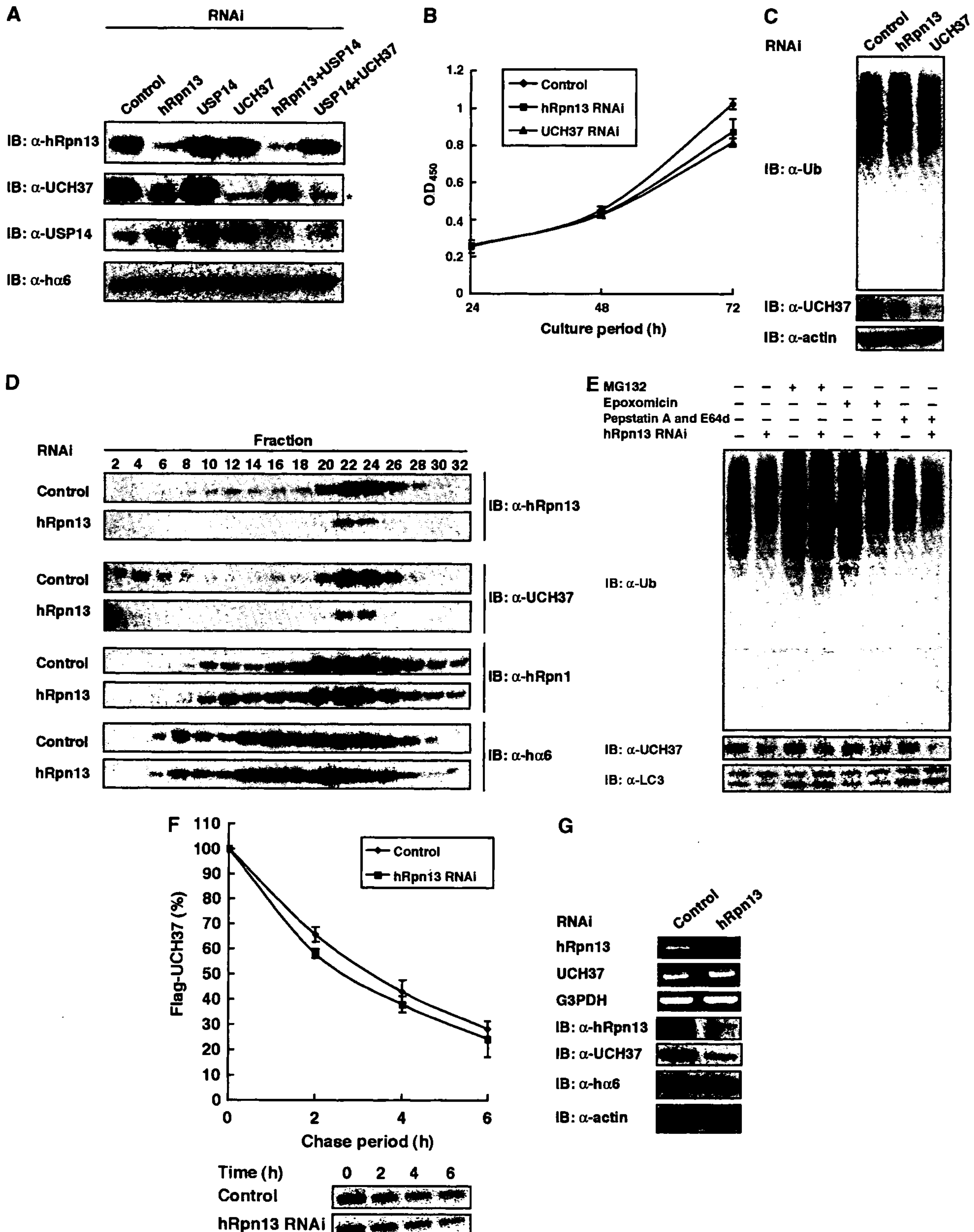
2006). The relatively low homology between Adrm1 and yeast Rpn13 may reflect this fact, as genuine proteasome subunits, but not PIPs, have much higher similarities between human and yeast subunits. Knockdown experiments further revealed that hRpn13 is not essential for proteolysis or viability of mammalian cells, whereas knockdown of hRpt2, a 19S ATPase subunit, was fatal (Figure 4). It is noteworthy that all subunits of RP, except Rpn9, Rpn10,



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and Rpn15/Sem1, are essential for proliferation of the budding yeast examined so far. Considered together, these results suggest that hRpn13 plays an auxiliary role in the proteasome-dependent protein degradation.

hRpn13 has no known functional motifs and does not seem to be necessary for the structural integrity of proteasomes. Then, what is the role of hRpn13 in proteasomes? hRpn13 can be divided into three functional regions: the N-terminal, C-terminal, and the inserted regions. The former two regions are well conserved from budding yeast to human, whereas the latter one is not found in budding yeast (Figure 1). We proved that the conserved N-terminal region is required for association with proteasomes (Figure 3). We also examined the biological role of the C-terminal region, and our results revealed that it serves as an acceptor for UCH37 (Figure 5). Moreover, loss of hRpn13 proteins caused concurrent loss of UCH37 proteins, indicating that hRpn13 recruits UCH37 to proteasomes and at the same time it is required for maintenance of UCH37 protein levels (Figure 6). As the half-life of UCH37 protein in hRpn13-knockdown cells was similar to that of control cell, the role of hRpn13 in maintaining UHC37 protein levels does not seem to be the stabilization of UCH37 protein. Consistent with this notion, neither the use of a proteasome inhibitor nor a lysosomal inhibitor resulted in accumulation of UCH37 proteins in knockdown cells. The amount of UCH37 mRNA was also unaltered in knockdown cells. At present, we do not know the reason for loss of UCH37 protein in knockdown cells, and the role of the insertion region is yet to be determined. No ortholog of UCH37 is found in budding yeast. Evolutionarily, UCH37 orthologs have emerged synchronously with the insertion region of Rpn13 orthologs. This region may have some role in the relationship with UCH37 and/or other proteins yet to be identified.

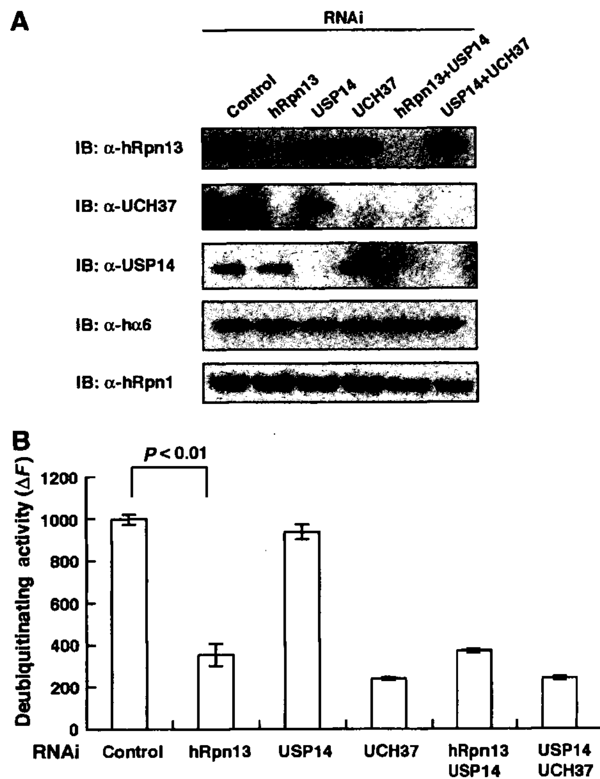

Figure 7 Knockdown of hRpn13 reduces deubiquitinating activities of 26S proteasomes. (**A**) hRpn13 and proteasome associating DUBs in knockdown cells. Cell extracts shown in Figure 6A were fractionated by 10–40% glycerol gradient centrifugation into 32 fractions. Immunoblot analysis of 26S proteasome fractions determined by Suc-LLVY-hydrolyzing activity was performed using the indicated antibodies. (**B**) The deubiquitinating activities of 26S proteasome fractions shown in panel A were measured using ubiquitin-AMC as a substrate. hRpn13- and UCH37-knockdown cells showed significantly reduced deubiquitinating activities ($P<0.01$). Data are mean±s.e.m. values of three independent experiments.

Ubp6 (USP14 in mammals) is another well-known DUB that associates with proteasomes. A mutation in USP14 in mice causes neurological disorders, demonstrating the importance of USP14 in mammals. Low expression of USP14 in mice is associated with reduced levels of free ubiquitin, suggesting its role in recycling ubiquitins (Anderson *et al*, 2005), as is also suggested in studies in budding yeast (Guterman and Glickman, 2004). However, the deubiquitinating activity of proteasomes is mainly attributed to UCH37 in fission yeast (Stone *et al*, 2004). Our present study also demonstrates that the deubiquitinating activities of 26S proteasomes are affected more profoundly by loss of UCH37 than USP14, indicating that UCH37 is the dominant DUB over USP14 in mammalian proteasomes. Recruitment of UCH37 by hRpn13 is essential for the activity of UCH37, as hRpn13 knockdown resulted in loss of UCH37, followed by a decrease in the deubiquitinating activity of 26S proteasomes at a level comparable to UCH37 knockdown. Further studies are required to distinguish the roles of USP14 (Ubp6) and UCH37 in organisms that have both molecules. It is plausible that the two molecules have distinct roles.

Despite the significant reduction of deubiquitinating activity of hRpn13-deficient proteasomes, they efficiently

Figure 6 Knockdown of hRpn13 causes loss of UCH37 proteins. (**A**) HEK293T cells were transfected with siRNA against hRpn13, UCH37, or USP14. Where indicated, cells were transfected with a mixture of siRNAs. After 96 h, cell extracts were subjected to SDS–PAGE, followed by immunoblotting with the indicated antibodies. (**B**) Growth rates of hRpn13 knockdown, UCH37 knockdown, and mock cells. The cells were seeded in triplicate in 96-well dishes on day 0 (72 h after transfection), cultured in normal growing medium, and their proliferation was measured every 24 h. Data are mean±s.d. values. (**C**) Cell extracts of hRpn13 knockdown, UCH37 knockdown, and mock cells were subjected to SDS–PAGE, followed by immunoblotting with the indicated antibodies. (**D**) Samples fractionated by 10–40% glycerol gradient centrifugation were immunoblotted with the indicated antibodies. (**E**) Lack of accumulation of UCH37 proteins in hRpn13-knockdown cells following inhibition of the proteasome or lysosomal cathepsins. HEK293T cells transfected with siRNA were treated with MG132 (50 μM, 2 h), epoxomycin (1 μM, 12 h), or E-64 and pepstatin A (10 μg/ml, 12 h). The cells were lysed and subjected to immunoblot. (**F**) Pulse-chase analysis of Flag-UCH37. HEK293T cells stably expressing Flag-UCH37 were pulse-labeled for 30 min with ^{35}S-labeled methionine and chased for the indicated time periods. After immunoprecipitation with M2 agarose, samples were separated by SDS–PAGE and autoradiographed (bottom panels). The upper panel shows the results of band quantitative analysis. Data are mean±s.d. values of three independent experiments. (**G**) hRpn13 knockdown does not alter UCH37 mRNA transcription. Semiquantitative RT–PCR (the upper three panels) and immunoblotting (the lower three panels) were performed using total RNA and proteins extracted from control and hRpn13-knockdown cells 48 h after transfection with siRNAs.

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degraded substrate proteins such as ODC, ubiquitinated c-IAP, and IκBα (Figure 4). These observations seem to be in marked contrast to the case of yeast Rpn13, whose deletion caused a complete defect in degradation of a UFD substrate (Verma *et al*, 2000). However, as yeasts that lack Rpn13 are viable (Winzeler *et al*, 1999), the proteolytic defect is probably specific to UFD substrates. Rpn13 may recruit a component essential for degradation of UFD substrates in yeast. The precise role of hRpn13 and UCH37 still remains elusive. Curiously, knockdown of hRpn13 significantly increased the degrading activity of ODC but not that of ubiquitinated cIAP1 protein *in vitro* and IκBα *in vivo*. As sole knockdown of UCH37 did not increase the degrading activity of ODC (data not shown), the results observed in hRpn13 knockdown are not simply due to loss of UCH37. hRpn13 may influence access of protein to the channel of ATPase rings of proteasomes by sitting in the space between the base and lid (Holzl *et al*, 2000), or may recruit proteins that are relevant to proteolysis other than UCH37. At least from these results, both hRpn13 and UCH37 do not seem to be generally required for protein degradation by 26S proteasomes. It is assumed that UCH37 disassembles polyubiquitin chains from the distal end, shortening it such that the attached proteins can be released from the proteasome if there is a delay in efficient degradation (Stone *et al*, 2004). hRpn13 and UCH37 may be important in some specific situations. Further studies are required to determine the specific functions of hRpn13 and UCH37.

Materials and methods

Plasmids and cloning

The complementary DNAs (cDNAs) used in the present study were obtained by RT-PCR from total RNA isolated from HeLa cells or mouse livers using Superscript III (Invitrogen, San Diego, CA) and Pyrobest DNA polymerase (Takara Shuzo, Ohtsu, Japan). All amplified fragments were cloned into pcDNA3.1 vector (Invitrogen) and sequenced for confirmation. Deletion mutants (hRpn13ΔC, ΔN, N, ΔKE1 and ΔKE1 + 2, and UCH37ΔKE) were generated by PCR from wild-type hRpn13 and UCH37 and the Flag tag was introduced at the N-terminus of the constructs. For expression of GST and 6xHis fusion proteins, the cDNAs were subcloned into pGEX6P-1 (Amersham Life Science, Buckinghamshire, UK) and pET-28a (Novagen, Madison, WI) vector, respectively.

Immunological analysis

For immunoprecipitation analysis, HEK293T cells were transfected with plasmids using Fugene 6 (Roche, Mannheim, Germany). After 36 h, the cells were lysed with ice-cold phosphate-buffered saline (PBS) containing 1% Nonidet P-40 (NP-40) and centrifuged at 20 000 *g* for 10 min at 4°C. The supernatant was added with M2-agarose (Sigma Chemical Co., St Louis, MO) and rotated for 1 h at 4°C. The immunoprecipitates were washed five times with ice-cold PBS containing 0.5% NP-40 and then boiled in SDS sample buffer in the presence of β-mercaptoethanol (β-ME). Samples were subjected to SDS–PAGE, transferred to polyvinylidene fluoride membrane, and analyzed by immunoblotting with anti-Flag (M2; Sigma). Polyclonal antibodies against hRpn1, hRpn10, hRpt2, hRpn13, UCH37, and USP14 were raised in rabbits using recombinant proteins expressed in and purified from BL21RIL strain (Novagen) as GST fusion proteins: mouse Rpn1 (full length), mouse Rpn10 (residues 1–251), hRpt2 (residues 1–82), hRpn13 (residues 361–407), mouse UCH37 (residues 228–329), and human USP14 (full length). Anti-LC3 antibody was described previously (Komatsu *et al*, 2005). The antibodies for polyubiquitin and actin were purchased (FK2; MBL, Ina, Japan, Chemicon International Inc., Temecula, CA). All experimental protocols described in this study were approved by the Ethics Review Committee for Animal Experimentation of Tokyo Metropolitan Institute of Medical Science.

GST pull-down assay

Recombinant GST- or 6xHis-tagged proteins were produced in *Escherichia coli* and purified with glutathione Sepharose 4B (Amersham) or Ni-NTA Sepharose (Qiagen, Hilden, Germany). After elution of proteins from the beads, the preparations were dialyzed against buffer A (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM β-ME, and 10% glycerol). In GST pull-down analysis, 5 μg of each sample was mixed in 600 μl of buffer A and constantly rotated for 1 h at 4°C, and then 30 μl of glutathione Sepharose 4B was added and further rotated for 1 h. After washing with buffer A, bound proteins were eluted with 10 mM glutathione and subjected to SDS–PAGE, followed by immunoblot and silver staining (Wako Pure Chemical Industries, Osaka, Japan).

RNAi experiments

siRNAs targeting hRpt2, hRpn13, UCH37, and USP14 with the following 25-nucleotide sequences were purchased from Invitrogen: Rpt2, 5′-GGAGUACGAUGUGUAAGUGCCCAUU-3′; Rpn13, 5′-GGAGGGUCUACGUGCUGAAGUUCAAA-3′; UCH37, 5′-ACCGAGCTCATTAAAGGATTCGGTT-3′; USP14, 5′-UCAGCAUCGUAACACCAGAAGAUAU-3′. siRNAs were transfected into HEK293T cells with Lipofectamine 2000 (Invitrogen) at a final concentration of 2 nM in six-well dishes. The cells were analyzed 96 h (hRpn13, UCH37, USP14, hRpn13 + USP14, and USP14 + UCH37) or 48 h (hRpt2) after transfection. For protease inhibition assay, HEK293T cells transfected with siRNA (96 h) were cultured in the presence or absence of protease inhibitors (50 μM MG132 for 2 h, 10 μg/ml E64d and 10 μg/ml pepstatin A for 12 h, or 1 μM epoxomycin for 12 h). Cell growth was measured using Cell Counting Kit-8 (Wako) according to the instructions provided by the manufacturer. Briefly, cells were seeded at 1×10^3 cells/well in 96-well plates. Absorbance was measured using Microplate reader (Bio-Rad).

Glycerol-density gradient analysis

Mouse livers were homogenized in a Potter-Elvehjem Homogenizer in buffer B (in mM, 25 Tris–HCl (pH 7.5), 2 ATP, 5 $MgCl_2$, and 1 dithiothreitol). HEK293T cells were lysed in buffer B containing 0.2% NP-40. The homogenates and lysates were clarified by centrifugation at 20 000 *g* and subjected to 10–40% (v/v) or 8–32% (v/v) linear glycerol-density density gradient centrifugation (22 h, 83 000 *g*) as described previously (Hirano *et al*, 2005).

In vitro assay of proteasome activity

Proteasome peptidase activity was measured using a peptide substrate, succinyl-Leu-Leu-Val-Tyr-7-amino-4-methyl-coumarin (Suc-LLVY-MCA), and the degradation of the recombinant ^{35}S-labeled ODC was assayed in the presence of ATP, an ATP-regenerating system, and AZ, as described previously (Hirano *et al*, 2005). For the assay of cIAP1 degradation, cDNAs encoding Flag-cIAP1 subcloned into pcDNA3.1 were transcribed *in vitro*, translated, and radiolabeled as described previously (Hirano *et al*, 2005). The ^{35}S-labeled Flag-cIAP1 was purified using M2-agarose (Sigma) and eluted with Flag-peptide (Sigma). For ubiquitination of cIAP1, 3 000 000 c.p.m. of ^{35}S-labeled cIAP1, 0.25 μg of E1, 0.9 μg of UbcH5, and 33 μg of ubiquitin (Sigma) were mixed and incubated in a volume of 80 μl for 90 min at 30°C, as described previously (Murata *et al*, 2001). Finally, 2.5 μl of the ubiquitination mixture was added to 10 μl of cell lysates in the presence of 2 mM ATP, incubated at 37°C for 20 min, and then radioactivities of trichloroacetic acid-soluble fractions were measured.

TNF-α-dependent IκBα degradation

HEK293T cells transfected with siRNA were treated with 100 μg/ml cycloheximide (Sigma) for 10 min, and then human TNF-α (Genzyme, Cambridge, MA) was added at a final concentration of 10 ng/ml. Changes in the protein levels of endogenous IκBα after treatment with TNF-α were analyzed by immunoblotting with anti-IκBα (c-21) (Santa Cruz Biotechnology, Santa Cruz, CA).

Deubiquitination assay

For ubiquitin-7-amino-4-methylcoumarin (ubiquitin-AMC) (Boston Biochem) hydrolysis assays, 10 μl of 26S proteasome fraction separated by glycerol gradient centrifugation was incubated with 0.25 μM ubiquitin-AMC for 15 min at 37°C. The release of AMC was measured fluorometrically.

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Pulse–chase analysis
HEK293T cells stably expressing Flag-UCH37 were transfected with siRNA for hRpn13 or control siRNA. Pulse-chase experiments were performed as described previously (Hirano *et al*, 2005).

RT–PCR analysis
Total RNA (2.5 μg) was reverse transcribed using SuperScript III (Invitrogen) and oligo(dT)$_{20}$ primers. Specific primers for each gene were as follows: 5′-AAGGATCCATGAGCATCCTGGCCACG ATGAACG-3′ and 5′-TTCTCGAGTCAGTCCAGGCTCATGTCCTCC-3′ for hRpn13, 5′-AAGGATCCATGACGGGCAACGCCGGGGAG-3′ and 5′-TTCTCGAGTCATTTGGTTTCCTGAGCTTTC-3′ for UCH37, and 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCT GTA-3′ for G3PDH.

Supplementary data
Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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9
The Molecular Pathway to Neurodegeneration in Parkin-Related Parkinsonism

Ryosuke Takahashi

9.1
Introduction

Parkinson's disease (PD) is the most common neurodegenerative disease of the motor system amongst elderly people. The prevalence of PD is approximately 1% of people by the age of 70 years [1]. PD is characterized by a progressive loss of dopaminergic neurons in the pars compacta of the substantia nigra accompanied by the formation of Lewy bodies. Lewy bodies are intra-neuronal fibrillary inclusions mainly composed of α-synuclein [2]. They are regarded as the hallmark of idiopathic PD. Loss of neurons within the pars compacta of the substantia nigra causes progressive motor disturbances, classically tremor, rigidity, bradykinesia and postural instability. To date, there is no known effective therapy to prevent or retard neurodegeneration as a result of PD [1, 3].

Most cases of PD develop sporadically, however, fewer than 10% of cases are familial and presumably inherited [4]. Autosomal recessive juvenile parkinsonism (AR-JP) accounts for approximately 50% of cases of early-onset familial PD in affected European families [5]. It is characterized by several unique features, including young age of onset (usually under 40 years of age), dystonia, and a marked response to dopamine. The neuropathological hallmark of AR-JP is selective degeneration of dopaminergic neurons in the substantia nigra zona compacta, similar to that observed in the idiopathic form of PD. However, AR-JP is not usually associated with Lewy bodies [6, 7].

Mutations in the parkin gene are responsible for AR-JP [8]. In this chapter, the role of parkin in the ubiquitin–proteasome system will be focused and discussed in light of recent findings.

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9.2 Parkin is an E3 Ubiquitin Ligase

9.2.1 Parkin and the Ubiquitin-Proteasome System

Parkin is a 465-amino acid protein characterized by a ubiquitin-like domain at its NH_2-terminus, as well as two RING-finger motifs and an IBR (in-between RING fingers) motif at its COOH terminus (RING-IBR-RING or RBR domain) [9]. The RING domain has been shown to be a feature of ubiquitin ligase involved in the ubiquitination reaction [10]. Polyubiquitination involves a sequence of reactions performed by ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin ligating (E3) enzymes. E3 interacts with specific substrate(s) and facilitates the formation of covalent bonds between the COOH terminus of ubiquitin and ε-lysine, either on a target protein or on the last ubiquitin of a protein-bound polyubiquitin chain in concert with its partner E2s. Yeast protein UFD2 is a multi-ubiquitin chain elongation factor, also called E4, required for efficient multi-ubiquitination of a substrate [11]. Polyubiquitin chains are thought to be potent targeting signals for the degradation of proteins within 26S proteasomes.

Several groups have shown that wild-type parkin is an E3 ubiquitin ligase [12–14] (Figure 9.1). Parkin ubiquitinates substrate proteins or itself in concert with E2s, such as UbcH7, UbcH8, Ubc6 and Ubc7 [12–14]. Moreover, several AR-JP-related missense mutations have been identified in the ubiquitin-like

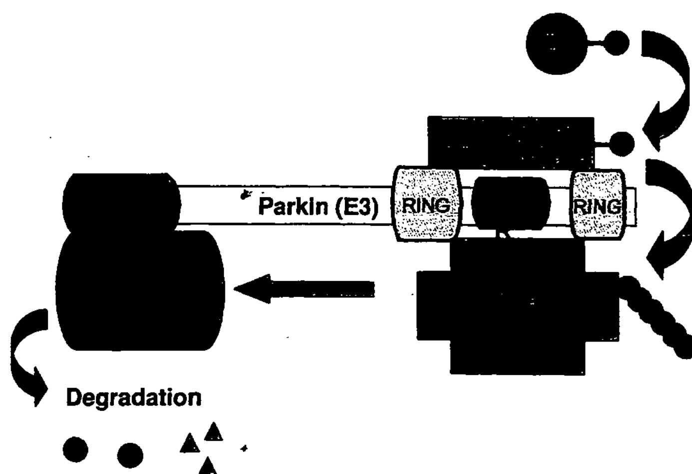

Fig. 9.1. Function of parkin in the ubiquitin proteasomal pathway. Parkin is an E3 ubiquitin ligase that recognizes substrate X and promotes ubiquitination in adjunct with two other ubiquitination enzymes, E1 and E2. Polyubiquitinated substrate X is recognized and degraded by the 26S proteasome. The N-terminal ubiquitin-like domain and the C-terminal RING-IBR-RING domain of parkin serve as recruitment domains for 26S proteasome and E2 enzymes, respectively. Some of the known substrates of parkin associate with its RING-IBR-RING domain.

domain of the parkin gene [15]. Furthermore, an NMR analysis has revealed binding between the ubiquitin-like domain of parkin and the Rpn 10 subunit of the 26S proteasome [16], strongly suggesting the link between Parkin and the UPS (Figure 9.1).

9.2.2
Proteasome-independent Role of Parkin

Polyubiquitin chains are formed through distinct types of linkages using one of the seven internal lysine residues (K6, K11, K27, K29, K33, K48 and K63) within the previous ubiquitin molecule [17].

Recently, parkin was shown to be a dual function ubiqutin ligase that mediates both K48- and K63-linked polyubiquitination [18]. K48-linked polyubiquitin chain, the best characterized form of polyubiquitin, leads the proteins to degradation via 26S proteasomes, constituting the ubiquitin–proteasome system as mentioned before. On the other hand, K63-linked chains act as proteasome-independent signals in several different cellular pathways [17]. Dual specificity seems to be determined by the E2 enzymes that parkin recruits. In the assembly of a K63-linked polyubiquitin chain, parkin interacts with the UbcH13/Uev1a heterodimer [18]. Parkin mediates K63-linked, proteasome-independent ubiquitination of its substrate synphilin 1 [19]. Although K63-linked ubiqitination is implicated in inclusion body formation, further study is required to clarify its physiological relevance [20].

9.2.3
Multiple Monoubiquitination is Mediated by Parkin

Surprisingly, two recent reports have shown that Parkin mediates multiple monoubiquitination *in vitro* [21, 22]. Both reports have demonstrated that the second RING finger is responsible for E3 activity in an *in-vitro* ubiquitination assay, where bacterially-produced recombinant Parkin was used. Previous findings that mutations in regions other than the second RING finger showed reduced E3 activity *in vivo* might be ascribed to their insolubility and sequestration [23–26]. Parkin itself as well as maltose binding protein (MBP) connected to Parkin as a pseudosubstrate and p38 as a substrate, have been shown to be monoubiquitinated *in vitro* and *in vivo* respectively. In contrast to a previous report that Parkin accelerates polyubiquitin chain formation [18], Parkin has been shown to mediate monoubiquitination in concert with Ubc13 as well as Ubc7 or Ubc H7 under pure *in vitro* conditions [21, 22]. These results suggest that Parkin may mediate monoubiquitination regardless of its partner E2s.

A recent report showed that Parkin mediates monoubiquitination of an adaptor protein Eps15 with two ubiquitin-interacting motifs (UIMs) [27]. Eps15 interacts with and positively regulates the endocytosis of ubiquitinated epithelial growth factor receptor (EGFR). Parkin-mediated ubiquitination of Eps15 inhibits its ability to bind with and promote endocytosis of EGFR, resulting in suppression of EGFR

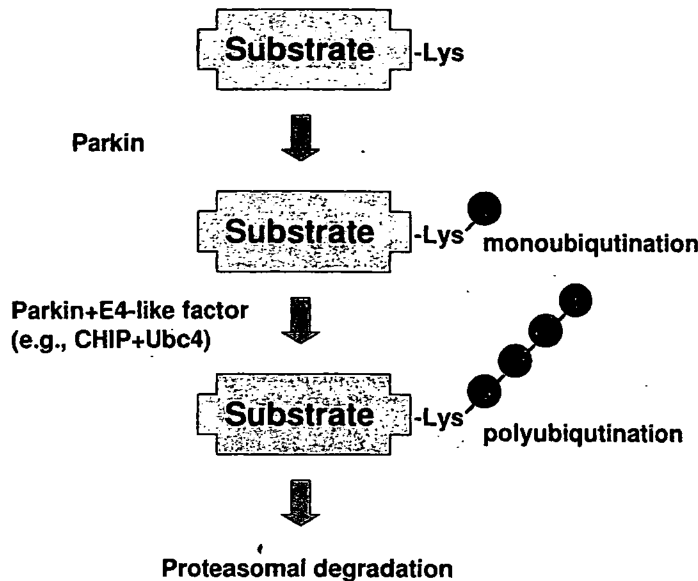

Fig. 9.2. Hypothetical two-step ubiquitination of Parkin substrates. At the first step, Parkin monoubiqutinates its substrate. Then, E4-like factors promote the elongation of polyubiquitin chain on the substrate molecules, thereby targeting the substrates to 26S proteasome. CHIP and its partner E2 Ubc4 are candidates for such E4-like molecules [28].

internalization and degradation, and promoting phosphoinositide 3-kinase (PI(3)K)-Akt signaling. Since Akt plays an important role in neuronal survival, this proteasome-independent function of Parkin may explain some aspects of neurodegeneration.

On the other hand, monoubiquitination may lead to proteasomal degradation. A previous report showed that the carboxy-terminus of Hsc70-interacting protein (CHIP), a U-box motif containing E3 protein, together with Ubc4, serves as an E4-like protein and cooperates with Parkin to form polyubiquitin chains [28]. Given the presence of E4-like factor, monoubiquitination catalyzed by Parkin may eventually target the proteins to degradation via the 26S proteasome (Figure 9.2). Whether Parkin can mediate both monoubiquitination and polyubiquitination should be re-examined and clarified in light of the recent findings.

9.2.4
Modulators of Parkin E3 Activity

Parkin is a component of a high molecular weight complex located in cells and the function of parkin seems to be modulated by its binding partners [28, 29]. Two parkin-associated proteins have been shown to promote the elimination of Pael-R by parkin: CHIP and Hsp70 [28].

CHIP contains a U-box motif, which is structurally similar to the RING-finger motif and exhibits U box-dependent E3 activity [30–32]. On the other hand, CHIP has been shown to downregulate chaperone ATPase activity [33]. Moreover, CHIP

has been shown to ubiquitinate improperly-folded protein in a chaperone-dependent manner [32]. When bound to parkin however, CHIP markedly enhances parkin-mediated ubiquitination of Pael-R *in vitro* [28]. Consistent with this observation, overexpression of CHIP accelerates Pael-R degradation in cultured cells, leading to a marked reduction in the steady-state level of Pael-R protein.

In contrast to CHIP, Hsp70 has been observed to inhibit ubiquitination of Pael-R *in vitro* and to increase levels of the soluble form of Pael-R *in vivo*, presumably by facilitating the proper folding of Pael-R. Moreover, Hsp70 inhibits CHIP-mediated degradation of soluble and probably functional Pael-R, so that only insoluble aggregates of the receptor are removed.

It has also been shown that bcl-2-associated athanogene 5 (BAG5), a BAG-family member, directly interacts with parkin and the chaperone Hsp70. BAG5, similar to CHIP, downregulates chaperone ATPase activity. Within this complex, BAG5 inhibits both parkin E3 ubiquitin ligase activity and Hsp70-mediated refolding of misfolded proteins. BAG5 enhances parkin sequestration within protein aggregates and attenuates parkin-dependent preservation of proteasome function [34].

Two binding partners of Parkin, 14-3-3 eta and Nrdp1/FLRF are also found to be negative regulators of Parkin E3 activity [35, 36].

9.3 Substrates of Parkin

9.3.1 Parkin Substrates and their Recognition Mechanisms

Although Parkin may mediate diverse forms of ubiquitination, it is likely that some, but not all, are involved in the UPS. Given that Parkin targets its ubiquitinated substrates to the 26S proteasome, Parkin dysfunction should lead to the accumulation of its substrate. Accumulation of toxic substrate(s) of Parkin (substrate-X) due to loss of parkin E3 activity or disruption of the parkin–proteasomal interaction in AR-JP patients with a genetic defect of parkin, should result in the development of dopaminergic neurodegeneration.

Based on this hypothesis, the identification of such toxic substrate(s) is the key to understanding the molecular mechanisms underlying AR-JP.

To date, 13 proteins have been identified as substrates of parkin [37]: CDCrel-1 , synaptotagmin XI [38], synphilin-1 [39], glycosylated α-synuclein [40], α/β-tubulin [41], the p38 subunit of an aminoacyl-tRNA synthetase (ARS) complex [42], Parkin-*a*ssociated *e*ndothelin receptor-like *r*eceptor (Pael-R) [43], the expanded form of polyglutamine [44], and cyclin E [45], SEPT5_v2/CDCrel-2 [46], misfolded dopamine transporter [47], far upstream element-binding protein 1 [48], RanBP2 [49] and Eps15 [27] (Table 9.1). It has been shown that the unmodified form of α-synuclein, a major component of Lewy body, is not a substrate for Parkin [39].

Although there are no apparent common properties among Parkin substrates, it has been noted that several different substrates are found within Lewy bodies.

Table 9.1. The reported substrates of parkin

Protein	Biological function	Lewy body
O-glycosylated α-synuclein	Septin family protein with unknown function	–
CDCrel-1	Isoform of α-synuclein with unknown function	N.D.
(Misfolded) Pael receptor	Orphan G-protein coupled receptor	+
p38 subunit of the aminoacyl-tRNA synthetase	Role in protein biosynthesis	+
Synaptotagmin XI	Regulation of exocytosis of neurotransmitters	+
Expanded polyglutamine(polyQ) proteins	Aberrant proteins responsible for polyQ diseases	–
α/β-Tubulins	Microtubule proteins	+
Synphilin-1	α-Synuclein-binding protein	+
Cyclin E	Cell cycle regulation of mitotic cells; unknown function in neurons	N.D.
SEPT5_v2/CDCrel-2	SEPT5_v2 is highly homologous with CDCrel-1	N.D.
Misfolded dopamine transporter	Regulation of dopamine uptake	N.D.
Far upstream element-binding protein-1	A binding partner of p38	N.D.
RanBP2	Small ubiquitin-related modifier (SUMO) E3 ligase family protein	N.D.
EPS15	Adaptor protein with ubiquitin-interacting motifs (UIMs)	N.D.

N.D., not detected.

Considering that the components of Lewy bodies consist of misfolded proteins, molecular chaperones and proteasome subunits, it is likely that Parkin ubiquitinates a subset of misfolded proteins. Consistent with this idea, an expanded form of polyglutamine, which is a causative agent of polyglutamine diseases such as Huntington's disease, has been identified as a parkin substrate [44]. Moreover, there is evidence to suggest that binding between parkin and polyglutamine is mediated by Hsp70, which is known to be a binding partner of Parkin. Hsp70-mediated substrate recognition explains the diversity of substrate specificity observed with parkin (Figure 9.3).

Among the various substrate molecules discussed above, the Pael receptor (Pael-R), CDC-rel1, cyclin E, synphilin-1, and the p38 subunit of aminoacyl tRNA synthetase have either been shown or suggested to promote cell death under certain conditions, and so represent the proteins which are most likely relevant to neurodegeneration in AR-JP.

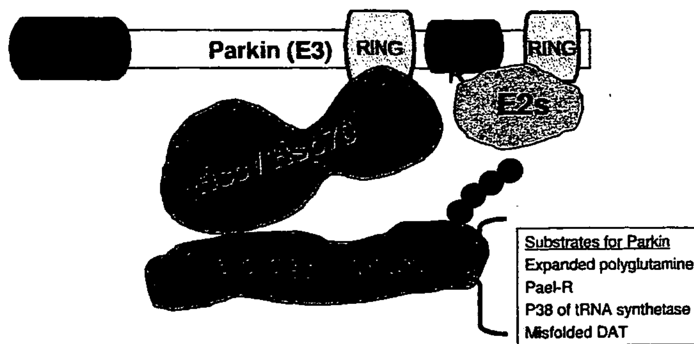

Fig. 9.3. Parkin may recognize a subset of misfolded proteins through Hsc/Hsp70. Parkin interacts with Hsc/Hsp70 through its first RING domain [28]. Parkin may recognize misfolded proteins including Pael-R, and p38 by using Hsc/Hsp70 as a substrate recognition subunit.

9.3.2 The Link between Substrate Accumulation and Cell Death: Pael-R

9.3.2.1 Pael-R and Endoplasmic Reticulum Stress

Pael-R is a putative G-protein-coupled orphan receptor, which is highly expressed in the central nervous system, especially in the substantia nigra [43, 50, 51]. Although the physiological function of Pael-R is implicated in dopamine metabolism, its ligand has yet to be identified [52]. It has been shown that misfolded Pael-R was ubiquitinated by parkin at the level of the endoplasmic reticulum and the disturbance of Pael-R degradation leads to ER stress-induced cell death.

The endoplasmic reticulum (ER) functions as a quality control regulator of membrane and secretory proteins [53]. Newly synthesized secretory proteins are transported to the ER. Inside its lumen, ER chaperones such as BiP/GRP78 bind to these newly synthesized proteins to facilitate their proper folding. After this, proteins enter the conventional secretory pathway. Proteins that are not properly folded are transported back to the cytosol where they are degraded via ubiquitin-proteasomal degradation, a process known as endoplasmic reticulum-associated degradation (ERAD) [54] (Figure 9.4). It has been shown that parkin is an ERAD-related E3 and that Pael-R is a substrate. When insoluble misfolded Pael-R is accumulated in the cells by the inhibition of the proteasome, Pael-R is first accumulated in the ER and then forms a special type of aggregate, known as an aggresome, in the cytoplasm [55]. As these aggresomes form, the cells undergo apoptosis, demonstrating cell death due to the accumulation of Pael-R.

Pael-R-induced cell death was assumed to be mediated by ER stress. Abnormal accumulation of unfolded protein in the ER is a major threat to cell viability, a phenomenon known as ER stress or unfolded protein stress. Cells attempt to adapt to ER stress in several different ways, including transcriptional upregulation of ER chaperones, and suppression of translation. These cellular responses are

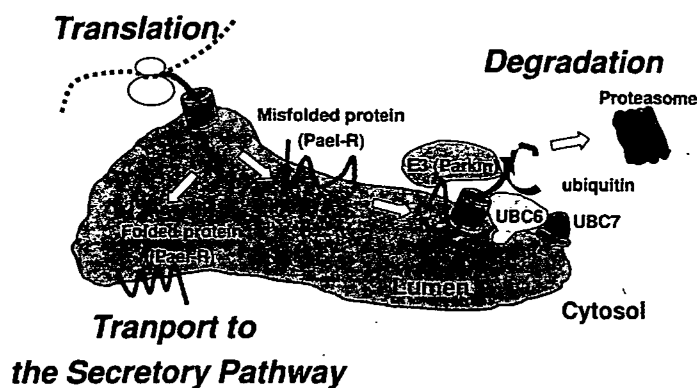

Fig. 9.4. Endoplasmic reticulum-associated degradation (ERAD). ERAD is a protein degradation system for unfolded secretory and membrane proteins. Improperly folded Pael-R is subject to ERAD, and parkin is an E3 involved in ERAD.

collectively known as unfolded protein responses (UPR) [53]. However, when the burden of accumulated protein exceeds these protective mechanisms, cells undergo a death process accompanied by the activation of JNK and caspases as well as upregulation of CHOP [56, 67].

Consistent with the idea that accumulation of misfolded Pael-R contributes to the pathogenesis of AR-JP, the level of detergent-insoluble Pael-R was elevated in the brains of AR-JP patients [43].

9.3.2.2 Pael-R Overexpressing Animals and Dopaminergic Neurodegeneration

The Drosophila model for AR-JP was created by overexpression of Pael-R [58]. When Pael-R was expressed in dopaminergic neurons in Drosophila, the number of dopaminergic neurons observed within the dorsomedial cluster fell to about 50% of that observed in control flies at 40 days of age. Equal numbers of dopaminergic neurons were observed in younger Pael-R and control flies, indicating that the observed cell loss was due to neurodegeneration occurring after birth. Moreover, even when Pael-R expression was driven by a pan-neuronal promoter, only dopaminergic neurons underwent degeneration. This suggests that dopaminergic neurons are selectively vulnerable to Pael-R toxicity.

A recent report showed that Pael-R overexpression in the substantia nigra of mouse brain through adenoviral vectors, resulted in induction of ER stress followed by dopaminergic neuronal death [59]. Pael-R-induced cell death was greatly enhanced in the parkin-deficient mouse and was suppressed by the overexpression of an ER chaperone, ORP150. Moreover, when the animal was pretreated with dopamine synthesis inhibitor, dopaminergic neuronal death was significantly attenuated, indicating that dopamine enhances Pael-R toxicity. It has been reported that dopamine covalently modifies and functionally inactivates Parkin [60]. Although the relationship between Pael-R toxicity and dopamine is still obscure, cellular protective mechanisms against Pael-R toxicity other than Parkin, might also be inactivated by dopamine.

9.3.3 The Link between Substrate Accumulation and Cell Death: CDC-rel1, Synphilin-1, Cyclin E and p38

When CDC-rel1 was introduced into the striatum and the substantia nigra of rat brain by using adeno-associated viral vectors, only dopaminergic cells in the substantia nigra underwent cell death [61]. Since the reduction of dopamine levels by pharmacological treatment alleviated nigral cell death and CDC-rel1 overexpression in PC12 cells decreased the extracellular dopamine level, the accumulation of dopamine by CDC-rel1-mediated exocytosis inhibition is thought to contribute to dopaminergic neuron-selective cell death.

Synphilin-1 is an α-synuclein-interacting protein that promotes the formation of Lewy body-like inclusions in cultured cells [62]. Parkin mediates K63-linked polyubiquitinanation of synphilin-1, apparently contributing to inclusion formation by α-synuclein and synphilin-1 in cultured cells [19]. Moreover, parkin can protect against the toxicity induced by α-synuclein plus synphilin-1 overexpression following proteasome inhibition [39].

Cyclin E has been implicated in glutamate-induced neuronal death, since it is accumulated in primary neuronal cultures in response to glutamanergic excitotoxin kainate. Interestingly, parkin overexpression inhibits the accumulation of cyclin E and cell death induced by kainate treatment, whereas RNAi-mediated parkin downregulation showed the opposite effects. The mechanism underlying cyclin E-induced cell death is not clear.

The p38 subunit plays an essential role in the *in vivo* assembly of the ARS complex [63]. When overexpressed in dopaminergic neuroblastoma cells, it forms aggresomes and induces cell death by unknown mechanisms. Parkin promotes the formation of ubiquitinated p38-positive inclusion bodies and suppresses the p38-induced cell toxicity [42]. It has been noted that only p38 is shown to be upregulated by 15% in the ventral midbrain of the parkin-null mouse among all the substrates identified [48]. The role of p38 in dopaminergic neuronal death should be validated in animal models in the future.

9.4 The Animal Models of AR-JP

To establish animal models of AR-JP, parkin gene deletion mutants for Drosophila and mouse were created [64–69]. However, the phenotypes of parkin-null mutant animals are very different from those of AR-JP patients.

9.4.1 Drosophila Model of AR-JP

The parkin gene deletion mutant Drosophila are small in size, have a short life span and become vulnerable to oxidative stress [64, 65]. The most remarkable

phenotypes of the mutant fly are apoptotic muscle degeneration and disturbances in spermatogenesis, which result in locomotor dysfunction and male sterility respectively [64, 65]. Ultrastructural analysis revealed abnormal mitochondrial morphology in both muscle and sperm [64]. On the other hand, the number of dopaminergic neurons was not reduced, although shrinkage of the cell bodies and decreased tyrosine hydroxylase immunostaining in proximal dendrites of dopaminergic neurons were observed [64].

9.4.2 Parkin-null Drosophila and Drosophila

Mutations in the PTEN-induced putative kinase 1(PINK1) are responsible for the autosomal recessive form of familial Parkinson's disease termed PARK6 [70]. PINK1 is a putative mitochondrial protein kinase, whose function is totally unknown. Three recent reports have shown that deletion of Drosophila PINK1 leads to an almost identical phenotype to that of Parkin-deficient Drosophila, i.e. it produces mitochondrial dysfunction resulting in male sterility, apoptotic muscle degeneration, and moderate loss of dopaminergic neurons [71–73]. Interestingly, the disease phenotype of PINK1-deleted Drosophila was rescued by Parkin overexpression, but not vice versa, suggesting that Parkin functions downstream of PINK1.

9.4.3 Mouse Model of AR-JP

Parkin knockout mice, in which exon 3 or 7 is deleted, have been described by three different groups [66–68]. Dopaminergic neuronal loss was not observed in any of the reports, even in aged mice. However, regarding the parkin exon-3 deletion mutant mouse, the dopamine level in the limbic system was elevated and the level of dopamine transporters was lowered according to one report, while the extracellular dopamine concentration in the striatum was increased according to a second report [66, 67]. These changes are accompanied by behavioral or electrophysiological alterations. In addition, in the mutant mouse reported by Goldberg et al., mild mitochondrial dysfunction and mild increase of oxidative stress were observed [74].

On the other hand, in exon-7-deleted parkin mutant mice, abnormalities in the nigrostriatal dopaminergic system were not detected [68]. However, noradrenergic neurons in the locus coeruleus were decreased by 20% in 70% of the total number in mice. These mild noradrenergic neuronal losses were detected as early as 2 months after birth and do not appear to progress with further aging. Consistent with this neuronal loss, the level of noradrenalin in the brain and spinal cord was reduced, accompanied by a significant reduction in the noradrenalin-dependent startle response.

In contrast to these reports, a recent extensive analysis of parkin exon-2 deletion mutant mouse revealed that the behavioral profile and cathecholamine levels in

the brain were not different from those of control mice [69]. Moreover these mutant mice were not more sensitive to 6-hydroxydopamine or methamphetamine neurotoxicity, indicating that Parkin-deficient mice are not a robust model of parkinsonism [75].

9.4.4
The Problems with Animal Models of AR-JP

The differences between parkin-deficient fly and mouse models may be explained by the difference in the endogenous substrates or the presence of redundant pathways dealing with parkin substrates in mice. Drosophila and human parkin shows a similar cell protective effect against human Pael-R- and alpha-synuclein-mediated toxicities, suggesting that the substrates are conserved to some extent [58, 76, 77]. It is particularly important to investigate whether the relationship between Parkin and PINK1 is conserved in mice and humans.

The reason why dopaminergic cell loss does not occur in the parkin knockout mouse may be due to the existence of redundant ubiquitination pathways in mice. For example, Pael-R is known to be ubiquitinated by an ER-resident E3, Hrd1 as well as by Parkin [78]. In addition, the absence of dopaminergic cell loss can also probably be attributed to the relatively short lifespan of mice (2–3 years), which would not provide enough time for the toxic substrates to accumulate in concentrations sufficient to cause cell death.

It has been noted that disturbances of the nigrostriatal system, which may represent the early signs of neurodegeneration, are suggested to occur in two exon 3-deletion mutant mice. However, the individual key findings in these papers are not in accord and no dopaminergic phenotype was detected in the exon 7- and exon 2-deletion mutant mice with respect to parkin [68, 69]. Some of the discrepancies in the detection of mild phenotypes might be caused by the different techniques employed or differences in the genetic backgrounds of the mice. Taking these possibilities into consideration, detailed and careful comparison of the phenotypes of these different parkin knockout mice should be carried to identify the real and reproducible phenotype.

9.5
Future Directions

Seven years have passed since parkin was identified as a ubiquitin ligase, and since then 13 different molecules have been isolated as parkin substrates. Some of the substrate molecules appear to explain the pathogenetic mechanisms underlying AR-JP. However, proof of accumulation of known substrates in the parkin knockout mouse brain has not been obtained except for p38, probably because of the relatively short lifespan of the mouse. So, what then is the next step?

One of the potentially promising approaches is to examine whether the nigral dopaminergic neurons in parkin-deficient mice are vulnerable to a specific stress

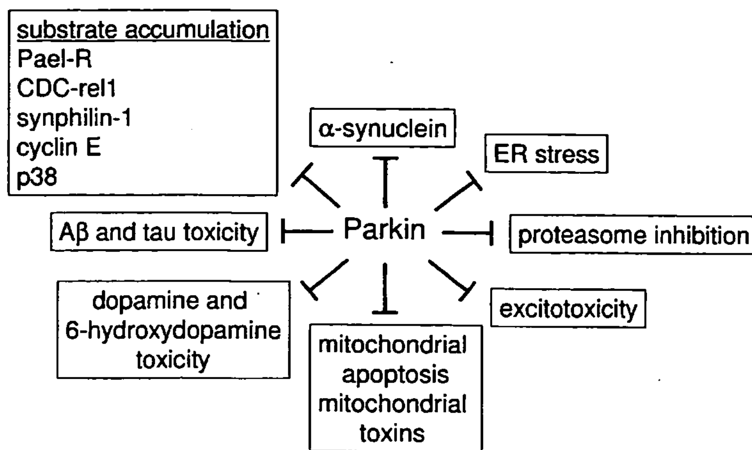

Fig. 9.5. Parkin protects cells from various stresses.

or overexpression of a specific substrate using virus vector or transgenic approaches, as has been applied to Pael-R [59]. On one hand, it is possible that the disease-causing substrate(s) has not been identified and further efforts to identify such a substrate(s) will also be important.

On the other hand, parkin appears to have cell-protective functions against various stresses (Figure 9.5). According to the reports to date, Parkin protects cells against ER stress [13], proteasomal inhibition [77], excitotoxicity [45], ceramide-induced mitochondrial apoptosis [79], mitochondrial toxins [80], intracellular Aβ [80], tau [81, 82], dopamine or 6-hydoxydopamine toxicity [47, 83] and α-synuclein-induced cell death [58, 76, 77, 84]. It is intriguing to ask whether clearance/sequestration of certain parkin substrate(s) contributes to such cell protective effects. Whether the pathways to neurodegeneration caused by parkin mutations are multiple or not should be clarified in the future.

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