells were transiently transfected with the target vector(s) using LipofectAMINE PLUS reagents according to the manufacturers' instructions (Invitrogen). To prepare stable transformed cells, SH-SY5Y cells were transfected with pCDNA3-HA-p38 and pCMV-flag-synphilin-1. Cells were selected post-transfection using 700 μg/ml geneticin (Invitrogen). Individual clones were isolated and characterized to confirm expression on a western blot.

Table 2. Summary of results

Parkin	Domain	Solubility	Inclusion	Ubn activity	p38 Binding	Synphilin binding	p38 Ubn	Synphilin Ubn	p38 Degradation	Synphilin degradation
WT		Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
R42P	UBL	Yes	Yes/p38+	↑	↓	↑	↑	↑	No	No
K161N	SH2-like	Yes	No	↓	↓	↓	↓	↓	No	No
T240R	RING 1	Yes	No	↓	↓	↓	↓	↓	No	No
R256C	RING 1	No	Yes	↑	↓	↑	↓	↓	No	No
R275W	RING 1	No	Yes/p38+	↑	↓	↑	↑	↑	No	No
Q311X	Truncate	No	Yes/p38+	↓	↑	↑	↓	↓	No	No
G328E	IBR	Yes	No	↑	↓	↓	↓	↓	No	No
T415N	Before R2	Yes	No	↓	↓	↓	↓	↓	No	No
G430D	RING 2	Yes (low)	No	↑	↓	↓	↑	↓	No	No
C431 F	RING 2	Yes (low)	Yes/p38+	↑	↓	↓	↓	↓	No	No
P437L	RING 2	Yes	No	↓	↓	↓	↓	↓	No	No
W453X	Truncate	No	Yes/p38+	↑	↑	↑	↓	↓	No	No

Summary of the physical and biochemical characterization of parkin mutants compared with wild-type. The mutants were profiled for their relative solubility and localization in the cell, ubiquitination activity, ability to bind, uniquitinate and degrade two substrates, the p38 subunit of aminoacyl-tRNA synthetase and synphilin-1.

Co-immunoprecipitation

SH-SY5Y cells were transfected with 1–2 μg of each plasmid. After 36 h, cells were washed with cold 1× PBS and harvested in immunoprecipitation buffer [(1% Triton X-100 or 1% SDS, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 100 μg/ml PMSF in Tris-buffered saline (TBS)]. The lysate was then rotated at 4°C for 1 h followed by centrifugation at 16 000*g* for 15 min (soluble fraction) or sonicated at 4°C (insoluble fraction). The supernatants were then combined with 50 μl Protein G–Sepharose (Amersham) pre-incubated with antibodies against FLAG or myc (Roche), followed by rotation at 4°C for 2 h. The Protein G–Sepharose was pelleted and washed three times using immoprecipitation buffer, followed by three washes with 50 mM NaCl in TBS and three washes with 1× TBS. The precipitates were resolved on SDS–PAGE gel and subjected to western blot analysis. Bands were visualized with chemiluminescence (Pierce, IL, USA).

Immunocytochemistry and confocal microscopy

About 5×10^4 SH-SY5Y cells were seeded onto polylysine-coated sterile glass cover slips in a 24-well culture plate a day prior to transfection. After attachment, cells were transfected as described earlier with 0.25 μg of the appropriate constructs. Two days later, cells were washed once with PBS and fixed in 3% paraformaldehyde (w/v) for 20 min. The fixed cells were washed three times with PBS before permeabilization in 0.2% (v/v) Triton X-100 in PBS for 5 min. Blocking was then carried out with 5% goat serum in PBS for 1 h. This was followed by incubation in primary antibodies for 1 h at 25°C and secondary antibodies for another hour at 25°C. Immunofluorescent images were acquired on an Olympus confocal microscope.

Statistical analysis

Densitometric analysis of protein bands was done using Alpha Imager 2000 (Alpha Inotech, Wohlen, Switzerland). Data are expressed as mean ± SEM. The results were subjected to the ANOVA test and statistical significance was determined.

ACKNOWLEDGEMENTS

Supported by grants from the NIH, NINDS NS 38377 and NS 48206, the Lee Martin Trust and the Sylvia Nachlas Trust. T.M.D. is the Leonard and Madlyn Abramson Professor in Neurodegenerative Diseases.

Conflict of Interest statement. None declared.

REFERENCES

1. Lang, A.E. and Lozano, A.M. (1998) Parkinson's disease. First of two parts. *N. Engl. J. Med.*, **339**, 1044–1053.
2. Lang, A.E. and Lozano, A.M. (1998) Parkinson's disease. Second of two parts. *N. Engl. J. Med.*, **339**, 1130–1143.
3. Siderowf, A. and Stern, M. (2003) Update on Parkinson disease. *Ann. Intern. Med.*, **138**, 651–658.
4. Jenner, P. and Olanow, C.W. (1998) Understanding cell death in Parkinson's disease. *Ann. Neurol.*, **44**, S72–S84.
5. Steventon, G.B., Sturman, S., Waring, R.H. and Williams, A.C. (2001) A review of xenobiotic metabolism enzymes in Parkinson's disease and motor neuron disease. *Drug Metab. Drug Interact.*, **18**, 79–98.
6. Sherer, T.B., Betarbet, R. and Greenamyre, J.T. (2002) Environment, mitochondria, and Parkinson's disease. *Neuroscientist*, **8**, 192–197.
7. Burn, D.J., Mark, M.H., Playford, E.D., Maraganore, D.M., Zimmerman, T.R., Jr., Duvoisin, R.C., Harding, A.E., Marsden, C.D. and Brooks, D.J. (1992) Parkinson's disease in twins studied with 18F-dopa and positron emission tomography. *Neurology*, **42**, 1894–1900.
8. Maher, N.E., Currie, L.J., Lazzarini, A.M., Wilk, J.B., Taylor, C.A., Saint-Hilaire, M.H., Feldman, R.G., Golbe, L.I., Wooten, G.F. and Myers, R.H. (2002) Segregation analysis of Parkinson disease revealing evidence for a major causative gene. *Am. J. Med. Genet.*, **109**, 191–197.
9. Maher, N.E., Golbe, L.I., Lazzarini, A.M., Mark, M.H., Currie, L.J., Wooten, G.F., Saint-Hilaire, M., Wilk, J.B., Volcjak, J., Maher, J.E. *et al.* (2002) Epidemiologic study of 203 sibling pairs with Parkinson's disease: the GenePD study. *Neurology*, **58**, 79–84.
10. Dawson, T.M. and Dawson, V.L. (2003) Rare genetic mutations shed light on the pathogenesis of Parkinson disease. *J. Clin. Invest.*, **111**, 145–151.
11. Brice, A. (2005) How much does dardarin contribute to Parkinson's disease? *Lancet*, **365**, 363–364.

12. Vila, M. and Przedborski, S. (2004) Genetic clues to the pathogenesis of Parkinson's disease. *Nat. Med.*, **10** (suppl) S58–S62.
13. Matsumine, H., Saito, M., Shimoda-Matsubayashi, S., Tanaka, H., Ishikawa, A., Nakagawa-Hattori, Y., Yokochi, M., Kobayashi, T., Igarashi, S., Takano, H. *et al.* (1997) Localization of a gene for an autosomal recessive form of juvenile parkinsonism to chromosome 6q25.2–27. *Am. J. Hum. Genet.*, **60**, 588–596.
14. Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y. and Shimizu, N. (1998) Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature*, **392**, 605–608.
15. Hayashi, S., Wakabayashi, K., Ishikawa, A., Nagai, H., Saito, M., Maruyama, M., Takahashi, T., Ozawa, T., Tsuji, S. and Takahashi, H. (2000) An autopsy case of autosomal-recessive juvenile parkinsonism with a homozygous exon 4 deletion in the parkin gene. *Mov. Disord.*, **15**, 884–888.
16. Gouider-Khouja, N., Larnaout, A., Amouri, R., Sfar, S., Belal, S., Ben Hamida, C., Ben Hamida, M., Hattori, N., Mizuno, Y. and Hentati, F. (2003) Autosomal recessive parkinsonism linked to parkin gene in a Tunisian family. Clinical, genetic and pathological study. *Parkinsonism Relat. Disord.*, **9**, 247–251.
17. Zhang, Y., Gao, J., Chung, K.K., Huang, H., Dawson, V.L. and Dawson, T.M. (2000) Parkin functions as an E2-dependent ubiquitin-protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. *Proc. Natl Acad. Sci. USA*, **97**, 13354–13359.
18. Shimura, H., Hattori, N., Kubo, S., Mizuno, Y., Asakawa, S., Minoshima, S., Shimizu, N., Iwai, K., Chiba, T., Tanaka, K. *et al.* (2000) Familial Parkinson disease gene product, parkin, is a ubiquitin–protein ligase. *Nat. Genet.*, **25**, 302–305.
19. Imai, Y., Soda, M. and Takahashi, R. (2000) Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin–protein ligase activity. *J. Biol. Chem.*, **275**, 35661–35664.
20. Chung, K.K., Zhang, Y., Lim, K.L., Tanaka, Y., Huang, H., Gao, J., Ross, C.A., Dawson, V.L. and Dawson, T.M. (2001) Parkin ubiquitinates the alpha-synuclein-interacting protein, synphilin-1: implications for Lewy-body formation in Parkinson disease. *Nat. Med.*, **7**, 1144–1150.
21. Corti, O., Hampe, C., Koutnikova, H., Darios, F., Jacquier, S., Prigent, A., Robinson, J.C., Pradier, L., Ruberg, M., Mirande, M. *et al.* (2003) The p38 subunit of the aminoacyl-tRNA synthetase complex is a Parkin substrate: linking protein biosynthesis and neurodegeneration. *Hum. Mol. Genet.*, **12**, 1427–1437.
22. Imai, Y., Soda, M., Inoue, H., Hattori, N., Mizuno, Y. and Takahashi, R. (2001) An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of parkin. *Cell*, **105**, 891–902.
23. Huynh, D.P., Scoles, D.R., Nguyen, D. and Pulst, S.M. (2003) The autosomal recessive juvenile Parkinson disease gene product, parkin, interacts with and ubiquitinates synaptotagmin XI. *Hum. Mol. Genet.*, **12**, 2587–2597.
24. Shimura, H., Schlossmacher, M.G., Hattori, N., Frosch, M.P., Trockenbacher, A., Schneider, R., Mizuno, Y., Kosik, K.S. and Selkoe, D.J. (2001) Ubiquitination of a new form of alpha-synuclein by parkin from human brain: implications for Parkinson's disease. *Science*, **293**, 263–269.
25. Staropoli, J.F., McDermott, C., Martinat, C., Schulman, B., Demireva, E. and Abeliovich, A. (2003) Parkin is a component of an SCF-like ubiquitin ligase complex and protects postmitotic neurons from kainate excitotoxicity. *Neuron*, **37**, 735–749.
26. Ren, Y., Zhao, J. and Feng, J. (2003) Parkin binds to alpha/beta tubulin and increases their ubiquitination and degradation. *J. Neurosci.*, **23**, 3316–3324.
27. Goldberg, M.S., Fleming, S.M., Palacino, J.J., Cepeda, C., Lam, H.A., Bhatnagar, A., Meloni, E.G., Wu, N., Ackerson, L.C., Klapstein, G.J. *et al.* (2003) Parkin-deficient mice exhibit nigrostriatal deficits but not loss of dopaminergic neurons. *J. Biol. Chem.*, **278**, 43628–43635.
28. Greene, J.C., Whitworth, A.J., Kuo, I., Andrews, L.A., Feany, M.B. and Pallanck, L.J. (2003) Mitochondrial pathology and apoptotic muscle degeneration in *Drosophila* parkin mutants. *Proc. Natl Acad. Sci. USA*, **100**, 4078–4083.
29. Itier, J.M., Ibanez, P., Mena, M.A., Abbas, N., Cohen-Salmon, C., Bohme, G.A., Laville, M., Pratt, J., Corti, O., Pradier, L. *et al.* (2003) Parkin gene inactivation alters behaviour and dopamine neurotransmission in the mouse. *Hum. Mol. Genet.*, **12**, 2277–2291.
30. Perez, F.A. and Palmiter, R.D. (2005) Parkin-deficient mice are not a robust model of parkinsonism. *Proc. Natl Acad. Sci. USA*, **102**, 2174–2179.
31. von Coelln, R., Thomas, B., Savitt, J.M., Lim, K.L., Sasaki, M., Hess, E.J., Dawson, V.L. and Dawson, T.M. (2004) Loss of locus coeruleus neurons and reduced startle in parkin null mice. *Proc. Natl Acad. Sci. USA*, **101**, 10744–10749.
32. Jiang, H., Jiang, Q. and Feng, J. (2004) Parkin increases dopamine uptake by enhancing the cell surface expression of dopamine transporter. *J. Biol. Chem.*, **279**, 54380–54386.
33. Choi, P., Snyder, H., Petrucelli, L., Theisler, C., Chong, M., Zhang, Y., Lim, K., Chung, K.K., Kehoe, K., D'Adamio, L. *et al.* (2003) SEPT5_v2 is a parkin-binding protein. *Brain Res. Mol. Brain Res.*, **117**, 179–189.
34. Ko, H.S., von Coelln, R., Sriram, S.R., Kim, S.W., Chung, K.K., Pletnikova, O., Troncoso, J.C., Johnson, B., Saffary, R., Goh, E.L. *et al.* (2005) Accumulation of the authentic parkin substrate, aminoacyl-tRNA synthetase cofactor, p38/JTV-1, Leads to Catecholaminergic Cell Death. *J. Neurosci.*, in press.
35. Petrucelli, L., O'Farrell, C., Lockhart, P.J., Baptista, M., Kehoe, K., Vink, L., Choi, P., Wolozin, B., Farrer, M., Hardy, J. *et al.* (2002) Parkin protects against the toxicity associated with mutant alpha-synuclein: proteasome dysfunction selectively affects catecholaminergic neurons. *Neuron*, **36**, 1007–1019.
36. Oluwatosin-Chigbu, Y., Robbins, A., Scott, C.W., Arriza, J.L., Reid, J.D. and Zysk, J.R. (2003) Parkin suppresses wild-type alpha-synuclein-induced toxicity in SHSY-5Y cells. *Biochem. Biophys. Res. Commun.*, **309**, 679–684.
37. Chung, K.K., Thomas, B., Li, X., Pletnikova, O., Troncoso, J.C., Marsh, L., Dawson, V.L. and Dawson, T.M. (2004) S-nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function. *Science*, **304**, 1328–1331.
38. Darios, F., Corti, O., Lucking, C.B., Hampe, C., Muriel, M.P., Abbas, N., Gu, W.J., Hirsch, E.C., Rooney, T., Ruberg, M. *et al.* (2003) Parkin prevents mitochondrial swelling and cytochrome c release in mitochondria-dependent cell death. *Hum. Mol. Genet.*, **12**, 517–526.
39. Fallon, L., Moreau, F., Croft, B.G., Labib, N., Gu, W.J. and Fon, E.A. (2002) Parkin and CASK/LIN-2 associate via a PDZ-mediated interaction and are co-localized in lipid rafts and postsynaptic densities in brain. *J. Biol. Chem.*, **277**, 486–491.
40. Kalia, S.K., Lee, S., Smith, P.D., Liu, L., Crocker, S.J., Thorarinsdottir, T.E., Glover, J.R., Fon, E.A., Park, D.S. and Lozano, A.M. (2004) BAG5 inhibits parkin and enhances dopaminergic neuron degeneration. *Neuron*, **44**, 931–945.
41. Yamamoto, A., Friedlein, A., Imai, Y., Takahashi, R., Kahle, P.J. and Haass, C. (2005) Parkin phosphorylation and modulation of its E3 ubiquitin ligase activity. *J. Biol. Chem.*, **280**, 3390–3399.
42. Yao, D., Gu, Z., Nakamura, T., Shi, Z.Q., Ma, Y., Gaston, B., Palmer, L.A., Rockenstein, E.M., Zhang, Z., Masliah, E. *et al.* (2004) Nitrosative stress linked to sporadic Parkinson's disease: S-nitrosylation of parkin regulates its E3 ubiquitin ligase activity. *Proc. Natl Acad. Sci. USA*, **101**, 10810–10814.
43. Lucking, C.B., Durr, A., Bonifati, V., Vaughan, J., De Michele, G., Gasser, T., Harhangi, B.S., Meco, G., Denefle, P., Wood, N.W. *et al.* (2000) Association between early-onset Parkinson's disease and mutations in the parkin gene. French Parkinson's Disease Genetics Study Group. *N. Engl. J. Med.*, **342**, 1560–1567.
44. West, A.B. and Maidment, N.T. (2004) Genetics of parkin-linked disease. *Hum. Genet.*, **114**, 327–336.
45. Mata, I.F., Lockhart, P.J. and Farrer, M.J. (2004) Parkin genetics: one model for Parkinson's disease. *Hum. Mol. Genet.*, **13**, R127–R133.
46. Hedrich, K., Eskelson, C., Wilmot, B., Marder, K., Harris, J., Garrels, J., Meija-Santana, H., Vieregge, P., Jacobs, H., Bressman, S.B. *et al.* (2004) Distribution, type, and origin of parkin mutations: review and case studies. *Mov. Disord.*, **19**, 1146–1157.
47. Gu, W.J., Corti, O., Araujo, F., Hampe, C., Jacquier, S., Lucking, C.B., Abbas, N., Duyckaerts, C., Rooney, T., Pradier, L. *et al.* (2003) The C289G and C418R missense mutations cause rapid sequestration of human parkin into insoluble aggregates. *Neurobiol. Dis.*, **14**, 357–364.

48. Rankin, C.A., Joazeiro, C.A., Floor, E. and Hunter, T. (2001) E3 ubiquitin-protein ligase activity of parkin is dependent on cooperative interaction of RING finger (TRIAD) elements. *J. Biomed. Sci.*, **8**, 421–429.
49. Wang, C., Tan, J.M., Ho, M.W., Zaiden, N., Wong, S.H., Chew, C.L., Eng, P.W., Lim, T.M., Dawson, T.M. and Lim, K.L. (2005) Alterations in the solubility and intracellular localization of parkin by several familial Parkinson's disease-linked point mutations. *J. Neurochem.*, **93**, 422–431.
50. Doss-Pepe, E.W., Chen, L. and Madura, K. (2005) Alpha-synuclein and parkin contribute to the assembly of ubiquitin lysine63-linked multiubiquitin chains. *J. Biol. Chem.*
51. Lim, K.L., Chew, K.C., Tan, J.M., Wang, C., Chung, K.K., Zhang, Y., Tanaka, Y., Smith, W., Engelender, S., Ross, C.A. *et al.* (2005) Parkin mediates nonclassical, proteasomal-independent ubiquitination of synphilin-1: implications for Lewy body formation. *J. Neurosci.*, **25**, 2002–2009.
52. von Coelln, R., Dawson, V.L. and Dawson, T.M. (2004) Parkin-associated Parkinson's disease. *Cell Tissue Res.*, **318**, 175–184.
53. Winklhofer, K.F., Henn, I.H., Kay-Jackson, P.C., Heller, U. and Tatzelt, J. (2003) Inactivation of parkin by oxidative stress and C-terminal truncations: a protective role of molecular chaperones. *J. Biol. Chem.*, **278**, 47199–47208.
54. Cookson, M.R., Lockhart, P.J., McLendon, C., O'Farrell, C., Schlossmacher, M. and Farrer, M.J. (2003) RING finger 1 mutations in parkin produce altered localization of the protein. *Hum. Mol. Genet.*, **12**, 2957–2965.
55. Henn, I.H., Gostner, J.M., Lackner, P., Tatzelt, J. and Winklhofer, K.F. (2005) Pathogenic mutations inactivate parkin by distinct mechanisms. *J. Neurochem.*, **92**, 114–122.
56. Ardley, H.C., Scott, G.B., Rose, S.A., Tan, N.G., Markham, A.F. and Robinson, P.A. (2003) Inhibition of proteasomal activity causes inclusion formation in neuronal and non-neuronal cells overexpressing parkin. *Mol. Biol. Cell*, **14**, 4541–4556.
57. Junn, E., Lee, S.S., Suhr, U.T. and Mouradian, M.M. (2002) Parkin accumulation in aggresomes due to proteasome impairment. *J. Biol. Chem.*, **277**, 47870–47877.
58. Capili, A.D., Edghill, E.L., Wu, K. and Borden, K.L. (2004) Structure of the C-terminal RING finger from a RING–IBR–RING/TRIAD motif reveals a novel zinc-binding domain distinct from a RING. *J. Mol. Biol.*, **340**, 1117–1129.
59. Sakata, E., Yamaguchi, Y., Kurimoto, E., Kikuchi, J., Yokoyama, S., Yamada, S., Kawahara, H., Yokosawa, H., Hattori, N., Mizuno, Y. *et al.* (2003) Parkin binds the Rpn10 subunit of 26S proteasomes through its ubiquitin-like domain. *EMBO Rep.*, **4**, 301–306.
60. West, A., Periquet, M., Lincoln, S., Lucking, C.B., Nicholl, D., Bonifati, V., Rawal, N., Gasser, T., Lohmann, E., Deleuze, J.F. *et al.* (2002) Complex relationship between parkin mutations and Parkinson disease. *Am. J. Med. Genet.*, **114**, 584–591.
61. Abbas, N., Lucking, C.B., Ricard, S., Durr, A., Bonifati, V., De Michele, G., Bouley, S., Vaughan, J.R., Gasser, T., Marconi, R. *et al.* (1999) A wide variety of mutations in the parkin gene are responsible for autosomal recessive parkinsonism in Europe. French Parkinson's Disease Genetics Study Group and the European Consortium on Genetic Susceptibility in Parkinson's Disease. *Hum. Mol. Genet.*, **8**, 567–574.
62. Oliveira, S.A., Scott, W.K., Martin, E.R., Nance, M.A., Watts, R.L., Hubble, J.P., Koller, W.C., Pahwa, R., Stern, M.B., Hiner, B.C. *et al.* (2003) Parkin mutations and susceptibility alleles in late-onset Parkinson's disease. *Ann. Neurol.*, **53**, 624–629.
63. Farrer, M., Chan, P., Chen, R., Tan, L., Lincoln, S., Hernandez, D., Forno, L., Gwinn-Hardy, K., Petrucelli, L., Hussey, J. *et al.* (2001) Lewy bodies and parkinsonism in families with parkin mutations. *Ann. Neurol.*, **50**, 293–300.
64. Lohmann, E., Periquet, M., Bonifati, V., Wood, N.W., De Michele, G., Bonnet, A.M., Fraix, V., Broussolle, E., Horstink, M.W., Vidailhet, M. *et al.* (2003) How much phenotypic variation can be attributed to parkin genotype? *Ann. Neurol.*, **54**, 176–185.
65. Bence, N.F., Sampat, R.M. and Kopito, R.R. (2001) Impairment of the ubiquitin–proteasome system by protein aggregation. *Science*, **292**, 1552–1555.
66. McNaught, K.S., Mytilineou, C., Jnobaptiste, R., Yabut, J., Shashidharan, P., Jennert, P. and Olanow, C.W. (2002) Impairment of the ubiquitin–proteasome system causes dopaminergic cell death and inclusion body formation in ventral mesencephalic cultures. *J. Neurochem.*, **81**, 301–306.
67. Tanaka, M., Kim, Y.M., Lee, G., Junn, E., Iwatsubo, T. and Mouradian, M.M. (2004) Aggresomes formed by alpha-synuclein and synphilin-1 are cytoprotective. *J. Biol. Chem.*, **279**, 4625–4631.
68. Johnston, J.A., Ward, C.L. and Kopito, R.R. (1998) Aggresomes: a cellular response to misfolded proteins. *J. Cell Biol.*, **143**, 1883–1898.
69. Terreni, L., Calabrese, E., Calella, A.M., Forloni, G. and Mariani, C. (2001) New mutation (R42P) of the parkin gene in the ubiquitinlike domain associated with parkinsonism. *Neurology*, **56**, 463–466.
70. Forloni, G., Terreni, L., Bertani, I., Fogliarino, S., Invernizzi, R., Assini, A., Ribizzi, G., Negro, A., Calabrese, E., Volontc, M.A. *et al.* (2002) Protein misfolding in Alzheimer's and Parkinson's disease: genetics and molecular mechanisms. *Neurobiol. Aging*, **23**, 957–976.
71. Hattori, N., Matsumine, H., Asakawa, S., Kitada, T., Yoshino, H., Elibol, B., Brookes, A.J., Yamamura, Y., Kobayashi, T., Wang, M. *et al.* (1998) Point mutations (Thr240Arg and Gln311Stop) [correction of Thr240Arg and Ala311Stop] in the Parkin gene. *Biochem. Biophys. Res. Commun.*, **249**, 754–758.
72. Oliveira, S.A., Scott, W.K., Martin, E.R., Nance, M.A., Watts, R.L., Hubble, J.P., Koller, W.C., Pahwa, R., Stern, M.B., Hiner, B.C. *et al.* (2003) Parkin mutations and susceptibility alleles in late-onset Parkinson's disease. *Ann. Neurol.*, **53**, 624–629.
73. Pramstaller, P.P., Kunig, G., Leenders, K., Kann, M., Hedrich, K., Vieregge, P., Goetz, C.G. and Klein, C. (2002) Parkin mutations in a patient with hemiparkinsonism-hemiatrophy: a clinical-genetic and PET study. *Neurology*, **58**, 808–810.
74. Khan, N.L., Graham, E., Critchlcy, P., Schrag, A.E., Wood, N.W., Lees, A.J., Bhatia, K.P., and Quinn, N. (2003) Parkin disease: a phenotypic study of a large case series. *Brain*, **126**, 1279–1292.
75. Periquet, M., Lucking, C., Vaughan, J., Bonifati, V., Durr, A., De Michele, G., Ilorstink, M., Parrer, M., Illarioshkin, S.N., Pollak, P. *et al.* (2001) Orgin of the mutations in the parkin gene in Europe: exon rearrangements are independent recurrent events, whereas point mutations may result from Founder effects. *Am. J. Hum. Genet.*, **68**, 617–626.
76. Foroud, T., Uniacke, S.K., Liu., L., Pankratz, N., Rudolph, A., Halter, C., Shults, C., Marder, K., Conneally, P.M. and Nichols,, W.C. (2003) Heterozygosity for a mutation in the parkin gene leads to later onset Parkinson disease. *Neurology*, **60**, 796–801.
77. Rawal, N., Periquet, M., Lohmann, E., Lucking, C.B., Teive, H.A., Ambrosio, G., Raskin, S., Lincoln, S., Hattori, N., Guimaraes, J. *et al.* (2003) New parkin mutations and atypical phenotypes in families with autosomal recessive parkinsonism. *Neurology*, **60**, 1378–1381.
78. Maruyama, M., Ikeuchi, T., Saito, M., Ishikawa, A., Yuasa, T., Tanaka, H., Hayashi, S., Wakabayashi, K., Takahashi, H. and Tsuji, S. (2000) Novel mutations, pseudo-dominant inheritance, and possible familial affects in patients with autosomal recessive juvenile parkinsonism. *Ann. Neurol.*, **48**, 245–250.
79. Nichols, W.C., Pankratz, N., Uniacke, S.K., Pauciulo, M.W., Halter, C., Rudolph, A., Conneally, P.M. and Foroud, T. (2002) Linkage stratification and mutation analysis at the Parkin locus identifies mutation positive Parkinson's disease families. *J. Med. Genet.*, **39**, 489–492.