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How does parkin ligate ubiquitin to Parkinson's disease?

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Recessive mutations in the human *PARKIN* gene are the most common cause of hereditary parkinsonism, which arises from the degeneration of dopaminergic neurons in the substantia nigra. However, the molecular mechanisms by which the loss of parkin causes dopaminergic neurodegeneration are not well understood. Parkin is an enzyme that ubiquitinates several candidate substrate proteins and thereby targets them for proteasomal degradation. Hypothesis-driven searches have led to the discovery of aggregation-prone protein substrates of parkin. Moreover, the enzyme is upregulated when under unfolded protein stress. Thus, loss-of-function mutations of parkin might impair the removal of potentially toxic protein aggregates. However, the limited neuropathological information that is available from parkin-proven patients, as well as the recent knockout of the parkin gene in fruit flies and mice, may indicate a more complex disease mechanism, possibly involving the misfolding of parkin itself or of additional substrates. The risk factors that predispose dopaminergic neurons to degenerate on parkin failure are yet to be identified.

Keywords: ubiquitin ligase; unfolded protein stress; parkinsonism; dopamine; neurodegeneration

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Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder in the elderly and affects approximately 1% of the population who are over 60 years old. PD symptoms include resting tremor, rigor and a generalized reduction of locomotor performance. The loss of dopaminergic neurons that project from the midbrain substantia nigra to the striatum underlies parkinsonian symptoms. Dopamine replacement therapy can ameliorate the locomotor dysfunction in patients for some time, but PD relentlessly proceeds until the patient dies.

The majority of idiopathic PD cases occur sporadically, but in some patients parkinsonism is inherited (Dawson & Dawson, 2003). Several gene loci are associated with familial parkinsonism. Of particular note is α-synuclein (α-SYN), which is not only genetically linked to rare cases of PD (Golbe & Mouradian, 2004), but also comprises the main constituent of Lewy bodies (LBs). These are the hallmark inclusions that form the basis of the neuropathological diagnosis of all PD patients (Goedert, 2001). LBs are often ubiquitin-immunoreactive, and failure of the ubiquitin-proteasome system is implicated in PD (McNaught & Olanow, 2003).

A logical link between parkinsonism and the ubiquitin-proteasome system can be deduced from the finding that the PD gene product parkin has ubiquitin ligase activity (Shimura *et al*, 2000). The gene that encodes parkin was originally associated with autosomal-recessive juvenile parkinsonism (AR-JP) in Japan (Kitada *et al*, 1998). A great variety of mutations in the parkin gene have subsequently been found worldwide in families with early-onset parkinsonism. It is estimated that up to 50% of hereditary parkinsonism cases are due to parkin mutations (for a review, see Mata *et al*, 2004). Although their phenotypes vary, these 'parkin patients' tend to develop parkinsonism at an early age with slow disease progression, and they respond very well to the standard PD drug levodopa, which increases the supply of dopamine.

What are the molecular and cellular consequences of parkin mutations, how do they impinge on dopaminergic neuron viability, and what does this tell us about the pathological mechanisms of sporadic PD? Here, we review recent progress in the investigation of parkin mutations and protein misfolding and of parkin substrates, with special emphasis on the recently developed knockout animal models.

Parkin ubiquitinates a diverse set of substrates

Parkin belongs to the 'really interesting new gene' (RING) finger class of E3 ubiquitin ligases (Shimura *et al*, 2000). The carboxy-terminal half of parkin comprises a specific arrangement of three zinc-finger domains: two RING fingers flank a domain known as the in-between RING (IBR) domain (Fig 1). This RING–IBR–RING configuration defines a protein superfamily, which includes dorfin and ariadne ubiquitin ligases (Marín & Ferrús, 2002). The extreme amino terminus of parkin is structurally related to ubiquitin (Sakata *et al*, 2003). This ubiquitin-like (UBL) domain is separated from the unique parkin domain (UPD; Kahle *et al*, 2000) and the

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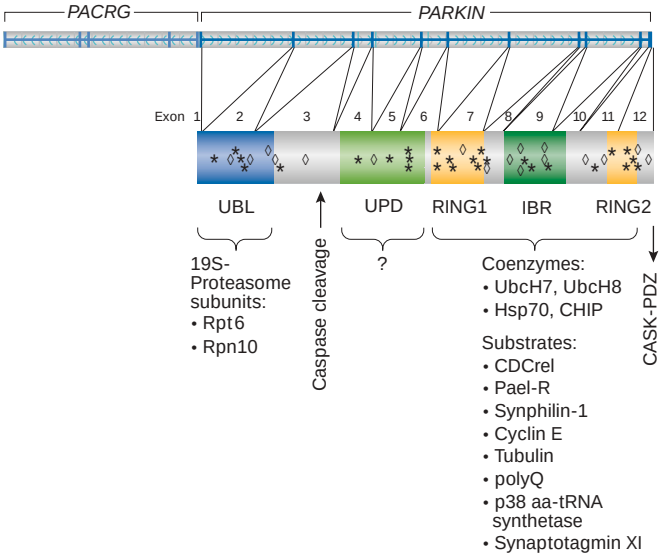

Fig 1 | Genomic and domain arrangement of parkin. Within the human chromosome band 6q23, *PARKIN* and the parkin co-regulated gene (*PACRG*) are located in a head-to-head configuration. The 12 exons of *PARKIN* are spliced and translated into a 457-amino-acid protein. The positions of disease-associated mutations are indicated using asterisks for missense mutations and diamonds for nonsense/frameshift mutations. Functional domains within the parkin protein include an amino-terminal ubiquitin-like (UBL) domain and the cysteine-rich unique parkin domain (UPD) followed by two really interesting new gene (RING) fingers flanking an in-between RING (IBR) domain. The UBL binds to 19S proteasome subunits, the RING–IBR–RING domain binds to specific co-enzymes and substrates. The extreme carboxyl terminus binds to the PDZ motif of calcium/calmodulin-dependent serine protein kinase and targets a proportion of parkin to the postsynaptic density.

RING–IBR–RING region by a linker (Fig 1) that contains cleavage sites for the pro-apoptotic caspases 1 and 8 (Kahns *et al*, 2003). The extreme C-terminus of parkin binds to the postsynaptic density 95, discs large, zona occludens (PDZ) motif of the calcium/calmodulin-dependent serine protein kinase, thereby promoting the synaptic transport of parkin (Fallon *et al*, 2002). All of these structural elements are essential for the functional integrity of parkin, because point mutations cluster in the UBL, UPD, RING–IBR–RING and the C-terminus (Fig 1).

The most straightforward mechanism by which the recessive loss of parkin could eliminate dopaminergic neurons would involve some neurotoxic substrate proteins, which would accumulate when there is insufficient parkin for its ubiquitin-proteasome system-dependent degradation (Kahle *et al*, 2000). Several proteins have been identified as parkin substrates (Fig 1), but the molecular mechanism of dopaminergic neuron loss in AR-JP patients remains obscure.

One of the best-characterized parkin substrates is the parkin-associated endothelin receptor-like receptor (Pael-R), which is a multipass endoplasmic reticulum (ER) transmembrane protein, the function of which is unknown (Imai *et al*, 2001). Folding of Pael-R is a formidable challenge to cells and is chaperoned by the interaction of the nascent Pael-R polypeptide with the 70-kDa heat-shock protein (Hsp70). Overexpression of Pael-R elicits a marked ER stress response (Imai *et al*, 2001). Parkin is then upregulated (Imai *et al*, 2000) and directly suppresses Pael-R-mediated toxicity through the ubiquitination and proteasomal degradation of Pael-R (Imai *et al*, 2001). When Pael-R misfolding exceeds the cellular chaperone capacity, a protein known as C-terminus of Hsp70-interacting protein (CHIP) is upregulated, which sequesters Hsp70 and facilitates parkin-mediated Pael-R ubiquitination (Imai *et al*, 2002). Thus, parkin contributes to the suppression of ER stress due to proteasomal targeting of the highly aggregative Pael-R protein. It remains to be shown to what extent misfolding of Pael-R contributes to the specific loss of dopaminergic neurons in AR-JP patients. Moreover, it should be established whether Pael-R is a unique parkin substrate, or whether other difficult-to-fold polytopic membrane proteins are subject to parkin-mediated ubiquitination.

Parkin has been generally implicated in the elimination of aggregation-prone cytosolic proteins, including polyglutamine polypeptides (Tsai *et al*, 2003). Ataxin 3 or synthetic green fluorescent protein fused to a stretch of 79 glutamines confers cytosolic protein-misfolding stress. The cell reacts to this condition with an upregulation of Hsp70, similar to the ER stress mediated by Pael-R. When the polyglutamine expansion of cytosolic proteins exceeds the chaperone capacity of Hsp70, the latter recruits parkin through its interaction with the RING–IBR–RING domain (Fig 1), which ubiquitinates the polyglutamine protein and thereby labels it for proteasomal degradation (Tsai *et al*, 2003). In fact, proteasomal delivery of polyglutamine proteins and other parkin substrates might be further facilitated by direct binding of the UBL domain of parkin to the regulatory 19S proteasome (Sakata *et al*, 2003; Tsai *et al*, 2003).

If parkin functions to ubiquitinate aggregation-prone proteins, α-SYN would be an obvious candidate substrate. Using a particular combination of immunoprecipitating/immunoblotting antibodies, a rather unusual cytosolic O-linked glycosylated α-SYN species (α-Sp22) has been detected. This modification was reported to be a prerequisite for parkin binding, eventually leading to the accumulation of soluble α-Sp22 in AR-JP brains (Shimura *et al*, 2001). In contrast to all other substrates (Fig 1), α-Sp22 interacts with the UBL of parkin (Shimura *et al*, 2001). However, little is known about α-Sp22, and its existence could not be confirmed by other groups (Giasson & Lee, 2001). It has been suggested that the interaction between parkin and α-SYN is bridged by synphilin 1, a protein of unknown function that can bind to parkin and α-SYN individually (Chung *et al*, 2001; Engelender *et al*, 1999). Co-transfection of α-SYN and synphilin 1 in human embryonic kidney 293 cells leads to the formation of inclusion bodies, which were found to become ubiquitinated in the presence of wild-type, but not AR-JP mutant parkin (Chung *et al*, 2001). These results support the view that parkin ubiquitinates protein aggregates. However, the impact on cell death was not resolved in these studies.

Parkin substrates that may exert a direct cytotoxic effect on accumulation include α/β-tubulins (Ren *et al*, 2003), which are toxic in free, monomeric form (Burke *et al*, 1989), and cyclin E (Staropoli *et al*, 2003), which might force postmitotic neurons into abortive cell cycling, a process known to promote apoptosis. A more generalized toxic effect might arise from the aberrant turnover of the aminoacyl-tRNA synthetase subunit p38 (Corti *et al*, 2003). Dysregulation of synaptic vesicle transport might be

caused by the accumulation of the septins CDCrel-1 and CDCrel-2, the steady-state levels of which are regulated by parkin-mediated ubiquitination (Choi *et al*, 2003; Zhang *et al*, 2000). Synaptotagmin XI is another synaptic protein substrate for parkin (Huynh *et al*, 2003). Taken together, the cell-culture models have provided clues, but have not resolved the toxic mechanism of parkin deficiency.

Dopaminergic neuron loss without Lewy body formation?

Autopsies have been performed on only four parkin-proven cases of PD, so we should be cautious in drawing conclusions from neuropathological examinations. Patients with homozygous parkin deletions showed depigmentation and loss of dopaminergic neurons in the substantia nigra, accompanied by gliosis (Hayashi *et al*, 2000; Mori *et al*, 1998). Dopaminergic neuron loss was also reported for compound heterozygous patients with exon 3 deletion and parkin missense mutations (Farrer *et al*, 2001; van de Warrenburg *et al*, 2001).

Despite massive neurodegeneration in the substantia nigra and also in the locus coeruleus of AR-JP patients, LBs were conspicuously absent from the brains of patients with homozygous parkin deletions (Hayashi *et al*, 2000; Mori *et al*, 1998; Takahashi *et al*, 1994). These observations give rise to two interpretations. The first is that the aetiology of AR-JP is distinct from idiopathic PD, which means that the loss of parkin causes the loss of dopaminergic neurons by a distinct pathway that differs from the classical α-synucleinopathy course. Alternatively, parkin expression might be a prerequisite for LB formation (Chung *et al*, 2001). The examination of compound heterozygous patients bearing exon 3 deletions and point mutations is revealing in this context. Whereas a patient with a K211N parkin allele had no LBs (van de Warrenburg *et al*, 2001), an individual expressing R275W parkin did display brainstem LBs (Farrer *et al*, 2001). In marked contrast to the non-aggregating K211N mutant, R275W parkin localized to aggresome-like structures in cultured cells (Cookson *et al*, 2003). Thus, the R275W parkin expressed in the only AR-JP patient with LBs is not a simple loss-of-function mutant, but might instead mediate protein misfolding. In fact, there have been several recent reports of aggresome formation elicited by specific parkin mutants (Table 1). Aggresome-forming parkin mutations reside in the RING fingers, the many disulphide bonds of which depend on well-controlled protein-folding trajectories. Moreover, proteasomal inhibition led to the recruitment of wild-type parkin to aggresomes (Muqit *et al*, 2004; Zhao *et al*, 2003), and a fraction of LBs contained parkin (Schlossmacher *et al*, 2002). Although aggresome formation is a fascinating cellular defence mechanism against unfolded protein stress, the relevance of this process in LB formation remains to be discovered. It will be interesting to investigate whether conditions that impair the folding of parkin actually cause LB formation, whereas mutations in other domains are not permissive.

Parkin knockout animal models

Parkin-null mutants have been generated in *Drosophila* by *P*-element insertion and transposon mutagenesis (Greene *et al*, 2003; Pesah *et al*, 2004). *parkin* mutant flies showed a reduced lifespan, as well as defects in spermatogenesis and muscle degeneration. Furthermore, these flies were sensitized to oxidative stress (Pesah *et al*, 2004). Mitochondrial dysfunction probably contributes to the phenotype (Greene *et al*, 2003). For example, mitochondria in the main flight muscles were grossly swollen and the myofibrillar arrangement was dispersed. Mitochondrial swelling is associated with apoptosis, and indeed, TUNEL staining revealed apoptotic nuclei in the indirect flight muscles. Consequently, wing posture and flight was significantly impaired in *parkin*-null mutants. Transgenic expression of *parkin* restored muscle integrity.

However, the nervous system was not affected in *parkin* mutant *Drosophila* and the dopaminergic neurons in the dorsomedial cluster were not impaired (Greene *et al*, 2003; Pesah *et al*, 2004). These neurons selectively degenerate upon expression of the PD gene *α-SYN* (Feany & Bender, 2000) as well as the parkin substrate Pael-R (Yang *et al*, 2003). Importantly, co-expression of parkin ameliorated dopaminergic neuron loss in the dorsomedial cluster of α-SYN and Pael-R transgenic flies (Yang *et al*, 2003). Conversely, knockdown of *Drosophila parkin* by RNA interference enhanced the toxicity of Pael-R expressed specifically in dopaminergic neurons. Primary neurons derived from such flies showed an accumulation of Pael-R (Yang *et al*, 2003). Similarly, parkin knockdown combined with unfolded protein stress (caused by proteasome inhibition and α-SYN overexpression) was neurotoxic to mammalian neurons (Petrucelli *et al*, 2002).

Table 1 | Aggresome-forming parkin mutants

Mutation	Domain	Aggresomes	Cell type	Reference
A82E	Linker	–	HEK293	Cookson *et al*, 2003
K161N	UPD	–	COS7 SH-SY5Y	Gu *et al*, 2003 Muqit *et al*, 2004
R256C	RING1	+ –	HEK293 COS7	Cookson *et al*, 2003 Gu *et al*, 2003
R275W	RING1	+	HEK293, primary neurons	Cookson *et al*, 2003
C289G	RING1	+	COS7	Gu *et al*, 2003
G328E	IBR	–	HEK293 SH-SY5Y	Cookson *et al*, 2003 Muqit *et al*, 2004
C418R	RING2	+	COS7	Gu *et al*, 2003
C431F	RING2	–	HEK293	Cookson *et al*, 2003
W453stop	C-terminus	+	N2a, SH-SY5Y	Winklhofer *et al*, 2003

The R275W mutation was validated in *post-mortem* human tissue. IBR, in-between RING domain; RING, really interesting new gene domain; UPD, unique parkin domain.

Conversely, wild-type but not mutant parkin rescued primary dopaminergic neurons from proteasome inhibitor toxicity (Petrucelli *et al*, 2002). Thus, parkin may preserve cellular viability under conditions of unfolded protein stress.

This concept was further evaluated in mice with the targeted disruption of *parkin* exon 3. Parkin-deficient mice showed reduced weight gain (Itier *et al*, 2003; Palacino *et al*, 2004), but as in flies, no loss of dopaminergic neurons was observed (Goldberg *et al*, 2003; Itier *et al*, 2003). *Parkin*$^{-/-}$ mice had elevated levels of extracellular dopamine (Goldberg *et al*, 2003), which might originate from the limbic system rather than the striatum (Itier *et al*, 2003). Dopamine metabolism appeared to be enhanced, as indicated by elevated amounts of 3,4-dihydroxyphenylacetic acid (Itier *et al*, 2003), which is produced by monoamine oxidase. This enzymatic reaction produces hydrogen peroxide, which might confer oxidative stress in the *parkin*$^{-/-}$ mice. Indirect markers of oxidative damage that appeared in ageing *parkin*$^{-/-}$ mice included protein carbonyls and 4-hydroxynonenal (Palacino *et al*, 2004). Although the mitochondrial ultrastructure appeared intact in *parkin*$^{-/-}$ mice, a proteomic analysis revealed reduced levels of several protein subunits of the respiratory chain, and the respiratory capacity of mitochondria isolated from the striatum of *parkin*$^{-/-}$ mice was decreased (Palacino *et al*, 2004).

Surprisingly, none of the known parkin substrates (CDCrel-1, synphilin 1, α-Sp22 and α-SYN) were found to accumulate in the brains of *parkin*$^{-/-}$ mice, when measured specifically (Goldberg *et al*, 2003) or through proteomic analysis (Palacino *et al*, 2004). Furthermore, no accumulation of any α-SYN species was detected in *quaking*^{viable} mice (Lorenzetti *et al*, 2004). This spontaneous mouse mutant bears a deletion that spans *PARKIN* and the parkin co-regulated gene (*PACRG*; Fig 1), both of which encode enzymes that might act synergistically to suppress unfolded protein stress (Imai *et al*, 2003). Therefore, the possible explanations of these results are that redundant ubiquitination pathways for parkin substrates may exist in the mouse brain, that parkin-mediated ubiquitination does not target substrate proteins for proteasomal degradation, or that the parkin substrates identified so far are erroneous.

Conclusions and outlook

Several parkin substrate proteins have been identified and validated to variable extents. Unfortunately, no straightforward common denominator of parkin substrates has emerged. An attractive hypothesis states that aggregation-prone proteins are cleared after parkin-mediated polyubiquitination followed by proteasomal degradation. Although most authors could detect their parkin substrates in LBs of sporadic PD patients, parkin-mutant cases tend to lack LBs. Rather than forming protein aggregates, parkin substrates showed only some increase in steady-state levels in the small number of AR-JP brains available. Thus, the question arises whether parkin mutations cause dopaminergic neuron loss by a distinct pathway that does not involve idiopathic PD neuropathology. If so, AR-JP might be considered as a separate disease entity.

The widespread expression of parkin and its substrates raises the question why dopaminergic neurons selectively fall prey to parkin mutations in human patients. The pigmented dopaminergic neurons in the human substantia nigra sustain a high basal level of oxidative stress. Although the disruption of the parkin gene in laboratory animals was not sufficient to elicit dopaminergic neuron loss, mitochondrial deficiencies were consistently observed. Thus, parkin might protect against mitochondrial failure. It will be interesting to study the effects of parkin on mitochondrial integrity. It will also be crucial to investigate whether the loss of parkin sensitizes mice to PD-relevant stressors, such as dopaminergic neurotoxins or protein folding stress due to α-SYN fibrillogenesis.

Finally, one should bear in mind the emerging complexity of ubiquitin biology (Schwartz & Hochstrasser, 2003). Does parkin monoubiquitinate key substrates and/or does it require accessory factors (such as the E4 enzyme CHIP) for polyubiquitination? Are the classical K48-linked polyubiquitin chains the primary product of the enzymatic activity of parkin, or can K63- or K29-linked polyubiquitin chains be attached to substrate proteins? Could parkin even act as an E3 ligase for members of the emerging family of small ubiquitin-like modifiers? And if multiple E3 ligase activities are exerted by parkin, how are they regulated in cells, especially the nigral dopaminergic neurons, in health and disease? After a highly productive phase that led to the discovery of several parkin-interacting proteins, the time might have come to take a fresh look at parkin beyond its putative function to ubiquitinate proteins for proteasomal targeting.

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