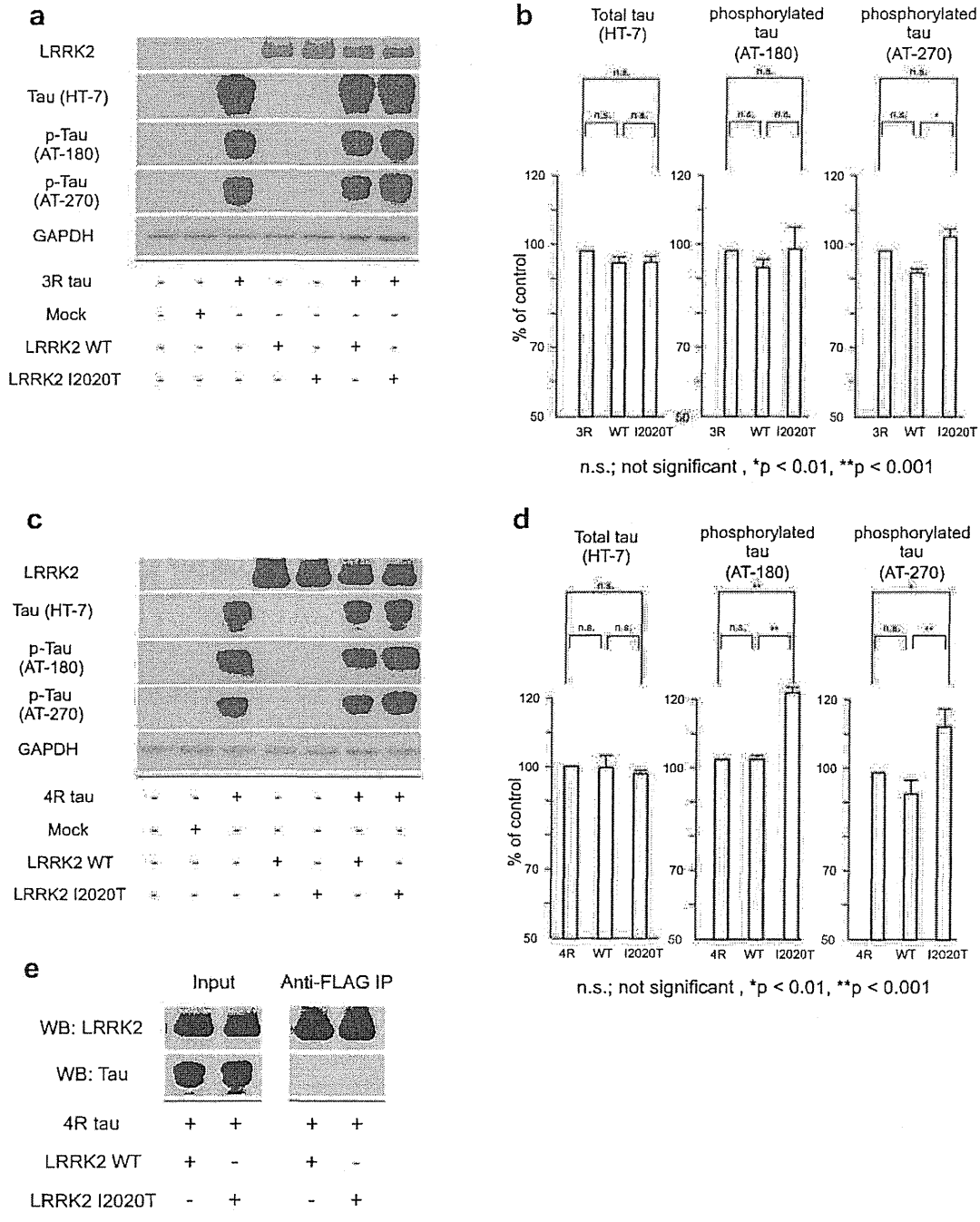

Fig. 3. LRRK2-I2020T induces increasing levels of phosphorylated tau compared with LRRK2-WT or mock transfected cells. (a, b) Lysate prepared from COS-1 cells co-expressing 3R tau and LRRK2-WT or I2020T, were subjected to anti-tau (HT-7) or anti-phosphorylated tau (AT-180 and AT-270) immunoblotting. LRRK2-I2020T increased expression levels of phosphorylated tau compared to LRRK2-WT, albeit modestly. (HT-7; 96.3 ± 1.8% with WT vs. 96.5 ± 1.9% with I2020T [mean ± SEM]; n.s., AT-180; 94.9 ± 2.4% with WT vs. 100.5 ± 6.5% with I2020T; n.s., AT-270; 93.5 ± 1.2% with WT vs. 104.1 ± 2.5% with I2020T; $p < 0.01$) (c, d). Lysate prepared from COS-1 cells co-expressing 4R tau and LRRK2-WT or I2020T, were subjected to anti-tau (HT-7) or anti-phosphorylated tau (AT-180 and AT-270) immunoblotting. LRRK2-I2020T significantly increased expression levels of phosphorylated tau compared to LRRK2-WT. (HT-7; 99.7 ± 3.5% with WT vs. 97.8 ± 1.1% with I2020T; n.s. AT-180; 100.0 ± 1.2% with WT vs. 118.5 ± 1.5% with I2020T; $p < 0.001$, AT-270; 93.7 ± 4.0% with WT vs. 113.8 ± 5.3% with I2020T; $p < 0.001$). (e) Lysate prepared from COS-1 cells transfected with Myc-4 repeats tau and FLAG-LRRK2-WT or FLAG-LRRK2-I2020T, were subjected to immunoprecipitation with anti-FLAG antibody followed by anti-tau (HT-7) immunoblotting. In the left panel, cell lysates were used to detect the expression of LRRK2 and tau. In the right panel, FLAG-LRRK2 was immunoprecipitated using FLAG antibody. Upper lanes show LRRK2 detected with anti-LRRK2 antibody. Lower lanes show that no bands were obtained with anti-HT-7 antibody. As a result, LRRK2 does not directly interact with 4R tau.

nucleus of the trochlear nerve in patients B and C, neither exhibited ophthalmoparesis. Consistent with these findings, Vitte et al. reported that LRRK2 protein is present throughout the human brain, with intense immunoreactivity in the neurons of several midbrain nuclei, including the nucleus of the trochlear nerve [28].

We then demonstrated the association between LRRK2 and tau hyperphosphorylation by using cultured cell models. Compared to LRRK2-WT or mock transfected, overexpression of LRRK2-I2020T in cultured cells resulted in increased levels of phosphorylated tau proteins. Furthermore, this increase in phosphorylated tau was

associated with upregulation of both 3R and 4R tau isoforms. These findings could provide support for abnormal hyperphosphorylated tau deposition in the pathological findings of patients with *LRRK2 I2020T* mutation.

Based on neuropathological findings and cultured cell models, we hypothesized that LRRK2 is able to enhance tau phosphorylation. Our immunoprecipitation studies showed no evidence of a direct interaction between either LRRK2-WT or I2020T mutant with tau, indicating that tau phosphorylation by LRRK2-I2020T involves the association of an intermediate, genetic, or environmental factor. Smith et al. also reported that LRRK2 failed to bind tau protein [30]. Furthermore, LRRK2 mutations have been reported to be associated with tau hyperphosphorylation without direct interaction in animal models. Li et al. reported that tau is hyperphosphorylated in brain tissues from LRRK2-R1441G overexpressing mice compared with LRRK2-WT mice [17]. Mice and drosophila overexpressing *LRRK2*-G2019S also exhibited tau alterations, including mislocalization and increased tau phosphorylation [18,19]. Therefore, we believe that LRRK2 mutations can be involved in the tau phosphorylation pathway.

How LRRK2 can participate in the tau phosphorylation pathway remains unclear. In addition, we failed to find that these abnormal tau deposits have any apparent spatial correlation with our observed region-specific neuronal degeneration in the Sagamihara family. Therefore, future work will need to evaluate the association between neurodegeneration and the tau hyperphosphorylation due to *LRRK2 I2020T* mutation.

Conflicts of interest

None declared.

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PINK1-mediated phosphorylation of the Parkin ubiquitin-like domain primes mitochondrial translocation of Parkin and regulates mitophagy

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Parkinson's disease genes *PINK1* and *parkin* encode kinase and ubiquitin ligase, respectively. The gene products PINK1 and Parkin are implicated in mitochondrial autophagy, or mitophagy. Upon the loss of mitochondrial membrane potential ($\Delta\Psi$m), cytosolic Parkin is recruited to the mitochondria by PINK1 through an uncharacterised mechanism – an initial step triggering sequential events in mitophagy. This study reports that Ser65 in the ubiquitin-like domain (Ubl) of Parkin is phosphorylated in a PINK1-dependent manner upon depolarisation of $\Delta\Psi$m. The introduction of mutations at Ser65 suggests that phosphorylation of Ser65 is required not only for the efficient translocation of Parkin, but also for the degradation of mitochondrial proteins in mitophagy. Phosphorylation analysis of Parkin pathogenic mutants also suggests Ser65 phosphorylation is not sufficient for Parkin translocation. Our study partly uncovers the molecular mechanism underlying the PINK1-dependent mitochondrial translocation and activation of Parkin as an initial step of mitophagy.

Mutations of the *PINK1* gene cause selective degeneration of the midbrain dopaminergic neurons in autosomal recessive juvenile Parkinson's disease (PD)[1]. The *PINK1* gene encodes a serine/threonine kinase with a predicted mitochondrial target sequence and a putative transmembrane domain at the N-terminus[2–5]. Loss of the *PINK1* gene in *Drosophila* results in the degeneration of mitochondria in cells with high energy demands, such as muscle and sperm cells, which is suppressed by the introduction of the *parkin* gene, another gene responsible for autosomal recessive juvenile PD[6–8]. The gene product Parkin encodes a RING-finger type ubiquitin ligase (E3) with a Ubl domain at the N-terminus[9–12].

A series of cell biological studies have provided strong evidence that there are important roles for PINK1 and Parkin in regulating mitochondrial homeostasis. PINK1 is constitutively proteolysed by the mitochondrial rhomboid protease, PARL, at the mitochondrial membrane of healthy mitochondria, resulting in processed forms of PINK1[13–16]. The processed PINK1 is rapidly degraded by the proteasome[2,17]. The reduction of $\Delta\Psi$m leads to the accumulation and activation of PINK1 in the mitochondria[17–19] through a currently unresolved mechanism[20]. The accumulation of PINK1 recruits Parkin from the cytosol to the mitochondria with decreased membrane potential, which stimulates Parkin E3 activity, promoting mitochondrial degradation via an autophagic event known as mitophagy[17,21–24]. The recruitment of cytosolic Parkin to the mitochondria upon disruption of $\Delta\Psi$m is believed to be the first step of mitophagy for the removal of damaged mitochondria. This recruitment is required for the kinase activity of PINK1[17,21–25]. Although two separate studies have proposed that Parkin is directly phosphorylated by PINK1[26,27], others have failed to detect Parkin phosphorylation by PINK1[21], suggesting that the kinase activity of PINK1 itself is relatively low. One reason biochemical analysis has been unable to obtain direct evidence is that recombinant human PINK1 purified from mammalian cultured cells or bacteria easily loses kinase activity, while insect PINK1 has significant autophosphorylation activity[28,29].

Very recently, Kondapalli, C. *et al.* reported that PINK1 directly phosphorylates Parkin at Ser65 in the Ubl domain[18]. However, the extent and consequences of Parkin phosphorylation by PINK1 in mitochondrial regulation are still not fully understood.

To address this issue, we attempted to independently monitor and compare the phosphorylation status of Parkin in wild-type and *PINK1*-deficient cells, thereby excluding the possibility of phosphorylations by uncharacterised kinases other than PINK1[30]. Here, we also report that Parkin is demonstrably phosphorylated at Ser65 in a PINK1-dependent manner. Furthermore, we show that this phosphorylation event is implicated in the regulation of mitochondrial translocation of Parkin and the subsequent degradation of mitochondrial surface proteins during mitophagy.

Results

Parkin is phosphorylated upon depolarisation in $\Delta\Psi$m. We used [^{32}P] orthophosphate to metabolically label mouse embryonic fibroblasts (MEFs) derived from *PINK1* deficient mice, in which HA-tagged Parkin together with FLAG-tagged wild-type or kinase-dead forms (triple mutant with K219A, D362A and D384A) of PINK1 were virally introduced (hereafter referred to as "PINK1-FLAG WT" or "KD/HA-Parkin/*PINK1*$^{-/-}$" MEFs) and then induced Parkin-mediated mitophagy via treatment with the protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP). As shown in Figure 1a, Parkin was specifically phosphorylated in CCCP-treated PINK1-FLAG WT/HA-Parkin/*PINK1*$^{-/-}$ MEFs, but not in PINK1-FLAG KD/HA-Parkin/*PINK1*$^{-/-}$ MEFs. Phos-tag Western blotting, in which phosphorylated proteins appear as slower migrating bands[28], revealed that Parkin was phosphorylated within 10 min following CCCP treatment (Fig. 1b). Phosphorylation of Parkin reached its maximum level approximately 40 min after CCCP treatment and was sustained at least until 6 hr (Supplementary Fig. S1). Under these conditions, slower migrating bands of PINK1 also appeared, which very likely reflects the autophosphorylation of PINK1 when activated (Fig. 1b)[18]. The suppression of PINK1 accumulation by RNA interference suggested that$\Delta\Psi$m depolarisation-dependent activation of PINK1 along with PINK1 accumulation is a key element for Parkin phosphorylation (Fig. 1c). Every PINK1 deletion and pathogenic mutant we tested failed to stimulate Parkin phosphorylation effectively, strongly suggesting that intact PINK1 is required for this action (Fig. 1d and e). Importantly, human fibroblasts from a patient with *PINK1*-linked parkinsonism also lacked the activity to phosphorylate Parkin (Fig. 1f). The phosphorylated Parkin disappeared within 30 min during the recovery of $\Delta\Psi$m depolarisation by the removal of CCCP from the culture medium (Fig. 1g). Further analysis using phosphatase and proteasome inhibitors suggested that phosphorylated Parkin is at least partly degraded by proteasomal activity in the mitochondria (Supplementary Fig. S2).

Phosphorylation of Ser65 in the Parkin Ubl domain primes the mitochondrial translocation of Parkin. To determine which residue(s) of Parkin are phosphorylated, we immunopurified HA-tagged Parkin from PINK1-FLAG WT or KD/HA-Parkin/*PINK1*$^{-/-}$ MEFs treated with or without CCCP and performed mass spectrometric analysis for phospho-peptides (Supplementary Fig. S3). Although Phos-tag Western blotting of Parkin mainly detected a single band shift, which represents a single phospho-modification, the mass spectrometric analysis identified Ser9 or Ser10 and Ser65, Ser101 and Ser198 as phosphorylated residues of Parkin. Among these residues, only Ser65 phosphorylation increased (33-fold) in CCCP-treated PINK1-FLAG WT/HA-Parkin/*PINK1*$^{-/-}$ MEFs (Supplementary Fig. S3). Phos-tag Western blotting with mutant forms of Parkin, in which the identified phospho-serine residues are replaced with alanine, revealed that the band shift represents Ser65 phosphorylation (Fig. 2a). An *in vitro* kinase assay with recombinant insect PINK1, which has marked kinase activity[28], strongly suggested that PINK1 directly phosphorylates Parkin at Ser65 (Supplementary Fig. S4). The Ser65 residue lies in the Ubl domain and is highly conserved from human to *Drosophila* (Fig. 2b). We next examined whether phosphorylation of Ser65 is required for Parkin-mediated mitophagy. GFP-tagged Parkin WT, which was localised both in the cytoplasm and in the nuclei of mock (DMSO)-treated cells (0 hr, Fig. 2c and d), was translocated to the mitochondria and induced the perinuclear aggregation of mitochondria 2 hr after CCCP treatment, as previously reported (2 hr, Fig. 2c and d)[17,23]. Replacement of Ser65 with alanine (S65A) did not affect the subcellular localisation of Parkin in mock-treated cells when compared with that of GFP-Parkin WT (0 hr, Fig. 2c and d). However, GFP-Parkin S65A almost completely inhibited the mitochondrial translocation of Parkin and the perinuclear rearrangement of mitochondria 0.5 hr after CCCP treatment (0.5 hr, Fig. 2c and d) and showed delayed translocation in 2 hr (2 hr, Fig. 2c and d). The expression of a putative phosphomimetic Parkin S65E also showed a subcellular localisation similar to that of GFP-Parkin WT in both DMSO- and CCCP-treated cells (Fig. 2c). However, GFP-Parkin S65E exhibited a mild translocation defect, suggesting that S65E does not fully mimic the phosphorylated Ser65 (Fig. 2d).

Parkin Ser65 phosphorylation is not sufficient for mitochondrial translocation upon depolarisation of $\Delta\Psi$m. As PINK1-mediated Ser65 phosphorylation appeared to be required for efficient translocation of Parkin, we next examined whether well-characterised pathogenic Parkin mutants were subjected to phosphorylation upon CCCP treatment. In this experiment, we used three kinds of Parkin mutants based on the previous and current studies (Supplementary Fig. S5)[17,22,23]. The first group, V15M, P37L, R42P and A46P, had intact or weakly impaired mitochondrial translocation activity. The second group, T415N and G430D, had mildly impaired translocation activity. The third group, K161N, K221N and T240R, almost completely lacked translocation activity (Fig. 3a). Surprisingly, all of the mutants possessed comparable phosphorylation efficiencies to those of WT (Fig. 3b). This result suggests that Ser65 phosphorylation is not sufficient for the mitochondrial translocation of Parkin.

Biochemical fractionation of endogenous Parkin from SH-SY5Y cells detected only the phosphorylated form of Parkin in the mitochondrial fraction upon CCCP treatment (Fig. 3c), which strongly suggests that phosphorylation of Parkin is required for mitochondrial translocation. There was a slight difference in the gel mobility of phosphorylated Parkin between the cytosolic and the mitochondrial fractions and between CCCP-treated periods of time. These differences very likely reflect differences in the complexity of the contents of each fraction rather than in the phosphorylation status of Parkin because a single shifted band appears in the mixed fractions (Mito + Cyto in Fig. 3c; CCCP 30 min + 60 min in Supplementary Fig. S6).

Effect of Parkin Ser65 phosphorylation on the autophagic reaction. We next examined whether Ser65 phosphorylation is required for the subsequent autophagic reaction, in which various ubiquitin-proteasome- and autophagy-related proteins are involved, including the 26S proteasome, p97/VCP, p62/SQSTM1, LC3, ATG5 and ATG7[22,23,31–35]. Parkin has been reported to be involved in the ubiquitin-proteasome-dependent degradation of a variety of mitochondrial outer membrane proteins, including Mitofusin1 (Mfn1)[32], Mfn2[32], Miro1[36,37], Miro2[37], VDAC1[22] and Tom20[31]. Degradation of Mfn1, VDAC1 and Tom20 at the mitochondrial outer membrane was observed in PINK1 WT/GFP-Parkin/*PINK1*$^{-/-}$ MEFs 1 to 4 hr after CCCP treatment (Fig. 4a). While GFP-Parkin harbouring S65A or S65E mutations was also capable of inducing Mfn1, VDAC1 and Tom20 degradation, the efficiency was impaired, especially in Mfn1 and VDAC1 (Fig. 4a). Long-term time course analysis revealed that in cells expressing Parkin with S65A or S65E mutations, Mfn1 and VDAC1 cannot be degraded effectively, and the mitochondrial outer membrane was likely more intact as indicated by the sustained

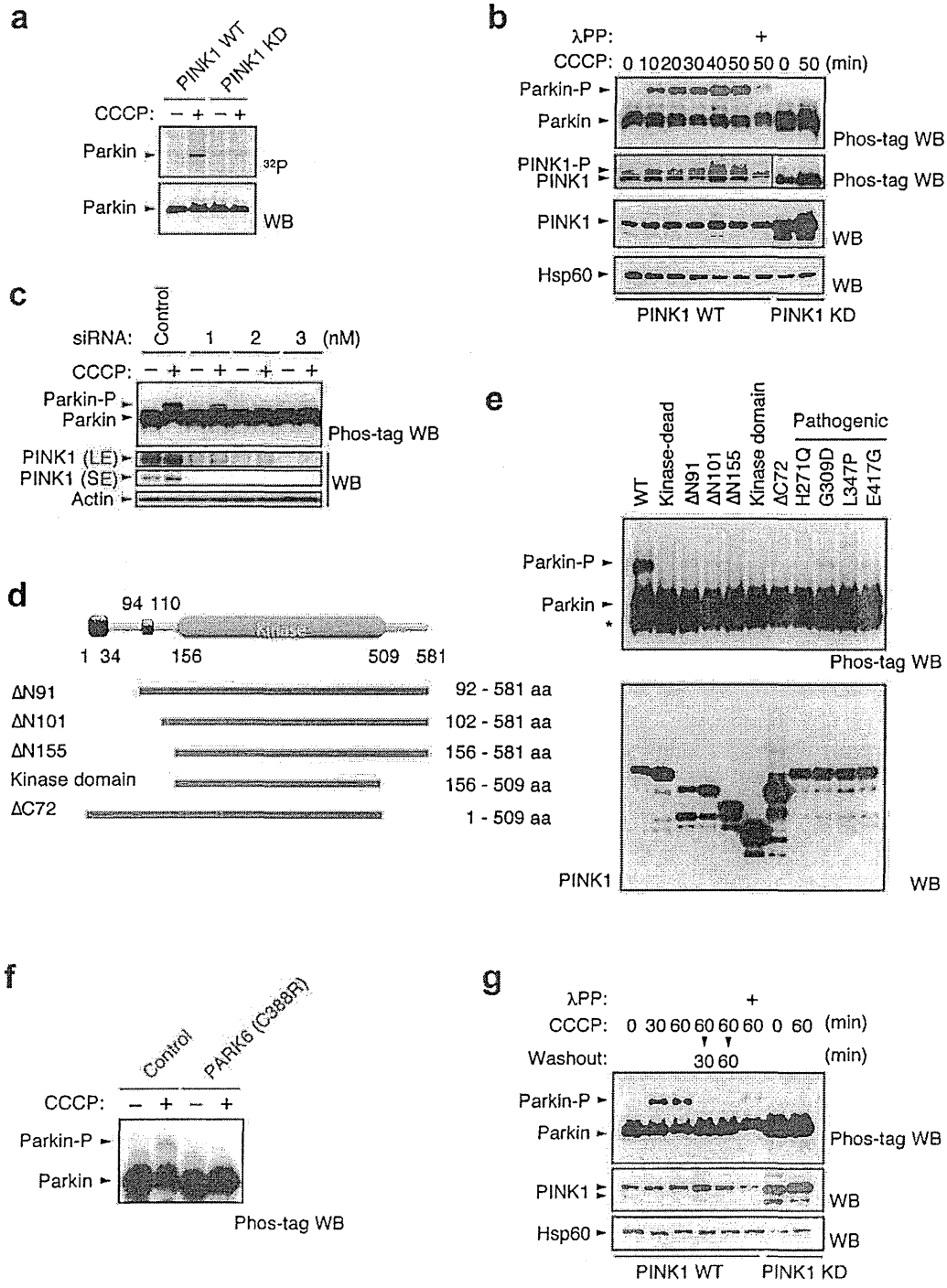

Figure 1 | PINK1-dependent phosphorylation of Parkin *in vivo*. (a) PINK1-FLAG WT or KD/HA-Parkin/*PINK1*$^{-/-}$ MEFs were labelled with [^{32}P] orthophosphate and treated with 30 μM CCCP for 1.5 hr. Phosphorylated Parkin was detected by autoradiography (^{32}P). Immunoprecipitated HA-Parkin was detected by Western blotting (WB) with anti-Parkin. (b) PINK1-FLAG WT or KD/HA-Parkin/*PINK1*$^{-/-}$ MEFs were treated with or without 30 μM CCCP for the indicated periods of time. Cell lysate was subsequently separated on a Phos-tag gel, followed by WB with anti-PINK1 or anti-Parkin antibodies (Phos-tag WB). Phosphorylated bands of Parkin and PINK1 were confirmed by their disappearance with lambda protein phosphatase (λPP) treatment. Mitochondrial Hsp60 was used as a loading control. (c) Suppression of endogenous PINK1 expression inhibits Parkin phosphorylation. HeLa cells stably expressing non-tagged Parkin were treated with the indicated concentrations of stealth siRNA duplex against PINK1 (Invitrogen) with or without 10 μM CCCP for 1 hr. Long- (LE) and short-exposure (SE) blot signals for PINK1 were shown. Actin was used as a loading control. (d) Truncated PINK1 mutants used in this study. Putative mitochondria-targeting sequence, 1–34 aa; transmembrane domain, 94–110 aa; kinase domain, 156–509 aa. (e) Full-length PINK1 is required for Parkin phosphorylation. *PINK1*$^{-/-}$ MEFs stably expressing non-tagged Parkin were transfected with various PINK1 constructs with C-terminal FLAG-tags. PINK1 expression was confirmed with anti-FLAG-HRP. (f) Human fibroblasts from a normal control and a *PARK6* case with a homozygous C388R mutation[41] were transfected with Parkin and were treated with or without 30 μM CCCP for 1 hr. (g) Cells treated with CCCP up to 60 min as in (b) were further incubated with fresh culture medium without CCCP for the indicated periods of time (Washout).

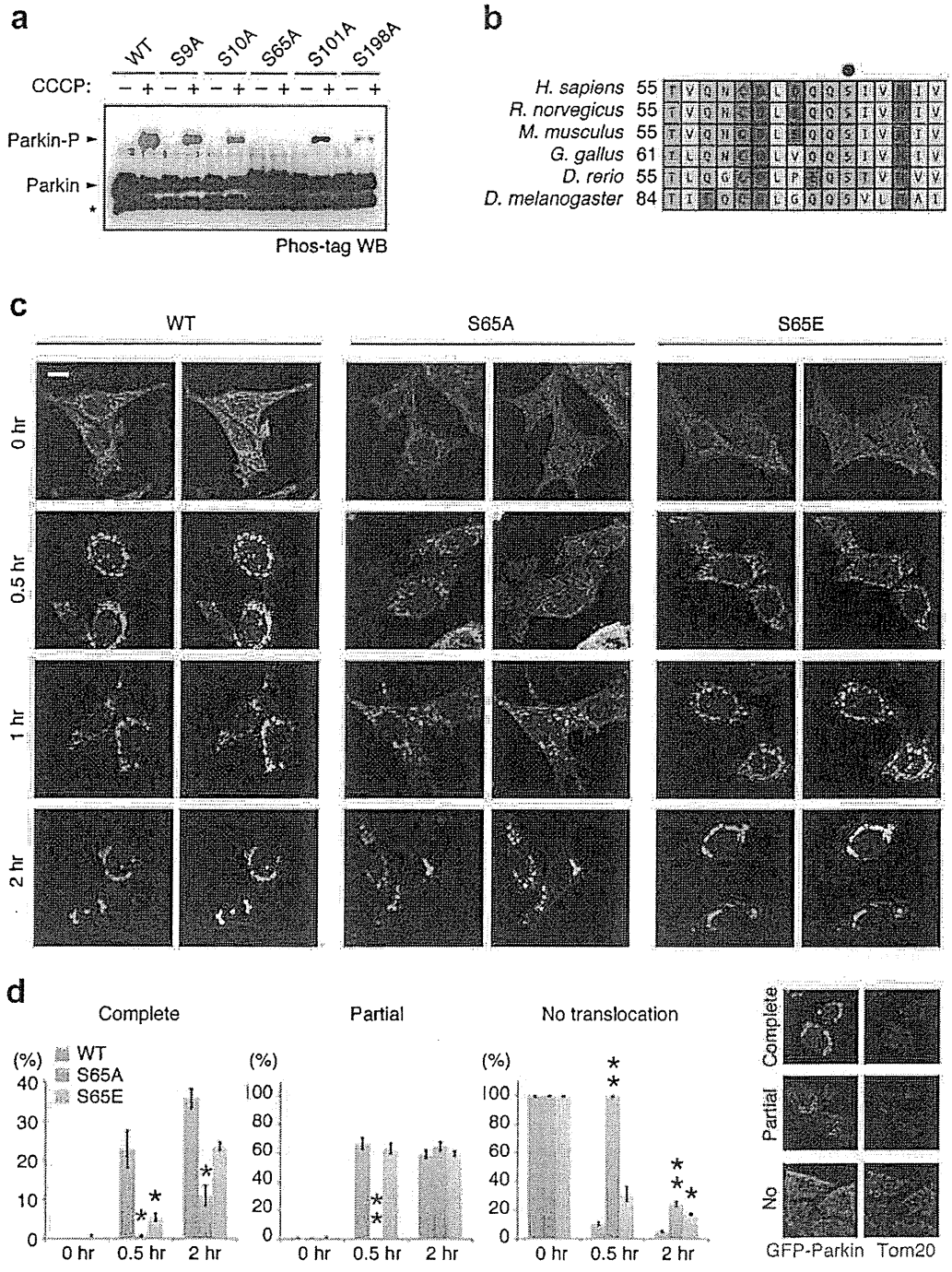

Figure 2 | Ser65 in the Ubl domain of Parkin is phosphorylated upon depolarisation of $\Delta\Psi$m. (a) Phos-tag Western blotting detected phosphorylation of Ser65. HeLa cells were transiently transfected with Parkin WT and a series of alanine mutants for the candidate phospho-residues followed by treatment with or without 20 μM CCCP for 1 hr. Cell lysates were analysed by Phos-tag Western blotting. An asterisk indicates degraded Parkin. (b) Alignment of the amino acid sequences surrounding Ser65 (marked by a black dot) from a variety of animal species. The numbers on the left correspond to the residue numbers of Parkin proteins. (c) Introduction of the S65A mutation delayed Parkin translocation to the depolarised mitochondria in PINK1 WT/GFP-Parkin/*PINK1*$^{-/-}$ MEFs. Cells retrovirally introduced with GFP-Parkin WT or its phospho-mutants (S65A and S65E) were treated with or without 30 μM CCCP for the indicated periods of time. GFP-Parkin and mitochondria were visualised with anti-GFP (green) and anti-Tom20 (red), respectively. Parkin signals are also shown as monochrome images. Scale bar = 10 μm. (d) Mitochondrial translocation efficiency of Parkin mutants. PINK1 WT/*PINK1*$^{-/-}$ MEFs stably expressing GFP-Parkin WT, S65A or S65E were treated as in (c). Cells expressing GFP-Parkin perfectly overlapped (Complete, examples are shown on the right), partially overlapped (Partial) or non-overlapped (No) with the Tom20 signal were counted. The data represent means ± SE from three experiments (n = 99–143 cells in each). ** $p < 0.01$, * $p < 0.05$ *vs.* WT at each time point.

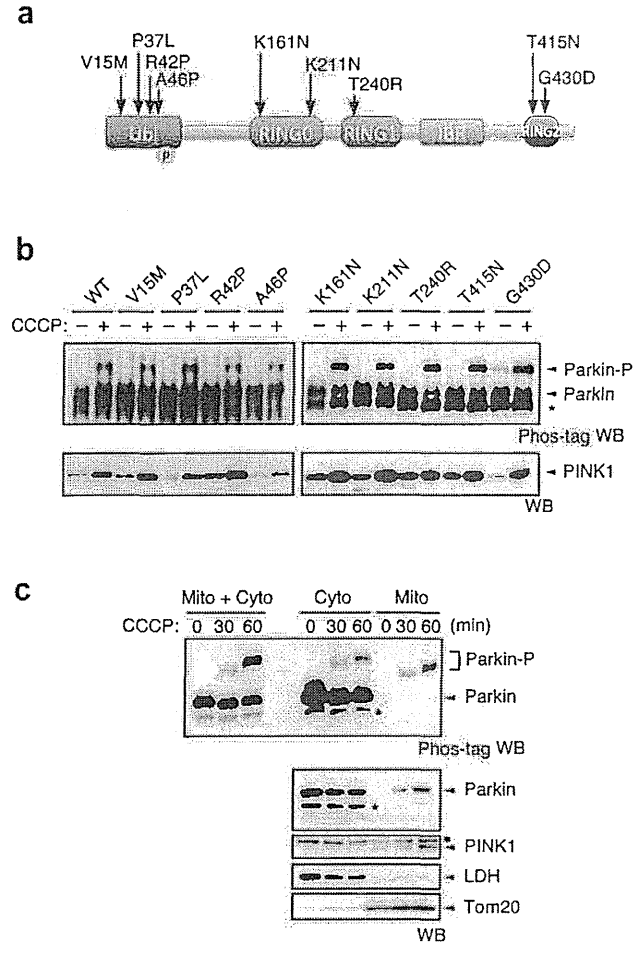

Figure 3 | Pathogenic mutants of Parkin are subjected to Ser65 phosphorylation. (a) Diagram of Parkin protein illustrating the pathogenic mutants used in this study. The Ser65 residue in the Ubl domain is shown as a yellow circle. RING, Ring-finger motif; IBR, in-between-Ring fingers domain. (b) Phos-tag Western blotting for Parkin and Western blotting for PINK1 were performed using Parkin WT and a series of pathogenic mutants as shown in Figure 2a. (c) Endogenous Parkin was also phosphorylated in SH-SY5Y cells after CCCP treatment. Post-nuclear cell lysates from SH-SY5Y cells treated with or without 10 μM CCCP for 30 and 60 min were fractionated into mitochondria-rich (Mito) and cytosolic (Cyto) fractions. These two fractions and their combination (Mito + Cyto) were subjected to Phos-tag or normal Western blotting analyses. Endogenous PINK1 was fractionated in the Mito fraction, as previously reported[45]. Lactate dehydrogenase (LDH) and Tom20 were used as cytosolic and mitochondrial marker proteins, respectively. Asterisks: putative cleaved Parkin; dots: non-specific bands.

accumulation of PINK1 (Fig. 4b). The impaired degradation cannot be explained simply by the delayed translocation of Parkin mutants because both mutants completed the mitochondrial translocation by the 6 hr time-point (data not shown and see Fig. 4c). In contrast, the profiles of Parkin expression and autoubiquitination in Parkin S65A- or S65E-expressing cells were comparable with those of WT (Fig. 4b). We also examined whether Ser65 mutations affect the accumulation of proteasome (Fig. 4c) and p62 (Supplementary Fig. S7) at the mitochondria during mitophagy via the immunostaining of the proteasome subunit alpha type 7 (α7) and p62. However, there was no evidence that Ser65 mutations inhibit or delay the recruitment of proteasome and p62 to the mitochondria. Finally, we tested whether the Parkin Ubl domain itself is indispensable for the mitochondrial translocation and the substrate degradation (Supplementary Fig. S8). Interestingly, Parkin mutant lacking the Ubl domain (ΔUbl) showed a mild delay in the mitochondrial translocation, slowed the mitochondrial reorganization to the perinuclear region (Supplementary Fig. S8b and c) and impaired the degradation of mitochondrial outer membrane proteins (Supplementary Fig. S8d). These results suggest that proper regulation of the Parkin Ubl domain through the Ser65 phosphorylation is required not only for efficient translocation to mitochondria as an initial step of mitophagy, but also for the degradation of mitochondrial outer membrane proteins during mitophagy through an as yet unknown mechanism.

Discussion

A series of *Drosophila* genetic and cell biological studies have clearly demonstrated that PINK1 is required for Parkin-mediated mitochondrial maintenance. The mitophagy of damaged mitochondria is a well-characterised event in which PINK1 and Parkin are involved. However, how PINK1 regulates Parkin is largely unclear. This study has shown that Ser65 in the Ubl domain of endogenous Parkin is phosphorylated in an activated PINK1-dependent manner. In addition to mitochondrial accumulation of PINK1, ΔΨm depolarisation-dependent PINK1 autophosphorylation has been reported to be an important element for PINK1 activation and Parkin recruitment[19,29]. Consistent with these observations, our investigation of PINK1 siRNA suggests that a lower level of PINK1 is able to phosphorylate Parkin after ΔΨm depolarisation (Fig. 1c, compare lanes 1 and 4). Our domain analysis of PINK1 demonstrates that intact PINK1 is required for CCCP-dependent Parkin phosphorylation, and the lack of phosphorylation in fibroblasts from a *PARK6* patient implies relevance to the pathogenesis of PD.

The biological significance of this phosphorylation event is suggested by the fact that replacement of Ser65 with alanine or glutamic acid impairs the mitochondrial translocation of Parkin and/or the subsequent mitophagy process. Our observation that maximal phosphorylation of Parkin occurs within 1 hr of CCCP treatment supports the idea that Ser65 phosphorylation is required for the early step of Parkin translocation. In contrast, PINK1 accumulation appears to last at least 6 hr (Fig. 4c and Supplementary Fig. S1b). The difference in time course between PINK1 accumulation and Parkin phosphorylation could be explained by the observation that phosphorylated Parkin is degraded by proteasomal activity. The biochemical evidence that only the phosphorylated form of endogenous Parkin is present in the mitochondrial fraction also implies that Parkin phosphorylation is an essential event for its mitochondrial translocation and subsequent activation (Fig. 3c and Supplementary Fig. S6). Overexpression of PINK1 and Parkin itself leads to mitochondrial translocation of Parkin independently of ΔΨm depolarization, which suggests that excessive amounts of PINK1 and Parkin do not faithfully reflect endogenous reactions. Our study using *PINK1*$^{-/-}$ MEFs stably co-expressing PINK1 and GFP-Parkin might also be saddled with such a problem. We believe that the endogenous observation in which phosphorylated Parkin is accumulated in mitochondria is a more reliable proposal as a molecular model. The delay of exogenous GFP-Parkin S65A in the mitochondrial translocation would indicate that modification of Ser65 is important for Parkin translocation at least. At the same time, another important finding is that pathogenic mutants that lose their translocation activity are also phosphorylated (Fig. 3b), raising the possibility that phosphorylation of Parkin at Ser65 is insufficient for translocation. Thus, Ser65 phosphorylation likely leads to other events in mitochondrial translocation, such as the association or dissociation of protein(s) involved in the mitochondrial translocation of Parkin or the modification of Parkin itself for activation at a different site(s).

Both the S65A and S65E Parkin mutants cannot undergo efficient mitophagy, as indicated by the incomplete degradation of

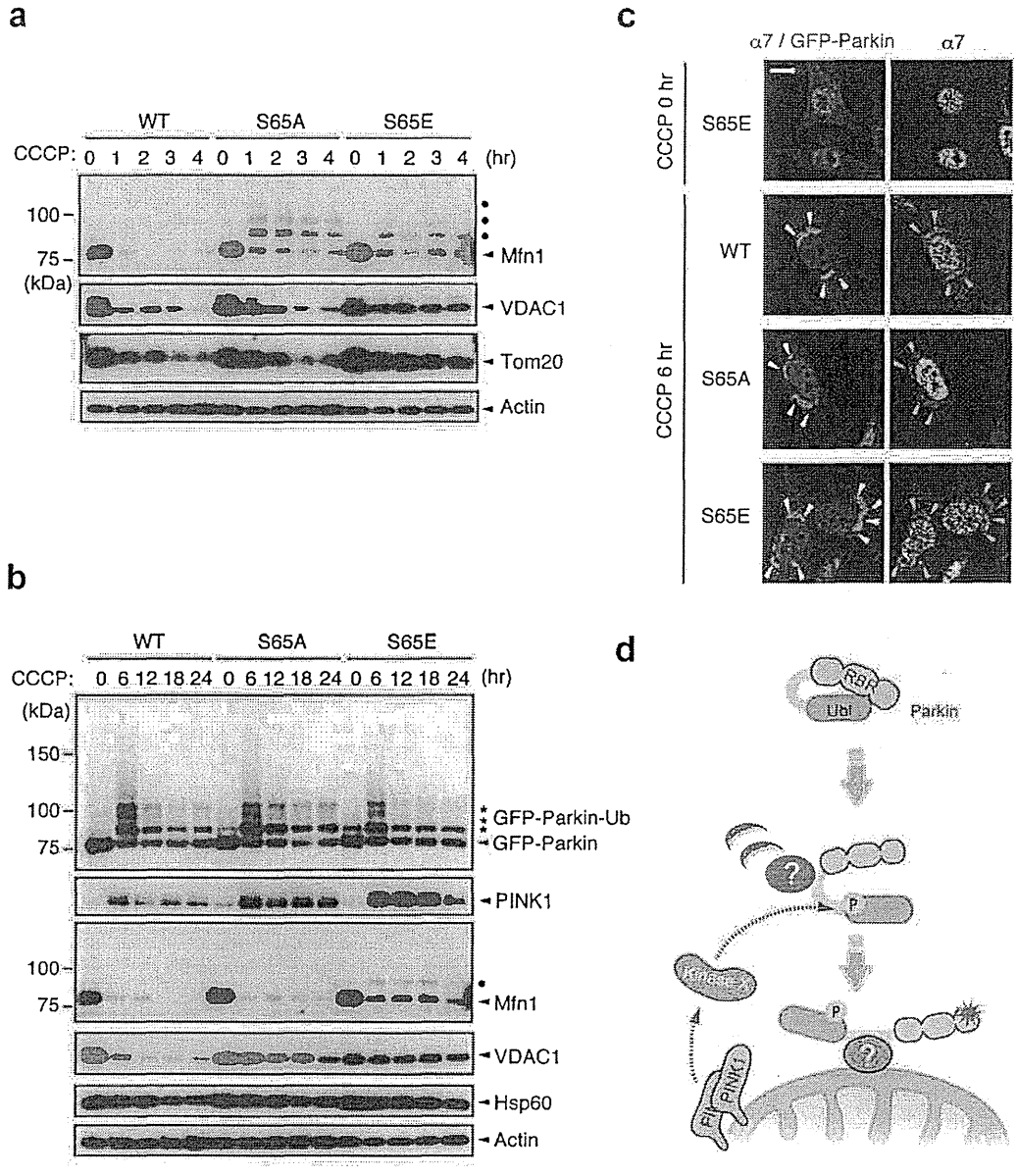

Figure 4 | Ser65 phosphorylation affects the subsequent autophagy reaction. (a) CCCP-dependent degradation of mitochondrial outer membrane proteins in PINK1 WT/*PINK1*$^{-/-}$ MEFs expressing WT or mutant forms of GFP-Parkin. Mfn1, VDAC1 and Tom20 were used as markers of mitochondrial outer membrane proteins. Actin: a loading control. Dots: ubiquitinated Mfn1. (b) Long-term time-course analysis of CCCP-dependent mitochondrial protein degradation. The degradation of outer membrane proteins was impaired in cells expressing GFP-Parkin S65A or S65E mutations. Hsp60 was used as a marker of mitochondrial matrix proteins. (c) S65A and S65E mutations do not affect proteasome recruitment to the mitochondria during mitophagy. PINK1 WT/*PINK1*$^{-/-}$ MEFs expressing WT or mutant forms of GFP-Parkin (green) were treated with 30 μM CCCP for 3 or 6 hr. Cells were stained with anti-proteasome subunit alpha type 7 (α7, red). α7-immunoreactivity was enriched in the nuclei of all three cell genotypes under normal conditions, as displayed in the representative image of S65E (CCCP 0 hr), and overlapped with the aggregated mitochondria (arrowheads) 6 hr after CCCP treatment irrespective of genotype. Similar results were obtained 3 hr after CCCP treatment. Scale bar = 10 μm. (d) Model for Parkin translocation and activation. The Parkin Ubl domain masks C-terminal RING-IBR-RING (RBR) domains for E3 activity[46]. A Parkin phosphorylation event at Ser65 (P), combined with unknown factor(s) (?), stimulates the mitochondrial translocation of Parkin, releasing the RBR domains from autoinhibition by the Ubl domain.

the mitochondrial outer membrane proteins. Because inhibition of the degradation of the mitochondrial outer membrane proteins by proteasome inhibitors is reported to block mitophagy[32,35], it may be that the modification of Parkin Ser65 has a greater than expected impact on the mitophagy process. Although our study does not demonstrate that the S65E mutant behaves exactly like the phosphorylated form of Parkin, the S65E mutant does translocate to the mitochondria in a similar way to WT, although with slightly impaired efficiency, suggesting that S65E has at least some properties that are similar to phosphorylated Parkin. Currently, it is unknown why S65E also inhibits the later processes of mitophagy. One possible explanation is that rapid degradation of phosphorylated Parkin is required for the proper progression of mitophagy, and S65E may not be degraded effectively. However,

there is no evidence that S65E is more stable than WT, as shown in Figure 4c.

Very recently, Kondapalli *et al.* proposed a model to explain the biological significance of Ser65 phosphorylation, in which Ser65 phosphorylation relieves autoinhibition of Parkin E3 activity by the Ubl domain[18]. This model may explain the depolarised $\Delta\Psi$m-dependent activation of Parkin. However, our data indicated that the Parkin S65A mutant is also autoubiquitinated (Fig. 4b) and that the ΔUbl mutant showed mild translocation defect and impaired substrate degradation (Supplementary Fig. S8). Moreover, if this is the case, the E3 activity of Parkin pathogenic mutants lacking mitochondrial translocational activity but harbouring intact E3 activity *in vitro* (such as K161N and K211N, which are subjected to the Ser65 phosphorylation) should be activated in the cytosol[38]. However, our previous data indicate that K161N and K211N are not activated by CCCP treatment[23]. Thus, it is conceivable that another step is required for depolarised $\Delta\Psi$m-dependent activation of Parkin E3. In addition, the Ubl domain might not only autoinhibit its E3 activity but also contribute to the mitochondrial translocation and the substrate degradation through an as yet unknown mechanism. We believe that an appropriate way to estimate Parkin E3 activity in the context of mitophagy is to evaluate the ubiquitination and degradation of substrates in cells with depolarised $\Delta\Psi$m. Mfn1 is a well-characterised direct substrate of Parkin[32], and Parkin-dependent poly-ubiquitination modification of Mfn1 can be detected by Western blotting upon $\Delta\Psi$m depolarisation[32,39,40]. Parkin S65A and S65E appear to ubiquitinate Mfn1, as poly-ubiquitinated forms of Mfn1 were observed (Fig. 4b). However, they cannot degrade it effectively, which suggests that the process of substrate degradation is also impaired in these mutants.

Kondapalli *et al.* have also shown that *T. castaneum* PINK1 (TcPINK1) directly phosphorylates human Parkin at Ser65[18]. We confirmed their finding using recombinant TcPINK1 produced from the same construct (Supplementary Fig. S4). The replacement of MBP-Parkin Ser65 with alanine completely abolished PINK1-mediated phosphorylation, indicating that Ser65 is the sole phosphorylation site *in vitro*. However, experiments in cultured cells showed that the replacement of Ser9, Ser10, Ser101 and Ser198 with alanine affects the Ser65 phosphorylation efficiency (Ser9, ~35% reduction; Ser10, ~76% reduction; Ser101, ~65% reduction; Ser198, ~92% reduction) (Fig. 2a). These residues might be priming phosphorylation sites for Ser65 phosphorylation.

Because PINK1 is believed to be activated in the mitochondria, a topological inconsistency arises from our cell-based data that cytosolic Parkin lacking the mitochondrial translocation activity is phosphorylated. Therefore, it is possible that PINK1 indirectly regulates Parkin phosphorylation. One possible explanation for this is the presence of another cytosolic kinase(s) regulated by PINK1 (Fig. 4d). Alternatively, because mitochondria are a dynamic organelle, cytosolic Parkin adjacent to the moving and fragmented mitochondria with depolarised $\Delta\Psi$m might be phosphorylated incidentally. The issue as to whether or not PINK1 directly phosphorylates Parkin in cells remains to be solved.

In conclusion, this study has suggested that PINK1-dependent Parkin phosphorylation at Ser65 accelerates the mitochondrial translocation of Parkin and showed that the introduction of mutations at this site also affects subsequent mitophagy processes. Concurrently, our data provide the possibility that there is an elaborate multi-step mechanism for the mitochondrial translocation of Parkin upon the loss of $\Delta\Psi$m (Fig. 4d), the clarification of which awaits further study.

Methods

Antibodies, plasmids and cell lines. Antibodies used in Western blot analysis were as follows: anti-Parkin (1 : 1,000 and 1 : 5,000 dilution for endogenous and exogenous Parkin, respectively; Cell Signaling Technology, clone PRK8), anti-PINK1 (1 : 1,000 dilution; Novus, BC100-494 or 1 : 1,000 dilution; Cell Signaling Technology, clone D8G3), anti-Mfn1 (1 : 1,000 dilution; Abnova, clone 3C9), anti-VDAC1 (1 : 1,000 dilution; Abcam, Ab15895), anti-Tom20 (1 : 500 dilution; Santa Cruz Biotechnology, FL-145), anti-FLAG-HRP (1 : 2,000 dilution; Sigma-Aldrich, clone M2), anti-GFP (1 : 5,000 dilution; Abcam, ab290), anti-Actin (1 : 10,000 dilution; Millipore, MAb1501), anti-LDH (1 : 1,000 dilution; Abcam, ab7639-1), anti-phospho-GSK3ß (1 : 1,000 dilution; Cell Signaling Technology, clone 5B3), anti-GSK3ß (1 : 1,000 dilution; Cell Signaling Technology, clone 27C10), and anti-Hsp60 (1 : 10,000 dilution; BD Biosciences, clone 24/Hsp60). Antibodies used in immunocytochemistry were as follows: FITC-conjugated anti-GFP (1 : 1,000 dilution; Abcam, ab6662), anti-Tom20 (1 : 1,000 dilution; Santa Cruz Biotechnology, FL-145), anti-Myc (1 : 500 dilution; Millipore, clone 4A6), anti-p62 (1: 500 dilution; Progen Biotechnik, GP62-C), anti-Parkin (1 : 1,000 dilution; Cell Signaling Technology, clone PRK8) and anti-proteasome α7 (1 : 250; a kind gift of Dr S. Murata at the University of Tokyo). cDNAs for human Parkin, PINK1 and its pathogenic and engineered mutants are as described in previous studies[23,41]. Parkin phospho-mutants were generated by PCR-based mutagenesis followed by sequencing confirmation of the entire gene. *PINK1*$^{-/-}$ MEFs, cultured as previously described[23], were retrovirally transfected with pMXs-puro harbouring non-tagged PINK1, PINK1-FLAG, non-tagged Parkin, HA-Parkin, GFP-Parkin and related cDNA; transfected cells were then selected with 1 μg/ml puromycin. HeLa cells maintained at 37°C in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS and 1x non-essential amino acids (GIBCO) were retrovirally transfected with pMXs-puro harbouring non-tagged Parkin along with pcDNA3Hyg-mSlc7a1-VSVG and pcDNA3Hyg-mSlc7a1-FLAG (a kind gift of Dr N. Fujita at UCSD). Stable cell lines were selected with 1 μg/ml puromycin and cloned. Transient transfections of cultured cells were performed using Lipofectamine 2000 (Invitrogen) for plasmids and Lipofectamine RNAiMAX (Invitrogen) for stealth siRNA duplexes (Invitrogen), which were used according to the manufacturer's instructions.

Tissue culture. Skin biopsies were obtained from a *PARK6* case and a control without mutations in any known PD genes. The study was approved by the ethics committee of Juntendo University, and all participants gave written, informed consent. Dermal primary fibroblasts established from biopsies were cultured in high glucose DMEM supplemented with 10% foetal bovine serum, 1x non-essential amino acids, 1 mM sodium pyruvate (GIBCO), 100 μM 2-mercaptoethanol, and 1% penicillin–streptomycin at 37°C in a 5% CO_2 atmosphere.

Mapping of Parkin phosphorylation sites. *PINK1*$^{-/-}$ MEFs (6.0 x 10^7) expressing HA-Parkin and PINK1-FLAG were treated with or without 30 μM CCCP for 30 min. HA-Parkin (~500 ng in each) immunopurified with anti-HA-conjugated agarose beads was eluted with 8 M urea buffered with 50 mM Tris-HCl at pH 9.0. Samples from two independent experiments were digested with trypsin or chymotrypsin and analysed by nano-scale liquid chromatography-tandem mass spectrometry (Dionex Ultimate3000 RSLCnano and ABSciex TripleTOF 5600) followed by MASCOT searching and Mass Navigator/PhosPepAnalyzer processing for identification and label-free quantitation, respectively[42]. Determination of phosphosite localisation was performed based on the presence of site-determining ions[43].

Phosphorylation assay and mitochondrial fractionation. *PINK1*$^{-/-}$ MEFs harbouring HA-Parkin along with wild-type or a kinase-dead form of PINK1-FLAG were metabolically labelled with 175 μCi/ml of [^{32}P] orthophosphate in phosphate-free DMEM (GIBCO) with 10% FBS at 37°C for 3 hr. The medium was then replaced with fresh DMEM containing 10% FBS. Cells were treated with CCCP for 1.5 hr and were lysed on ice with lysis buffer containing 0.2% NP-40, 50 mM Tris (pH 7.4), 150 mM NaCl and 10% glycerol supplemented with protease inhibitor (Roche Diagnostics) and phosphatase inhibitor (Pierce) cocktails, and HA-Parkin and PINK1-FLAG were immunoprecipitated with anti-HA (Wako Pure Chemical, clone 4B2)- or anti-FLAG (Sigma-Aldrich, clone M2)-conjugated agarose beads. Immunoprecipitates were separated by SDS-PAGE and transferred onto a PVDF membrane. Autoradiography and Western blotting were performed to visualise proteins. Phos-tag Western blotting was performed as previously described[28]. Briefly, phospho-Parkin and phospho-PINK1 were separated on 8% gels containing 50 μM Phos-tag. Mitochondrial and cytosolic fractionations were performed as previously described, with some modifications[20]. The cytosolic fractions were further clarified by a second centrifugation at 105,000 *g* for 60 min to remove residual organelle membranes.

Immunocytochemical analysis. Cells plated on 3.5 mm glass-bottom dishes (MatTek) were fixed with 4% paraformaldehyde in PBS and permeabilised with 50 μg/ml digitonin for anti-Tom20 and anti-p62 staining or with 0.1% NP-40 for anti-α7 staining in PBS. Cells were stained with anti-Tom20 or anti-α7 antibodies in combination with FITC-conjugated anti-GFP antibody and were counterstained with DAPI for nuclei. Cells were imaged using laser-scanning microscope systems (TCS-SP5, Leica or LSM510 META, Carl Zeiss).

Statistical analysis. A one-way repeated measures ANOVA was used to determine significant differences between multiple groups unless otherwise indicated. If a significant result was achieved ($p < 0.05$), the means of the control and the specific test group were analysed using the Tukey-Kramer test.

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Author contributions

K.S., Y. Imai and N.H. designed the research; K.S., Y. Imai, S.Y., T.K. and Y. Ishihama performed the experiments; S.S. contributed new reagents/analytic tools; K.S. and Y. Imai analysed the data; and Y. Imai and N.H. wrote the paper. K.S. and Y. Imai contributed equally to this work.

Additional information

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Digest シリーズ
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始まりは一人の患者から

New gene discovery from one patient

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1986年千葉大学医学部卒業。'97年より福岡大学神経内科所属。2000年から3年間米国Mayo Clinic留学後，'05年准教授，'11年より教授。研究テーマ：臨床神経学，運動障害学，特にパーキンソン病，レビー小体型認知症，前頭側頭型認知症，レストレスレッグズ症候群，プリオン病。

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Key Words: Perry症候群，家族性パーキンソン病，ダイナクチン，TDP-43

■はじめに

家族性パーキンソニズムは，1990年代後半に至るまでの神経学史において，稀な疾患とみなされ，遺伝型，臨床症候，画像所見，病理所見の特徴を記述し，分類学的試みが行われるにとどまり，病態解明には至らなかった。近年遺伝子学の発達により，この分野がパーキンソン病病態解明の足がかりとして，一気に神経科学分野でのトピックスに躍り上がった。その発端はαシヌクレイン遺伝子変異の発見であったと思われるが，これはイタリアのContursi家系の地道な臨床研究が結実した結果である。この蛋白が孤発性パーキンソン病脳にみられるLewy小体の構成蛋白であることから，この蛋白の機能解析，凝集の意義について研究が盛んになり，現在，凝集物の前段階であるαシヌクレインオリゴマーに細胞毒性があることが判明し，そこをターゲットとした新規治療薬の開発までに至っている。

また，αシヌクレイン蛋白凝集は細胞から細胞へ伝達するプリオン病の感染様式に類似するといったパーキンソン病の病勢進展におけるプリオン仮説まで登場している。ここにもうひとつ重要な蛋白が，パーキンソニズムの病態に関わる可能性が見えてきた。Perry症候群の研究から見出された，微小管を通じて細胞内の物質輸送に関わるダイナクチンである。

■Perry症候群と家族性のパーキンソン病研究

1975年にカナダのPerryらが，常染色体優性遺伝の家族性パーキンソニズムにうつ，体重減少，中枢性低換気を伴う疾患を初めて報告した。その後，類似の症候を呈するもう1家系がカナダに存在することが判明した。その後，約10年間忘れられていたが，1988年に米国，1992年にフランスより同様家系の報告があり，フランス家系の報告者，LechevalierによりPerry症候群（Perry-Purdy症候群）と名付けられた。これらの報告は，その臨床経過があまりにも特徴的で，他の家族性パーキンソニズムとは独立した疾患であることを報告者は確信していた。さらに1993年英国から報告されたのち，この疾患はさらに10年間空白の時期を迎える。

■始まりは一人の患者さん

私は1999年に43歳，若年性のうつ症状を呈した

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患者のコンサルトを受けたが，その時の最初の記述が以下のようである。

「身長163 cm，体重63 kg。軽度仮面様顔貌で精神活動の低下（無気力）がみられる。四肢の筋緊張がやや亢進し，Westphal現象が陽性，下肢はやや痙性が疑われる。歩行は腕の振りが欠如している。」

この当時の症状をまとめると，軽度のパーキンソン症候群があるも，目立つ症状は無気力であった。ちなみにベックのうつ病調査表では正常範囲であった。頭部のMRIでも異常はみられず，軽度パーキンソニズムに対してL-DOPAを内服し，軽度の改善をみたとの記載がある。

その後この患者は，仕事に対する意欲がなくなり，他の精神科病院に入院し抗うつ剤による治療を受けるが，そこで明らかなパーキンソニズムの悪化を示した。この状態は先の入院中に軽度錐体外路症状がみられたことから，薬剤誘発性パーキンソニズムではなく，薬剤によりパーキンソニズムが顕在化したものと解釈したほうが良いと思われる。その後薬剤の中断でいったん軽快するも，以前より明らかに進行した状態を呈していた。2000年X月に精神科病院に入院中の朝に，意識障害で発見された。その時の動脈血液ガス分析ではPCO2が72 Torrと高値で，救急病院に搬送され，CO2ナルコーシスと判断された。その時は人工呼吸器管理で意識は回復し，呼吸器からの離脱も可能であった。さらに当院に転院したが，驚いたことに，体重が初回入院時の63kgから38kgまで減少していた。その間の食事摂取に関しては，それ以前と大きく変わっていなかったものと考えられた。当然，この低換気に関して諸検査が行われたが，心機能，肺機能に異常はなく，Perryの原著にもある中枢性換気不全という病態が考えられた。このパーキンソニズム，うつ，体重減少，中枢性低換気はPerry症候群の4徴候といえる。この時点で我々はこの家系が本邦に未だ報告のないPerry症候群であると確信した。

さらに原著の記載以外に我々の臨床的検討から様々なことがわかってきた。まずL-DOPAに対する効果は良い点，しかしすぐにその効果が減弱し，薬の効果が血中濃度と主に変動するウエアリング・オフ現象，ジスキネジアの早期出現などで，うつはあまり強くなく中核症状は無気力（apathy）であること，抗精神病薬の薬剤感受性が強いことなどであった。

■パーキンソン病遺伝学黎明期

この間1997年以来に家族性パーキンソン病の研究はいつの間にか黎明期を迎えていた。この年にCuntursi家系からαシヌクレイン遺伝子の異常が発見され，臨床遺伝研究が盛んになった。さらに前頭側頭型認知症パーキンソニズムでもタウ遺伝子変異が発見。その後さらに拍車がかかり，常染色体劣性パーキンソニズムで*Parkin*, *DJ-1*, *PINK1*遺伝子の変異が見つかり，常染色体優性型においても新たに，*LRRK2*, *SCA2*遺伝子の関与が相次いで判明した（表）。この時に，私はフロリダのMayo Clinic Jacksonvilleに籍をおき，時の神経内科医のWszolek先生と遺伝学者のFerrer先生と共に，Perry症候群の臨床病理学的研究，および原因遺伝子の探求を目的に，国際共同研究が2001年に始まった。

その後，それまで報告のあった6家系から5つの家系の主治医に連絡を取ることに成功し，その間，新たにハワイと本邦から未発表の家系が発見された。我々はなるべく詳細な臨床経過を集め検討したほか，すでに報告された家系の剖検脳組織をMayo Clinicに集め，さらにDNAサンプル（計8家系から17人の発症者を含む74人）も集約され，結果としてこの疾患の臨床病理的特徴と新たな遺伝子変異の検索が始まった。

■おわりに

比較的小さい家系が多かったPerry症候群において，責任遺伝子の検討作業は難航した。しかし，その後も発症者や家系内未発症者のDNAサンプルが徐々に集まり，責任遺伝子の連鎖解析はいくつかの染色体部位に絞られてきた。また2006年に，

表　パーキンソン病の原因遺伝子/染色体と臨床症候および病理所見

名称	遺伝型	染色体	蛋白	臨床症候	病理所見
PARK1&4	AD	4q21	SNCA	EOPD	Synucleinopathy
PARK 2	AR	6q25.2-27	Parkin	Juvenile and EOPD	
PARK 3	AD	2p13	unknown	LOPD	
PARK 5	AD	4p14	UCHL1	LOPD	
PARK 6	AR	1p35-36	PINK 1	EOPD	
PARK 7	AR	1p36	DJ-1	EOPD	
PARK 8	AD	12p12	LRRK2	LOPD	Synucleinopathy/Tauopathy
PARK 9	AR	1p36	ATP13A2	Kufor-Rakeb syndrome	
PARK 10	S	1p32	ELAV4	Not clear	
PARK 11	AD	2q36-q37	GIGYF2	LOPD	
PARK 12	S	Xq21-q25	unknown	Not clear	
PARK 13	AD	2p12	Omi/HTRA2	Not clear	
PARK 14	AR	22q13.1	PLA2G6	Adult onset dystonia-parkinsonism	
PARK 15	AR	22q12-13	FBXO7	Early onset parkinsonian-pyramidal syndrome	
SCA2	AD	12q24.1	Ataxin2	LOPD	Synucleinopathy
FTDP-17	AD	17q21-22	MAPT	FTDP-17	Tauopathy
Perry	AD	2p12-14	DCTN1	Perry syndrome	TDP-43 proteinopathy

AD：常染色体優性遺伝，AR：常染色体劣性遺伝，EOPD：若年発症パーキンソン病　S：孤発性
LOPD：高齢発症パーキンソン病，FTDP-17：17番染色体関連前頭側頭型認知症パーキンソニズム

TDP-43蛋白の異常な凝集が筋萎縮性側索硬化症および前頭側頭型認知症の中枢神経から発見されたが，これもPerry症候群の病態解明に偶然の発見を導いた。このように1人の患者さんから古くから知られていた疾患が再び注目され，遺伝学的，分子生物学的な偶然が重なり一気に新しい発見へと進むことができた。

News(学会情報)

●第45回日本臨床腎移植学会

開催日：2月1日（水）～3日（金）
代表者：服部　元史（東京女子医科大学教授）
会場：軽井沢プリンスホテルウエスト
テーマ：ハーモニー
事務局連絡先：東京女子医科大学腎臓小児科
TEL：03-3353-8111（内38317）
FAX：03-3359-4877
常設事務局URL：http://www.jscrt.jp/
開催案内URL：http://www.pcoworks.jp/jscrt45/

●第8回日本消化管学会総会学術集会

開催日：2月10日（金）～11日（土祝）
代表者：本郷　道夫（東邦大学病院教授）
会場：仙台国際センター，江陽グランドホテル
テーマ：「消化管学不楽是如何」
事務局連絡先：勁草書房コミュニケーション事業部
TEL：03-5840-6339
FAX：03-3814-6904
常設事務局URL：http://www.jpn-ga.jp/
開催案内URL：http://www.keisocomm.com/8jga/index.html

《トピックス》

1 パーキンソン病の基礎研究最前線：実地医家のための minimum requirement

舩山 学* 富山弘幸**

ポイント

- パーキンソン病の原因遺伝子として *α-synuclein*, *parkin*, *PINK1*, *LRRK2* などがあるが，最近新規原因遺伝子 *VPS35* も報告され，分子遺伝学的研究は日進月歩である．
- *α-synuclein*, *LRRK2*, *GBA* は重要な感受性遺伝子であり，孤発性パーキンソン病の病態においても密接に関与している．
- ミトコンドリアの品質管理不全がパーキンソン病に関与している．
- パーキンソン病の基礎研究と日常診療の進歩のなかで，研究者および実地医家の双方の果たすべき役割がますます大きくなってきている．

キーワード アルファシヌクレイン，ベータグルコシダーゼ，エクソーム解析，次世代シークエンサー，ミトコンドリア，オートファジー・リソソーム

＊順天堂大学大学院医学研究科 老人性疾患病態・治療研究センター ＊＊順天堂大学医学部 脳神経内科/神経変性疾患病態治療探索講座

パーキンソン病の基礎研究は 90 年代後半から分子遺伝学的手法によってさまざまな病因遺伝子が単離され始めてから飛躍的に進み，正に日進月歩の勢いで次々に新しい重要な知見が明らかになってきている．本稿では基礎研究分野における最新の話題を中心に紹介する．

◉パーキンソン病の遺伝子研究

1997 年に *α-synuclein* 遺伝子変異が報告されて以来，家族性パーキンソン病の分子遺伝学的解析が進み，現在までに多くの遺伝子が単離されている（表）．また近年は，*α-synuclein* 遺伝子や *parkin* 遺伝子のようなメンデル遺伝型の原因遺伝子のほかに，パーキンソン病が発症しやすくなる発症感受性遺伝子もゲノムワイド関連解析の研究成果等から次々と報告されている（表）．

1．メンデル遺伝型原因遺伝子

家族性パーキンソン病はパーキンソン病の 5〜10%を占めると考えられており，実地医家が病歴，家族歴を詳細に聴取し，家系図を記載していくことは大変重要である．

常染色体優性遺伝性の原因遺伝子として *α-synuclein*, *leucine-rich repeat kinase 2* (*LRRK2*) 等が報告されている．臨床像は中〜高齢発症で典型的な孤発性パーキンソン病と同様の症例が多い．わが国においては *α-synuclein* の重複変異と *LRRK2* の点変異が報告されている．いずれも頻度はそれほど高くないが，地域差があり，地域集積性，遺伝性疾患が疑われる（もしくは否定できない）場合，前の世代も含め出身地を聴取することも遺伝子解析，診断上有用である．

表　パーキンソン病関連遺伝子の一覧

	染色体上の位置	遺伝子	遺伝形式
PARK1	4q22.1	*SNCA*	常優/感受性
PARK2	6q25.2-27	*parkin*	常劣
PARK3	2p13	unknown	常優
PARK4	4q22.1	*SNCA*（重複）	常優
PARK5	4p14	*UCH-L1*	常優
PARK6	1p36-p35	*PINK1*	常劣
PARK7	1p36	*DJ-1*	常劣
PARK8	12p11.2-q13.1	*LRRK2*	常優/感受性
PARK9	1p36	*ATP13A2*	常劣
PARK10	1p	unknown	感受性
PARK11	2q37.1	*GIGYF2*	常優
PARK12	Xq21-q25	unknown	感受性
PARK13	2p12	*HTRA2*	常優
PARK14	22q13.1	*PLA2G6*	常劣
PARK15	22p12.3	*FBXO7*	常劣
PARK16	1q32	unknown	感受性
	4p16	*GAK*	感受性
	6p21.3	*HLA-DRB5*	感受性
	17q21.1	*MAPT*	常優/感受性
	1q21	*GBA*	感受性
	4p15	*BST1*	感受性

常染色体劣性遺伝性の原因遺伝子としては *parkin*，*PTEN induced putative kinase 1*（*PINK1*），*DJ-1*，*ATPase type 13A2*（*ATP13A2*），*phospholipase A2, group VI*（*PLA2G6*）等が報告されている．特に *parkin* はわが国から 1998 年に初めて報告された遺伝子で非常に重要である．*parkin* 遺伝子変異の頻度は常染色体劣性遺伝性のなかで約 50％と頻度が高く，若年発症の孤発例でも約 15％に認めたとする報告もある．したがって血族婚がある等劣性遺伝が疑われる患者，特に若年発症の患者については積極的に *pakin* 遺伝子検査を行うべきであろう．*PINK1* は臨床的には *parkin* 変異陽性例とほとんど変わりないが，若干発症年齢が高い印象がある．

若年性パーキンソニズムで最近注目されているのが *PLA2G6* である．*PLA2G6* は 2006 年に neurodegeneration with brain iron accumulation（NBIA）の原因遺伝子として報告されていたが，2009 年に若年性ジストニア-パーキンソニズム（*PARK14*）の原因遺伝子としても報告された．自験例では，認知症あるいは精神発達遅滞，または幻覚，妄想などの精神症状を呈する若年性パーキンソニズム患者 30 例について解析した結果，3 名に *PLA2G6* 遺伝子変異を認めた．これらの患者には既報とは異なりジストニアは認めず *PLA2G6* 陽性パーキンソニズムは臨床的に variable であるが，発症年齢，家族歴，認知症や精神症状の合併，大脳萎縮や鉄沈着のような MRI 所見など，臨床像をよく検討した後に変異解析をすれば比較的高頻度で変異を同定できる可能性が

ある．変異症例はレビー小体を認めたことも報告されており，鉄沈着とあわせパーキンソン病の病態との関連が興味深いところである．

2．エクソーム解析による新規原因遺伝子 *VPS35* の同定

最近，メンデル遺伝型の新規原因遺伝子として *vacuolar protein sorting 35*（*VPS35*）遺伝子が報告された．*VPS35* は16番染色体上腕にある遺伝子でレトロマー複合体という細胞内輸送に関与している蛋白の一つである．この遺伝子変異はスイス人とオーストリア人の常染色体優性遺伝性パーキンソン病家系から見出された．臨床像は孤発性パーキンソン病と変わらず，50歳前後発症でL-dopa反応性のパーキンソニズムであった．この報告は全エクソン解析（エクソーム解析）という次世代シークエンサーを利用した方法で変異が見出されたという点で非常に重要である．これまでメンデル遺伝型の遺伝子を単離しようとする場合，家系内に患者が多数存在している多発家系を複数世代の検体を用いて連鎖解析の手法で解析する必要があったが，遺伝子解析技術が進み現在は理論的には親子または同胞発症者の2名の塩基配列の解析をすれば遺伝子を単離することが可能になった．この技術革新により，臨床医に遺伝子解析が非常に身近になってきているとともに，遺伝情報の扱いなど倫理的問題に対する臨床医の役割も大きくなってきている．

3．感受性遺伝子—Common disease-common variant 仮説と common disease-multiple rare variant 仮説—

パーキンソン病は遺伝要因と環境要因が複雑に絡み合って発症する多因子疾患である．パーキンソン病のような頻度の高い疾患（common disease）の遺伝要因を考えるにあたり，頻度の高い共通の遺伝子多型（common variant）によるという common disease-common variant 仮説と，複数のまれな遺伝子多型（rare variant）の組み合わせによるという common disease-multiple rare variant 仮説が考えられており，事実，多くの遺伝要因がそれぞれ単独または相互作用して発症に関与していることが示されてきている．遺伝要因としての感受性遺伝子の知見のなかで特に注目すべきは，わが国の研究グループから発表されたSatakeらの報告とMitsuiらの報告である．Satakeらは common variant を利用したゲノムワイド関連解析で *α-synuclein*，*LRRK2*，*bone marrow stromal cell antigen 1*（*BST1*），*PARK16*（遺伝子未同定）の関連を報告した．Mitsuiらは *acid-β-glucosidase*（*GBA*）の rare variant が強い関連を示すことを報告した．これらの遺伝子群については欧米人において同様の結果が得られるものと，アジア人特有のものがあることがわかっている．遺伝子変異，多型については創始者効果などに基づく人種差があることが指摘されており，日本人やアジア人の大規模な集団でさらに研究を進めていくことが重要である．一般に rare variant は疾患に与える影響度が大きく，今後次世代シークエンサーなどを用いた解析で多くの遺伝的要因が同定され，病態解明から治療法の開発へとつながることが期待される．このような時代の流れ，研究の目覚ましい進歩のなかで，家族例のみならず孤発例も含めた症例の臨床的・遺伝学的知見を蓄積することで，病態解明・治療法の開発へと寄与していけるということからも，実地医家の果たす役割は大きいと思われる．

◉遺伝子から蛋白へ

遺伝学的研究の成果からさまざまな分子がパーキンソン病に関与していることが明らかになり，それぞれの遺伝子産物（＝蛋白質）の機能や病態生理が精力的に研究されてきている．近年は個々の蛋白の研究から複数の蛋白の機能的関連（分子間相互作用）へと研究の中心が移行しつつある．言い換えれば「遺伝子変異」という原因と「パーキンソン病発症」という結果をつなぐブラックボックスが徐々に開かれてきつつある（図）．

1．Parkin と PINK1 によるミトコンドリア品質管理

Parkin と *PINK1* はそれぞれ劣性遺伝性の若年性パーキンソニズムの原因遺伝子である．最近，この2つの分子がともにミトコンドリアの品質管

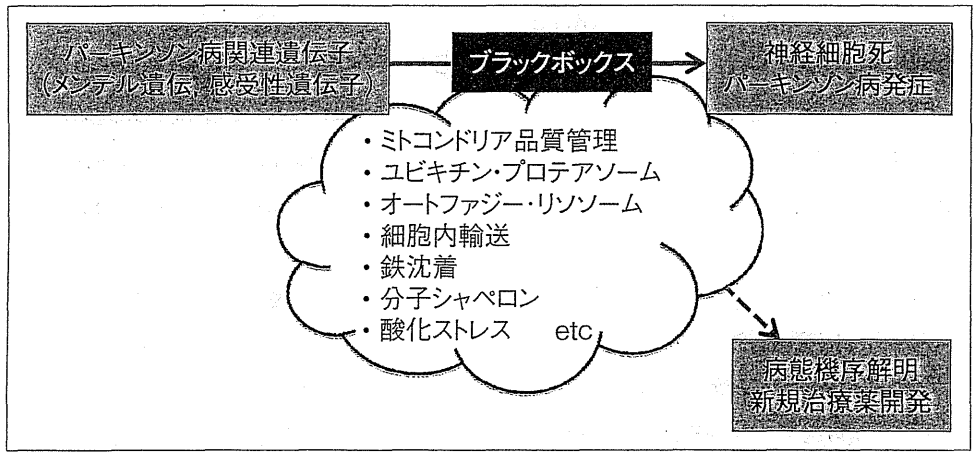

図　遺伝子研究から蛋白機能解析・病態機序解明の現状

理を行っていることが明らかになってきた．PINK1はその蛋白構造からミトコンドリアで働く酵素であることが予想されていたが，Parkinとミトコンドリアの関連は長年謎であった．しかしながらここ2〜3年で急速に研究が進み，Parkinが機能不全に陥ったミトコンドリアを速やかに分解経路へと導くことがわかり，その経路のきっかけをPINK1が作っていることが明らかとなった．*Parkin*や*PINK1*に変異のある遺伝性パーキンソニズム患者ではこのような機能が失われているため，不要なミトコンドリアを処理できずに発症に至っていると考えられている．

麻薬の副産物であるMPTPの曝露によってパーキンソン様症状を呈することが報告され，これがミトコンドリア電子伝達系を阻害していることからパーキンソン病とミトコンドリアは密接な関係にあることが長年示唆されてきた．ParkinやPINK1のような遺伝学から見出された分子の研究によってその病態生理が裏打ちされた功績は大きい．ParkinやPINK1以外にもパーキンソン病患者脳において質的・量的変化のあるミトコンドリア関連蛋白が次々に報告されてきており，ミトコンドリアは新たなパーキンソン病治療標的として今後ますます注目を浴びていくことが予想される．

2．α-synucleinとGBAの関連

先述のように*α-synuclein*と*GBA*はパーキンソン病の感受性遺伝子として報告された．α-synucleinはレビー小体の主要構成成分であり，*GBA*の遺伝子産物のβ-グルコシダーゼはリソソーム酵素である．*GBA*は両親から一つずつ受け継いだ遺伝子両方に変異があるとゴーシェ病を発症することが知られている．この2つの感受性遺伝子が互いに影響し合っていることがごく最近報告されている．ゴーシェ病やパーキンソン病患者でみつかっているGBAの変異体を培養細胞に導入したり，人工的にβ-グルコシダーゼの量を減少させるとα-synucleinの蛋白量が増加し，結果的にα-synucleinの蓄積が促進される．また，α-synuclein蛋白量の増加によってβ-グルコシダーゼの活性が抑制されるという負の連鎖が起こっていることがわかってきた．このα-synuclein蛋白量の増加はオートファジー誘導物質によって抑制され，さらにα-synucleinはリソソームプロテアーゼであるカテプシンDの基質であることから，オートファジー・リソソーム系とα-synucleinの量的恒常性の維持が密接に関係している可能性が高い．したがって，今後パーキンソン病の新薬としてオートファジー・リソソーム系を調節する化合物がターゲットとなる可能性がある．

3．14-3-3蛋白質とパーキンソン病関連分子

14-3-3蛋白質は脳に豊富に存在している多機能分子シャペロン様蛋白質ファミリーである．遺伝性パーキンソン病の原因遺伝子産物であるα-synuclein，Parkin，LRRK2はそれぞれ14-3-3

蛋白質と結合することが明らかになっている．14-3-3蛋白質はリン酸化された標的蛋白質に結合することが知られており，おそらくリン酸化-脱リン酸化を介した14-3-3蛋白質の結合と乖離がα-synuclein，Parkin，LRRK2の機能維持に重要な役割を演じていると考えられている．さらに14-3-3蛋白質はレビー小体に存在していることもわかっており，パーキンソン病をはじめとするレビー小体病にも14-3-3蛋白質が関与している可能性が高く，これらの疾患の病態生理の解明と創薬の重要分子の一つであるといえる．

まとめ

パーキンソン病の基礎研究についてできる限り最新の情報を紹介した．まだ十分に検証がなされていない知見もいくつかあり，すぐに日常診療に役立つことは少ないかもしれないが，目を見張るスピードでどんどん研究が進んでいるということを感じていただき，患者さんの希望の一助になる機会があれば幸いである．

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Digest シリーズ
—Perry症候群—
Vol. 2

Perry症候群と *DCTN1*遺伝子変異

Perry syndrome and *DCTN1* mutations

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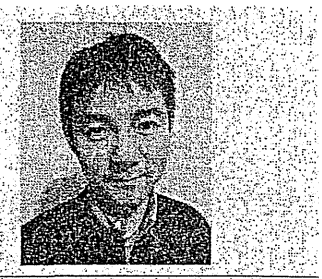

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Key Words:Perry syndrome, familial parkinsonism, *DCTN1*, TDP-43 proteinopathy, amyotrophic lateral sclerosis (ALS)

Abstract

Perry（ペリー）症候群はパーキンソニズム，うつ，体重減少，低換気をきたし，5-10年以内に急激に進行し突然死に至ることも多い，予後不良の稀な遺伝性疾患である。これまでの報告では30-40歳代の比較的若年で発症することが多く，浸透率が高い常染色体優性遺伝性疾患と考えられている。日本の家系も含み，2009年に*DCTN1*（ダイナクチン1）が原因遺伝子であると報告された。これまで5つのミスセンス変異が報告され，本邦では4家系，世界で10家系が遺伝子診断により確定されており，患者の報告が少しずつ増えてきているが，世界でもまだ稀な疾患といえる。日本においても患者の実態，地域特異性，有病率等疫学的知見には乏しいが，九州地方に複数家系が存在することがわかってきている。本疾患を鑑別せず遺伝子解析をしない場合，非典型的パーキンソニズムやパーキンソン病（PD）の診断で患者が見逃される可能性がある。
Perry症候群は低換気を呈するとともにTDP-43の蓄積を示し，*DCTN1*変異はTDP-43プロテイノパチーとしての筋萎縮性側索硬化症（ALS）を引き起こすことも知られている。したがってPerry症候群は，稀で特殊と考えられている疾患から，広くPDやALSなどの主要な神経変性疾患の根本的な病態解明の橋渡しとなる可能性をもつ極めて重要な位置付けにある疾患といえ，国際的な臨床診断基準の確立，遺伝子解析にも基づく疫学調査から，今後の研究が広がっていくことが期待される。

Perryらによる疾患の報告から原因遺伝子同定まで

1975年，Perryらは若年発症で常染色体優性遺伝形式をとるパーキンソニズム，うつ，急激な体重減少，低換気から全経過4-8年で突然死に至る症候群を報告した[1]。その後，本疾患はPerry症候群と呼ばれるようになり，福岡大学の坪井教授らにより日本の家系でも報告された[2]。福岡大学とMayo Clinicのグループの国際共同研究の結果，2009年に*DCTN1*（ダイナクチン1）が原因遺伝子として報告された[3]。そのため，本疾患において日本人の果たしている役割は多大であるといえる。

遺伝子変異

*DCTN1*は，32のエクソンと1,278のアミノ酸からなる比較的大きな遺伝子であり，その遺伝子解析が本疾患の診断において重要である。これまで，ALSでみつかったp.G59Sの他に[4]，世界のp.Q74P，p.T72P，p.G71A，p.G71E，p.G71Rの5つの病的変異は全てCAP-Glyドメインにあり，exon 2の狭い領域に存在している[3, 5, 6]（図）。G71A変異は複数の家系で報告され，共通祖先による創始者効果の可能性がある。家族内で世代間発症があり，浸透率が高い常染色体優性遺伝性疾患と考えられている[3]。

欧米でパーキンソン病（PD），筋萎縮性側索硬化症（ALS），前頭側頭葉型認知症例について大規模な*DCTN1*全exon解析が行われたが病的な*DCTN1*変異は認めず，今のところこれらの臨床病型でも，またexon 2以外においても*DCTN1*変異は稀と考えられてきている[7]。

遺伝子解析に基づく疫学

原因遺伝子*DCTN1*の同定後，世界で遺伝子診断により確定されたのは10家系で，白人で5家系，ハワイ日系人で1家系（Prof. Wszolekとのpersonal

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communication)，日本人で4家系が同定されてきている[3, 5, 6]。変異解析結果から地域差，共通祖先の存在の可能性も考えられてきている。本疾患の疫学的分布や頻度は未だ明らかでないが，本邦では西日本に複数の家系を認めており，まだまだ日本人でも患者家系が隠れている可能性があり今後大規模な疫学調査の結果が待たれる。

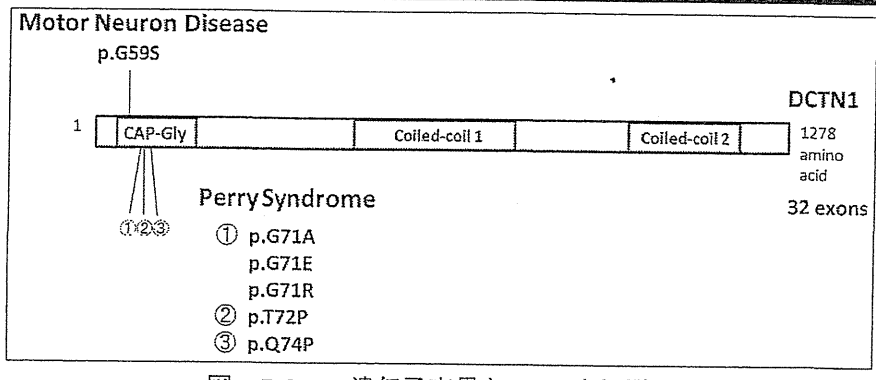

図　*DCTN1* 遺伝子変異とPerry 症候群
DCTN1 遺伝子は32 のexon からなるが，変異は全てCAP-Gly ドメイン中のexon 2 に集中しており，まず変異のhot spot であるexon 2 から変異解析を行うのがよいと考えられる。CAP-Gly ドメインは微小管結合ドメインであり，細胞内輸送に関わりMotor Neuron DiseaseおよびPerry 症候群の病態に関わる重要な機能的ドメインと考えられる。

■臨床像・検査所見と遺伝子変異

これまでの症例からは，パーキンソニズム（振戦，無動，筋強剛，姿勢反射障害)，うつ，体重減少，低換気が主症状であると報告されている。30-40歳代に運動障害やうつで初発し，急激な体重減少を伴い，運動障害の進行とともに夜間の無呼吸，呼吸不全を併発することが多く，全経過は5-10年くらいのことが多い[3, 5, 6]。認知症は伴いにくく，呼吸補助，人工呼吸器管理で生命予後の改善が期待できる。L-ドーパにより長期に渡りパーキンソニズムの改善が得られることもあることから，若年発症の家族性atypical parkinsonismのみでなく，PDと診断した症例の中にもPerry症候群が隠れている可能性があり，突然の呼吸不全に対する治療の観点からもPDとの鑑別が大きな問題となる。したがって遺伝子診断の後，中枢性低換気をきたす前に，ALSのように病名告知，人工呼吸管理の適応の選択について検討していく必要があると思われる。しかしながら一方，現時点で若年発症の予後不良の疾患であり，浸透率も比較的高いことが想定され，遺伝子診断の適応，説明と同意，病名告知においても難しい問題が伴う。

呼吸障害の観点からはALSと類似している部分があるが，運動ニューロン徴候（深部腱反射亢進，病的反射，筋萎縮，線維束攣縮など）は明らかでなく，筋電図は正常のことが多い。頭部CT，MRIは基本的には正常で，MIBG心筋シンチで取り込みの低下を認める症例も多い[5, 6]。起立性低血圧など自律神経障害，夜間呼吸困難/無呼吸，日中の過度な眠気，ポリソムノグラフィーで中枢性無呼吸/低換気を認めることがある。

合併症としては，精神症状（自殺企図，病的賭博，無為など)，転倒骨折，誤嚥，肺炎，尿路感染，褥創，呼吸不全などがある。主要症候の急激な進行のなかで，合併症も問題になってくる可能性があり，主要症候，合併症ともに症状の変化を注意深く観察していく必要がある。

病理像では黒質と青斑核，基底核のグリオーシス，神経細胞脱落がみられ，Lewy小体は基本的に認めない一方，TDP-43が黒質，淡蒼球に沈着することが報告されている[5, 6]。そのため，Perry症候群は新しいTDP-43プロテイノパチーとしての位置づけにある。

今のところ遺伝子変異ごとの臨床像、病理像の差異は十分に検討されていず，今後の変異症例の蓄積により明らかにされることが期待される。

以上のようにPerry症候群は，突然の呼吸障害から突然死に至るまでPDとの鑑別が困難なことがあるため，臨床疫学的，臨床分子遺伝学的，病理学的検討から，その臨床診断基準，指針を作成・確立し，病態の解明，治療法の開発に繋げていくことが急務である。

■治療法

パーキンソニズムに対し，L-ドーパの効果が（減弱することが多いものの）長期に渡ってあることがあり，抗パーキンソン病薬が適応になる。一方，病的賭博や妄想など精神症状の合併にも十分留意する必要があり，ドパミンアゴニストなど抗