研究成果の刊行に関する一覧表（斉木　臣二）

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Saiki S, Sato S, Hattori N.	Molecular pathogenesis of Parkinson disease: update.	***J Neurol Neurosurg Psychiat***	83	430-436	2012
Matsui H, Sato F, Sato S, Koike M, Taruno Y, Saiki S, Funayama M, Ito H, Taniguchi Y, Uemura N, Toyoda A, Sakaki Y, Takeda S, Uchiyama Y, Hattori N, Takahashi R.	ATP13A2 deficiency induces a decrease in cathepsin D activity, fingerprint-like inclusion body formation, and selective degeneration of dopaminergic neurons.	***FEBS Lett***	587	1316-1325	2013

研究成果の刊行に関する一覧表（舩山　学）

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Funayama M, Yoshino H, Li Y, Kusaka H, Tomiyama H, Hattori N.	Pseudo-heterozygous rearrangement mutation of parkin.	***Mov Disord.***	27	552-555	2012
Okatsu K, Oka T, Iguchi M, Imamura K, Kosako H, Tani N, Kimura M, Go E, Koyano F, Funayama M, Shiba-Fukushima K, Sato S, Shimizu H, Fukunaga Y, Taniguchi H, Komatsu M, Hattori N, Mihara K, Tanaka K, Matsuda N.	PINK1 autophosphorylation upon membrane potential dissipation is essential for Parkin recruitment to damaged mitochondria.	***Nat Commun.***	21	1016	2012
Ando M, Funayama M, Li Y, Kashihara K, Murakami Y, Ishizu N, Toyoda C, Noguchi K, Hashimoto T, Nakano N, Sasaki R, Kokubo Y, Kuzuhara S, Ogaki K, Yamashita C, Yoshino H, Hatano T, Tomiyama H, Hattori N.	VPS35 mutation in Japanese patients with typical Parkinson disease.	***Mov Disord.***	27	1413-1417	2012
Imaizumi Y, Okada Y, Akamatsu W, Koike M, Kuzumaki N, Hayakawa H, Nihira T, Kobayashi T, Ohyama M, Sato S, Takanashi M, Funayama M, Hirayama A, Soga T, Hishiki T, Suematsu M, Yagi T, Ito D, Kosakai A, Hayashi K, Shouji M, Nakanishi A, Suzuki N, Mizuno Y, Mizushima N, Amagai M, Uchiyama Y, Mochizuki H, Hattori N, Okano H.	Mitochondrial dysfunction associated with increased oxidative stress and alpha-synuclein accumulation in PARK2 iPSC-derived neurons and postmortem brain tissue.	***Mol Brain.***	5	35	2012
Matsui H, Sato F, Sato S, Koike M, Taruno Y, Saiki S, Funayama M, Ito H, Taniguchi Y, Uemura N, Toyoda A, Sakaki Y, Takeda S, Uchiyama Y, Hattori N, Takahashi R.	ATP13A2 deficiency induces a decrease in cathepsin D activity, fingerprint-like inclusion body formation, and selective degeneration of dopaminergic neurons.	***FEBS Lett***	587	1316-1325	2013

研究成果の刊行に関する一覧表（井本　正哉）

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Shinjo S, Mizotani Y, Tashiro E, Imoto M.	Comparative analysis of the expression patterns of UPR-target genes caused by UPR-inducing compounds.	***Biosci Biotechnol Biochem.***	77	729-735	2013
Magi S, Shitara T, Takemoto Y, Sawada M, Kitagawa M, Tashiro E, Takahashi Y, Imoto M.	Novel derivatives of aclacinomycin A block cancer cell migration through inhibition of farnesyl transferase.	***J Antibiot (Tokyo)***	66	165-170	2013
Magi S, Tashiro E, Imoto M.	A chemical genomic study identifying diversity in cell migration signaling in cancer cells.	***Sci Rep***	2	823	2012
Kobayashi H, Harada H, Nakamura M, Futamura Y, Ito A, Yoshida M, Iemura S, Shin-Ya K, Doi T, Takahashi T, Natsume T, Imoto M, Sakakibara Y.	Comprehensive predictions of target proteins based on protein-chemical interaction using virtual screening and experimental verifications.	***BMC Chem Biol***	12	2	2012
Sasazawa Y, Kanagaki S, Tashiro E, Nogawa T, Muroi M, Kondoh Y, Osada H, Imoto M.	Xanthohumol impairs autophagosome maturation through direct inhibition of valosin-containing protein.	***ACS Chem Biol.***	7	892	2012
Yamamoto K, Makino M, Watanapokasin R, Tashiro E, Imoto M.	Inostamycin enhanced TRAIL-induced apoptosis through DR5 upregulation on the cell surface.	***J Antibiot (Tokyo).***	65	295-300	2012

研究成果の刊行に関する一覧表（田代　悦）

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Shinjo S, Mizotani Y, Tashiro E, Imoto M.	Comparative analysis of the expression patterns of UPR-target genes caused by UPR-inducing compounds.	***Biosci Biotechnol Biochem.***	77	729-735	2013
Magi S, Shitara T, Takemoto Y, Sawada M, Kitagawa M, Tashiro E, Takahashi Y, Imoto M.	Novel derivatives of aclacinomycin A block cancer cell migration through inhibition of farnesyl transferase.	***J Antibiot (Tokyo)***	66	165-170	2013
Magi S, Tashiro E, Imoto M.	A chemical genomic study identifying diversity in cell migration signaling in cancer cells.	***Sci Rep***	2	823	2012
Sasazawa Y, Kanagaki S, Tashiro E, Nogawa T, Muroi M, Kondoh Y, Osada H, Imoto M.	Xanthohumol impairs autophagosome maturation through direct inhibition of valosin-containing protein.	***ACS Chem Biol.***	7	892	2012
Yamamoto K, Makino M, Watanapokasin R, Tashiro E, Imoto M.	Inostamycin enhanced TRAIL-induced apoptosis through DR5 upregulation on the cell surface.	***J Antibiot (Tokyo).***	65	295-300	2012

研究成果の刊行物・別刷

include interactions with calmodulin and that PLA2G6 might also be associated with calcium/calmodulin dependent protein kinase II-β.[110 111]

FBXO7 (PARK15)

Clinicogenetics

Only three families with mutations in *FBXO7* have been reported.[112 113] Affected individuals had juvenile onset (10–19 years old) of progressive parkinsonism associated with spasticity, and variable response to L-dopa. No pathological studies have been reported.

Molecular biology

Fbox7 is a member of the F box containing protein (FBP) family with an F box domain. F box containing proteins are expected to function as molecular scaffolds in the formation of the protein complex; however, the exact function of FBXO7 remains unclear.

OTHER GENES ASSOCIATED WITH PARKINSON'S DISEASE

GWAS have uncovered a number of candidate genes involved in PD in European and Japanese populations, indicating a substantial contribution of genetics underlying susceptibility to both early onset and late onset PD.[6 7 114–119] These studies have shown repeatedly a common variation in *SNCA* and an inversion of the region containing the *MAPT*. Recent genetic studies revealed mutations in the *GBA* gene, the most widespread genetic risk factor for parkinsonism identified to date.[120–124] In this section, we summarise the molecular mechanisms of the two genes, MAPT and GBA.

MAPT

Mutations in *MAPT*, encoding microtubule associated tau, result in tauopathies, including progressive supuranuclear palsy, corticobasal degeneration and frontotemporal lobar degeneration.[125] Tau is a soluble protein, but insoluble aggregates are produced during the formation of neurofibrillary tangles which disrupts microtubule associated dynamics and neuronal functions. Considering the interplay between α-synuclein and tau reported previously,[126] it is interesting that there would be a common pathogenesis associated with aggregation formations.

GBA

Early observed patients with Gaucher disease and their heterozygous relatives present with parkinsonism.[127] In addition, autopsy studies have shown the presence of mutant glucocerebrosidase (GCase) in α-synuclein positive Lewy bodies in Gaucher disease patients and carriers with α-synucleinopathies.[128] GCase is a lysosomal hydrolase with 497 amino acids that catalyses the metabolism of the glycolipid glucosylceramide to ceramide and glucose. Cells overexpressing mutant GCase promoted α-synuclein accumulation in a dose and time dependent manner.[129] α-Synuclein GCase interacts selectively under lysosomal solution conditions (pH 5.5) and the interaction site was mapped to the α-synuclein C terminal residues 118–137.[130] Insufficient functions of the lysosomes may have an effect on chaperone mediated autophagy or macroautophagy.

CONCLUDING REMARKS

In the 14 years since the first causative gene (α-synuclein) in PD was discovered, great advances have been made in understanding the biology of the disease. Recent evidence shows that the environment plays no role in the aetiology of PD.[131] In addition, GWAS suggest that a number of genes influence susceptibility.[3] The PD associated genes provide valuable clues regarding the molecular pathogenesis of PD because the pathomechanism for sPD would have certain pathways in common with those of hPD. Importantly, basic biological studies in PD have led to numerous potential therapeutic strategies. For example, a specific inhibitor for LRRK2 phosphorylations at Ser910 and Ser935 was recently developed.[132] In the future, it becomes more important to translate laboratory data, including molecular pathogenesis as well as genetic associations, into clinical treatments, leading to disease modifying therapies to conquer the disease onset and/or progression.

Funding The authors are very grateful for the CREST Grant from the Japan Science and Technology Agency (NH), grants from the Ministry of Health, Labour and Welfare (NH) and the Ministry of Education, Culture, Sports, Science and Technology (NH), Grant-in-Aid for Young Scientists (A) (S Saiki), a promoted grant from Juntendo University (S Saiki) and grants from the Takeda Scientific Foundation (S Sato, S Saiki) and the Life Science Foundation (S Saiki).

Competing interests None.

Contributors All authors contributed to this work, including interpretation of the references and manuscript writing.

Provenance and peer review Commissioned; externally peer reviewed.

REFERENCES

1. **Hatano T,** Kubo S, Sato S, *et al*. Pathogenesis of familial Parkinson's disease: new insights based on monogenic forms of Parkinson's disease. *J Neurochem* 2009;**111**:1075–93.
2. **Nuytemans K,** Theuns J, Cruts M, *et al*. Genetic etiology of Parkinson disease associated with mutations in the SNCA, PARK2, PINK1, PARK7, and LRRK2 genes: a mutation update. *Hum Mutat* 2010;**31**:763–80.
3. **Gasser T.** Molecular pathogenesis of Parkinson disease: insights from genetic studies. *Expert Rev Mol Med* 2009;**11**:e22.
4. **Polymeropoulos MH,** Lavedan C, Leroy E, *et al*. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* 1997;**276**:2045–7.
5. **Venda LL,** Cragg SJ, Buchman VL, *et al*. α-Synuclein and dopamine at the crossroads of Parkinson's disease. *Trends Neurosci* 2010;**33**:559–68.
6. **Simon-Sanchez J,** Schulte C, Bras JM, *et al*. Genome-wide association study reveals genetic risk underlying Parkinson's disease. *Nat Genet* 2009;**41**:1308–12.
7. **Satake W,** Nakabayashi Y, Mizuta I, *et al*. Genome-wide association study identifies common variants at four loci as genetic risk factors for Parkinson's disease. *Nat Genet* 2009;**41**:1303–7.
8. **Farrer M,** Maraganore DM, Lockhart P, *et al*. α-Synuclein gene haplotypes are associated with Parkinson's disease. *Hum Mol Genet* 2001;**10**:1847–51.
9. **Maraganore DM,** de Andrade M, Elbaz A, *et al*. Collaborative analysis of alpha-synuclein gene promoter variability and Parkinson disease. *JAMA* 2006;**296**:661–70.
10. **Giasson BI,** Duda JE, Murray IV, *et al*. Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. *Science* 2000;**290**:985–9.
11. **Kruger R,** Eberhardt O, Riess O, *et al*. Parkinson's disease: one biochemical pathway to fit all genes? *Trends Mol Med* 2002;**8**:236–40.
12. **Masliah E,** Rockenstein E, Veinbergs I, *et al*. Dopaminergic loss and inclusion body formation in alpha-synuclein mice: implications for neurodegenerative disorders. *Science* 2000;**287**:1265–9.
13. **Kahle PJ,** Neumann M, Ozmen L, *et al*. Subcellular localization of wild-type and Parkinson's disease-associated mutant alpha-synuclein in human and transgenic mouse brain. *J Neurosci* 2000;**20**:6365–73.
14. **Giasson BI,** Duda JE, Quinn SM, *et al*. Neuronal alpha-synucleinopathy with severe movement disorder in mice expressing A53T human alpha-synuclein. *Neuron* 2002;**34**:521–33.
15. **Cookson MR,** van der Brug M. Cell systems and the toxic mechanism(s) of alpha-synuclein. *Exp Neurol* 2008;**209**:5–11.
16. **Falsone SF,** Kungl AJ, Rek A, *et al*. The molecular chaperone Hsp90 modulates intermediate steps of amyloid assembly of the Parkinson-related protein alpha-synuclein. *J Biol Chem* 2009;**284**:31190–9.
17. **Webb JL,** Ravikumar B, Atkins J, *et al*. Alpha-synuclein is degraded by both autophagy and the proteasome. *J Biol Chem* 2003;**278**:25009–13.
18. **Cuervo AM,** Stefanis L, Fredenburg R, *et al*. Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy. *Science* 2004;**305**:1292–5.
19. **Martinez-Vicente M,** Talloczy Z, Kaushik S, *et al*. Dopamine-modified alpha-synuclein blocks chaperone-mediated autophagy. *J Clin Invest* 2008;**118**:777–88.
20. **Winslow AR,** Chen CW, Corrochano S, *et al*. α-Synuclein impairs macroautophagy: implications for Parkinson's disease. *J Cell Biol* 2010;**190**:1023–37.
21. **Brundin P,** Li JY, Holton JL, *et al*. Research in motion: the enigma of Parkinson's disease pathology spread. *Nat Rev Neurosci* 2008;**9**:741–5.

inhibits TRAIL induced apoptosis by blocking Fas associated protein death domain mediated pro-caspase-8 activation.[76] Along with parkin and PINK1, DJ-1 has various cellular functions such as regulation of mitochondrial morphology as well as misfolded protein degradation by forming an E3 ligase complex with those proteins.[77]

LRRK2 (PARK8)

Clinicogenetics

Clinical features of PARK8 are essentially similar to those of sPD except for earlier onset age. The disease gene was identified as the leucine rich repeat kinase 2 gene (*LRRK2*) linked to autosomal dominant inherited PD encoding 2517 amino acids.[78–80] PARK8 is the most common form of hPD in the world. Until now, 20 missense or nonsense mutations have been reported.[81] *LRRK2* mutations were also found in some sPD cases; neuropathological findings were heterogeneous.[82 83] Most of the cases with *LRRK2* mutations showed various degrees of Lewy bodies but intraneuronal aggregations positive to tau were rarely detected.[79 84 85] The G2019S mutation in *LRRK2* is the most common genetic cause of PD, accounting for a significant proportion of both autosomal dominant and sPD cases.

Molecular biology

LRRK2 protein, containing a GTPase domain, a Ras of complex domain, a C terminal of Ras complex domain and a mitogen activated kinase domain, is highly expressed in the brain, and mRNA levels are rich in the striatum and hippocampus compared with other regions.[86] Intracellular LRRK2 is mainly distributed in the plasma membrane and vesicular structures.[87 88] Immunoprecipitation techniques have revealed that LRRK2 interacts with parkin.[89] In transgenic flies, neurodegeneration by LRRK2 with or without a mutation is modified by overexpression or siRNA knockdown of parkin, PINK1 or DJ-1, suggesting genetic interaction between them.[90 91] Activity changes of LRRK2 kinase and GTPase have been suspected as a key factor in LRRK pathogenesis. Changes in LRRK2 activity cause alterations in mitogen activated protein kinase, translational control, tumour necrosis factor α/Fas ligand and Wnt signalling pathways with the cell biological functions of LRRK2 such as vesicle trafficking.[80] The most common pathological mutation in LRRK2, G2019S LRRK2, causes neurite retraction by activation of Rac1 small GTPase.[92] LRRK2 mutations inhibit an endogenous peroxidase by phosphorylation promoting dysregulation of mitochondrial function and oxidative damage.[93] G2019S human LRRK2 transgenic rat models specifically expressed in the nigrostriatal system have shown progressive degeneration of nigral dopaminergic neurons.[94] In terms of LRRK2 control, PKA has been identified as a potential upstream kinase of LRRK2 at S935, on which binding of 14-3-3 with LRRK2 depends.[95] However, the exact biological function of LRRK2 remains largely unclear because no physiological substrates have been identified to date.

ATP13A2 (PARK9)

Clinicogenetics

PARK9, also known as Kufor–Rakeb syndrome, is an autosomal recessive parkinsonian disorder characterised by early onset (14–16 years old), good response to L-dopa treatment, pyramidal feature, supranuclear gaze palsy and dementia.[96] The gene locus was mapped to 1p36 and the disease gene was identified as *ATP13A2*, which localises in lysosomal membranes.[97] Various types of mutations in the *ATP13A2* have been reported.

Molecular biology

ATP13A2 is predicted to be a lysosomal P5-type ATPase that plays important roles in regulating cation homeostasis. Although ATP13A2 function remains unclear, it might be involved in protecting cells against manganese and mutant α-synuclein toxicity.[98] Wild-type ATP13A2 localises mainly in lysosomes whereas three separate mutants with a mutation involved in PD cause retention of the protein in the endoplasmic reticulum, and are eliminated by the endoplasmic reticulum associated degradation pathway.[99] Wild-type ATP13A2, but not pathogenic mutants, reduced intracellular manganese concentration and prevented cytochrome C release from the mitochondria.[100]

Omi/HtrA2 (PARK13)

Clinicogenetics

Missense mutations in the gene coding for Omi/HtrA2 were reported to be associated with four patients with sPD, presenting with typical parkinsonism.[55] G399S and A141S mutations were detected and resulted in defective activation of the protease activity of Omi/HtrA2. Pathologically, accumulation of Omi was found in neuronal and glial inclusions in brains with α-synucleinopathies as well as in Lewy bodies.[101] The largest association study revealed no overall strong association of Omi/HtrA2 variants with sPD in populations worldwide.[102]

Molecular biology

Omi/HtrA2 is a nuclearly encoded mitochondrial protein consisting of 458 amino acids, originally identified as a proapoptotic protein binding with an apoptosis inhibiting protein.[103 104] Omi knockout mice presented with neuronal loss in the striatum and died within 30 days of birth.[105] Cells overexpressing Omi mutant with G399S have shown mitochondrial morphological changes followed by dysfunction and increased susceptibility against oxidative stress.[55] Interestingly, wild-type Omi/HtrA2, not protease defective mutant, activates autophagy through digestion of Hax-1, a Bcl-2 family related protein that represses autophagy via Beclin-1 inhibition, suggesting an insufficient protein degradation system may play a key role.[106]

PLA2G6 (PARK14)

Clinicogenetics

PARK14 is an autosomal recessive parkinsonian syndrome characterised by early onset rapidly progressive parkinsonism, dystonia, cognitive decline, and cerebral and cerebellar atrophy. Through homozygosity mapping and direct sequencing, two different homozygous mutations in *PLA2G6*, which also causes infantile neuroaxonal dystrophy and neurodegeneration with brain iron accumulation, were identified.[107 108] Cranial MRI did not detect iron accumulation in the basal ganglia in most cases with this disorder.[108 109]

Molecular biology

The PLA2G6 gene encodes a group VIA calcium independent phospholipase A2, also known as calcium independent phospholipase A2 β, which hydrolyses the sn-2 acyl chain of phospholipids, generating free fatty acids and lysophospholipids. In an in vitro assay, wild-type PLA2G6 associated with infantile neuroaxonal dystrophy/neurodegeneration with brain iron accumulation failed to catalyse fatty acid release from phospholipids, while PARK14 associated mutations ((R741Q, R747W and R632W) did not, implying that other functions of PLA2G6

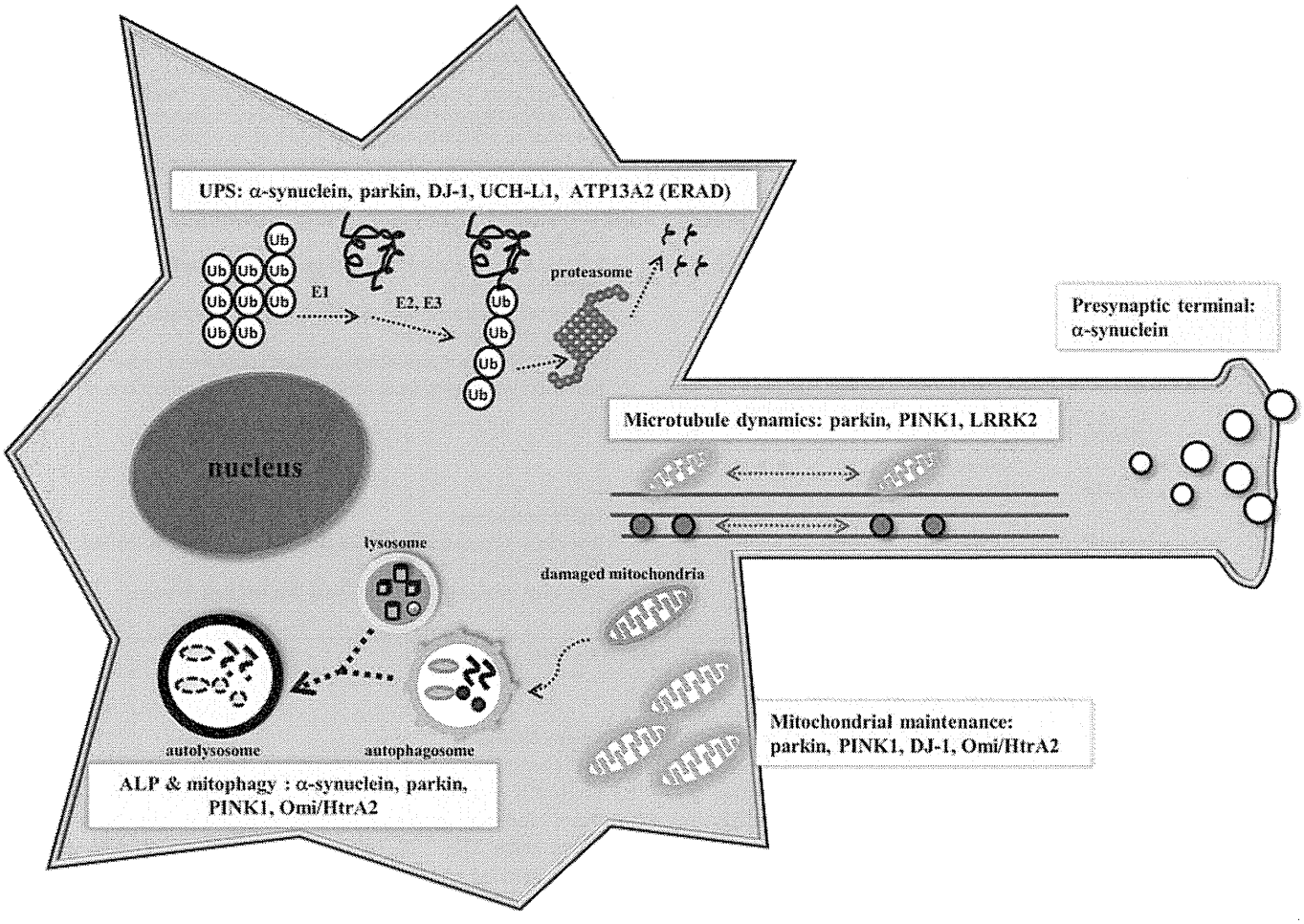

Figure 1 Schematic representation of the possible pathogenesis in hereditary Parkinson's disease. ALP, autophagy—lysosome pathway; ERAD, endoplasmic reticulum associated degradation; Ub, ubiquitin; UPS, ubiquitin proteasome system.

kinase domain.[48] The gene product is ubiquitously expressed in the brain and systemic organs. The protein mainly localises in mitochondria, especially in the outer membrane. PINK1 is a serine—threonine kinase and several pathological mutations in PINK1 have been reported to change their kinase activities.[49–52] In addition, Rictor (a component of mTORC2),[53] tumour necrosis factor receptor associated protein 1 (TRAP1; a mitochondrial chaperone),[50] Omi (PARK13 gene product) and parkin (PARK2 gene product) were identified as substrates for PINK1.[54 55]

PINK1 regulates mitochondrial dynamics and respiratory functions.[38 53 56–58] Mitochondrial fission is accelerated by PINK1 overexpression accompanied by parkin.[59 60] PINK1 ablation with siRNA in neurons reduces resistance against oxidative stress while its overexpression provides resistance.[61] Using genetically modified Drosophila models, we see that PINK1 deficiency causes the same phenotype as parkin deficiency and the PINK1 deficiency phenotype is rescued by parkin complementation, suggesting that parkin is downstream of PINK1.[62–64] Several lines of evidence have provided new aspects of the PINK1/parkin pathway associated with mitochondrial elimination via macroautophagy (mitophagy). When mitochondrial membrane potentials are lost, endogenous PINK1 is accumulated followed by parkin recruitment, and subsequently the depolarised mitochondria were eliminated by mitophagy.[40 41 65 66] Mitochondrial targeting sequence, kinase activity of PINK1 and the linker domain of parkin are indispensable for the PINK1/parkin mediated mitophagy.

DJ-1 (PARK7)

Clinicogenetics

Clinical features of *PARK7* are characterised by early onset parkinsonism with scoliosis, blephalospasm and psychiatric symptoms, similar to those of *PARK2* and *PARK6*. The disease gene was identified as *DJ-1*, which has eight exons encoding 189 amino acids. Three missense mutations (L166P, M26I, E64D) in exons 1—5 of the gene have been identified in Italian, Dutch and Uruguayan families. *DJ-1* protein was detected around Lewy bodies, suggesting *DJ-1* is not in the main structure of Lewy bodies. However, the protein was detected in astrocytes and in a part of the cytoplasmic inclusions positive to tau in brains with corticobasal degeneration, progressive supranuclear palsy and multiple system atrophy.[67–69]

Molecular biology

DJ-1 is almost ubiquitously expressed in organs, including the brain. Endogenous DJ-1 is present in synaptic terminals, mitochondria and membranous organelles.[70 71] DJ-1 with the L166P mutation lost more stability compared with the wild-type and mutant DJ-1 (M26I, E64D).[72] In DJ-1 knockout mice, no significant loss of dopaminergic neurons and decreased susceptibility to oxidative stress were noted.[73] DJ-1 is a multifunctional redox sensitive protein regulating mitochondrial oxidative stress and increases expression levels of SOD1 in an Erk1/2-Elk1 pathway dependent manner,[74] and facilitates prosurvival factor Akt, leading to suppression of apoptosis.[75] Also, the protein

Table 1 Genetic and clinical characteristics of hereditary Parkinson's disease

Locus	Inheritance	Gene	Type of mutation	Clinical features
PARK1/PARK4	AD	SNCA	Missense, duplication, triplication	A30P: late onset, L-dopa responded parkinsonism; A53T: typical parkinsonism with rapid progression; E64K: DLB-like symptoms; duplication: typical parkinsonism; triplication: early onset parkinsonism with rapid progression
PARK2	AR	PRKN	Nonsense, frameshift, missense	Early onset, symmetric, slowly progressed parkinsonism with spasticity and sleep benefits
PARK3	AD	Unknown	–	–
PARK5	AD	UCH-L1	Missense	Similar to sporadic PD
PARK6	AR	PINK1	Nonsense, frameshift, missense	Early onset typical parkinsonism with psychiatric symptoms and L-dopa associated dyskinesia
PARK7	AR	DJ-1	Missense	Early onset parkinsonism with psychiatric symptoms, occasionally with scoliosis and blephalospasm
PARK8	AD	LRRK2	Missense	Middle to late onset typical parkinsonism with response to L-dopa
PARK9	AR	ATP13A2	Missense, deletion, insertion, duplication	Rapidly progressed parkinsonism with dementia and pyramidal features
PARK10	Sporadic	Unknown	–	–
PARK11	AD	Unknown	–	–
PARK12	Sporadic	Unknown	–	–
PARK13	AD	Omi/HtrA2	Missense	Typical parkinsonism
PARK14	AR	PLA2G6	Missense	Early onset parkinsonism with rapid progression, cognitive decline and brain atrophy (cerebellum and cerebrum)
PARK15	AR	FBXO7	Missense, frameshift	Early onset parkinsonism with spasticity and response to L-dopa
PARK16	Sporadic	Unknown	–	–

AD, autosomal dominant; AR, autosomal recessive; DLB, dementia with Lewy bodies; PD, Parkinson's disease.

dependent manner by restricting conformational fluctuations of α-synuclein.[16] Recent advances in research on the protein degradation system associated with PD revealed the importance of ubiquitin proteasome and the autophagy–lysosome pathway in disease pathogenesis.[17] Wild-type α-synuclein is degraded by both chaperone mediated autophagy and macroautophagy, while A30P and A53T are degraded mainly by the latter.[17–19] Furthermore, macroautophagy itself is blocked by α-synuclein via Rap1a dysregulation.[20]

Several lines of evidence have shown that permeabilised α-synuclein from a neuron may be toxic to neurons and/or glias they are next to. Actually, grafted healthy neurons can gradually develop the same pathology as host neurons in PD brains.[21] These findings have suggested that non-cell autonomous cell death as well as cell autonomous cell death may have an important role in disease pathogenesis.

Parkin (PARK2)

Clinicogenetics

The first genetic locus for autosomal recessive juvenile parkinsonism was mapped to chromosome 6, and the disease gene named parkin (*PRKN*) was identified in consanguineous families.[22–24] Mutations in the *PRKN* gene are most common in autosomal recessive juvenile parkinsonism and many mutations have been reported.[3] The clinical picture is similar to that of sPD except for earlier onset, dystonic features, brisk reflexes and sleep benefit. Pathologically, no Lewy bodies were seen in most cases.[25–27] Whether or not heterozygous *PRKN* mutations may cause or increase the susceptibility to late onset typical PD remains controversial. [18F]Fluorodopa uptake by positron emission tomography was reduced in heterozygous carriers without symptoms.[28 29] In addition, heterozygous carriers of *PRKN* mutations have been reported to have either minor motor signs or present with late onset parkinsonism, suggesting a link between heterozygous mutations and disease pathogenesis.[27 30 31] On the other hand, screening for *PRKN* mutations in late onset PD and healthy controls revealed similar frequencies of genetic variants.[32 33]

Molecular biology

Parkin is associated with the ubiquitin proteasome system as an E3 ubiquitin ligase.[34] The C terminal binds with ubiquitin E2 enzymes and recognises a substrate whereas the N terminal interacts with the 19S subunit of proteasome. A nonsense mutation lacking the rear RING finger motif had no E3 activity and sole IBR-RING2 retained E3 activity, and thus most parkin mutations do not lead to loss of kinase activity.[35] α-Synuclein and synphilin-1 were identified as parkin substrates and consist of Lewy bodies.[36 37] Parkin mainly localises in the cytoplasm as well as in plasma membranes and partly in mitochondria. Under physiological or pathological conditions, parkin is involved in mitochondrial maintenance and recent evidence revealed that parkin with PINK1 physically associate and functionally cooperate to identify and label damaged mitochondria for selective degradation via autophagy (mitophagy).[38–42] Protein–protein interactions between parkin and other PD related genes are detailed in each gene section.

PINK1 (PARK6)

Clinicogenetics

PARK6 was first identified on chromosome 1p36.[43] The disease gene was identified as *PINK1* (PTEN induced kinase 1) containing eight exons.[44] The clinical characteristics are autosomal recessive, early onset, slow disease progression and L-dopa responsive parkinsonism. Most mutations were missense mutations, but whole gene deletions were also reported.[45 46] Many putative pathogenic mutations were also observed in a heterozygous state in familial and sPD patients as well as in healthy controls. However, most of the studies have not checked the copy number variants, causing the mutation pathogenicity to remain controversial.[2] Lewy bodies, neuronal loss and astrocytic gliosis in the substantia nigra were detected in a patient with *PINK1* compound heterozygous mutations.[47]

Molecular biology

PINK1 has eight exons encoding 581 amino acids, including a mitochondrial targeting sequence, transmembrane domain and

REVIEW

Molecular pathogenesis of Parkinson's disease: update

Shinji Saiki, Shigeto Sato, Nobutaka Hattori

Department of Neurology, Juntendo University School of Medicine, Bunkyo, Tokyo, Japan

Correspondence to
Professor N Hattori, Department of Neurology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan;
nhattori@juntendo.ac.jp

Received 16 August 2011
Revised 6 November 2011
Accepted 9 November 2011
Published Online First
3 December 2011

ABSTRACT

Parkinson disease (PD) is a neurodegenerative disease characterised by progressive disturbances in motor, autonomic and psychiatric functions. Much has been learnt since the disease entity was established in 1817. Although there are well established treatments that can alleviate the symptoms of PD, a pressing need exists to improve our understanding of the pathogenesis to enable development of disease modifying treatments. Ten responsible genes for PD have been identified and recent progress in molecular research on the protein functions of the genes provides new insights into the pathogenesis of hereditary as well as sporadic PD. Also, genome wide association studies, a powerful approach to identify weak effects of common genetic variants in common diseases, have identified a number of new possible PD associated genes, including PD genes previously detected. However, there is still much to learn about the interactions of the gene products, and important insights may come from chemical and genetic screens. In this review, an overview is provided of the molecular pathogenesis and genetics of PD, focusing particularly on the functions of the PD related gene products with marked research progress.

INTRODUCTION

Parkinson's disease (PD) is the second most common progressive neurodegenerative disease, named after James Parkinson's who provided a classic account of the condition in 1817. Affecting 1–2% of the population over the age of 65 years, the prevalence of PD increases by approximately 4% in those older than 85 years. Ten genes that contribute to the genetic aetiology of hereditary PD (hPD) were identified, mainly through positional cloning strategies in inherited PD patients and families (table 1).[1 2] Several responsible genes for hPD have been identified, and based on functional studies in vitro and in vivo of gene products, some have been found to interact with each other in various cellular systems for homeostasis, such as synaptic homeostasis (α-synuclein), mitochondrial maintenance (PINK1, parkin, DJ-1, Omi/HtrA2), autophagy–lysosome pathway (α-synuclein, parkin, PINK1, Omi/HtrA2), axonal transport (LRRK2) and ubiquitin proteasome systems (α-synuclein, parkin, DJ-1, UCH-L1). Impairments in a number of cellular systems have been suggested to underlie hPD (figure 1). Also, more recent studies revealed that mutations in the same genes can be involved in familial PD and be risk factors for sporadic PD (sPD), suggesting that inherited and sPD could have common pathological mechanisms.[3] Therefore, understanding the function of the proteins encoded by hPD genes will hopefully further our understanding of the mechanisms leading to inherited and sPD.

In this review, we will summarise the latest research progress in the molecular mechanisms of hPD and genetic association studies of sPD.

HEREDITARY PD

α-Synuclein (PARK1 and PARK4)

Clinicogenetics

SNCA was the first causal PD gene identified in a large Italian family.[4] Mutations (A30P, E46K and A53T), duplications and triplications of the *SNCA* gene have been reported.[2] Clinical features of patients with the E46K mutation are similar to those of dementia with Lewy bodies, while A30P is not associated with severe dementia. Individuals with *SNCA* triplication developed an early onset form of PD with rapid progression and more extended neurodegeneration.[5]

Recent genome wide association studies (GWAS) have demonstrated a strong association between common single nucleotide polymorphism within the *SNCA* locus and PD in European and Japanese population, consistent with the finding that variation at the *SNCA* locus increases PD susceptibility.[6–9] Although the *SNCA* single nucleotide polymorphism associated with sPD show a low OR (1.2–1.4), these findings are consistent with α-synuclein aggregation pathology.

Molecular biology

α-Synuclein is mainly expressed in the presynaptic terminal of the CNS. The protein binds with lipids and unfolds in the steady state. Although the exact function remains unclear, it regulates dopamine homeostasis in presynaptic vesicle cycling.[5] The phenotype of α-synuclein knockout mice is unremarkable and only shows a mild decrease in dopamine levels in the striatum and a mild decrease in synaptic vesicles in the hippocampus. Compared with the wild-type α-synuclein, mutant forms easily aggregate in neuronal cells in vitro and in vivo.[10 11] Transgenic mice with wild or mutant α-synuclein under various promoters have shown neuronal inclusions, mitochondrial abnormalities and neurodegeneration.[12–14] Which type of α-synuclein species is the most toxic to cells remains unclear but some studies assert that mature aggregates are not themselves the toxic moiety but rather an attempt by the cell to clear small toxic oligomers.[15] Hsp90 modulates the assembly of α-synuclein in an ATP

of *VPS35* mutation and the effects of interaction with other known pathogenic gene products on PD.

In conclusion, we have reported Asian PD patients with the *VPS35* p.D620N mutation. The p.D620N substitution may be a mutational hot spot across different ethnic populations. The frequency of *VPS35* mutation was low in ADPD; however, it is relatively high in Japanese patients compared with that reported in other populations.[3,4,6–8] Based on the clinical features of patients with *VPS35* mutation, *VPS35* should be analyzed in patients with PD, especially ADPD or tremor-predominant PD. ■

Acknowledgments: We thank all the participants in this study.

References

1. Lang AE, Lozano AM. Parkinson's disease. First of two parts. N Engl J Med 1998;339:1044–1053.
2. Lesage S, Brice A. Parkinson's disease: from monogenic forms to genetic susceptibility factors. Hum Mol Genet 2009;18: R48–R59.
3. Vilariño-Güell C, Wider C, Ross OA, et al. VPS35 mutations in Parkinson disease. Am J Hum Genet 2011;89:162–167.
4. Zimprich A, Benet-Pagès A, Struhal W, et al. A mutation in VPS35, encoding a subunit of the retromer complex, causes late-onset Parkinson disease. Am J Hum Genet 2011;89:168–175.
5. Bonifacino JS, Hurley JH. Retromer. Retromer. Curr Opin Cell Biol 2008;4:427–436.
6. Sheerin UM, Charlesworth G, Bras J, et al. Screening for VPS35 mutations in Parkinson's disease. Neurobiol Aging 2012;4: 838.e1–e5.
7. Guella I, Soldà G, Cilia R, et al. The Asp620asn mutation in VPS35 is not a common cause of familial Parkinson's disease. Mov Disord 2012;27:800–801.
8. Zhang Y, Chen S, Xiao Q, et al. Vacuolar protein sorting 35 Asp620Asn mutation is rare in the ethnic Chinese population with Parkinson's disease. Parkinsonism Relat Disord 2012;18:638–640.
9. Nishioka K, Hayashi S, Farrer MJ, et al. Clinical heterogeneity of alpha-synuclein gene duplication in Parkinson's disease. Ann Neurol 2006;59:298–309.
10. Kitada T, Asakawa S, Hattori N, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 1998;392:605–608.
11. Kumazawa R, Tomiyama H, Li Y, et al. Mutation analysis of the PINK1 gene in 391 patients with Parkinson disease. Arch Neurol 2008;65:802–808.
12. Tomiyama H, Li Y, Yoshino H, et al. Mutation analysis for DJ-1 in sporadic and familial parkinsonism: screening strategy in parkinsonism. Neurosci Lett 2009;455:159–161.
13. Tomiyama H, Li Y, Funayama M, et al. Clinicogenetic study of mutations in LRRK2 exon 41 in Parkinson's disease patients from 18 countries. Mov Disord 2006;21:1102–1108.
14. Yoshino H, Tomiyama H, Tachibana N, et al. Phenotypic spectrum of patients with PLA2G6 mutation and PARK14-linked parkinsonism. Neurology 2010;75:1356–1361.
15. Hughes AJ, Daniel SE, Kilford L, Lees AJ. Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. J Neurol Neurosurg Psychiatry 1992;55:181–184.
16. Funayama M, Tomiyama H, Wu RM, et al. Rapid screening of ATP13A2 variant with high-resolution melting analysis. Mov Disord 2010;25:2434–2437.
17. Lesage S, Condroyer C, Klebe S et al. Identification of VPS35 mutations replicated in French families with Parkinson disease. Neurology 2012;78:1449–1450.
18. Ibáñez P, Lesage S, Janin S, et al. Alpha-synuclein gene rearrangements in dominantly inherited parkinsonism: frequency, phenotype, and mechanisms. Arch Neurol 2009;66:102–108.
19. Seki N, Takahashi Y, Tomiyama H, et al. Comprehensive mutational analysis of LRRK2 reveals variants supporting association with autosomal dominant Parkinson's disease. J Hum Genet 2011;56:671–675.
20. Di Fonzo A, Rohe C. F, Ferreira J, et al. A frequent LRRK2 gene mutation associated with autosomal dominant Parkinson's disease. Lancet 2005;365:412–415.
21. Gilks WP, Abou-Sleiman PM, Gandhi S, et al. A common LRRK2 mutation in idiopathic Parkinson's disease. Lancet 2005;365:415–416.
22. Orimo S, Amino T, Yokochi M, et al. Preserved cardiac sympathetic nerve accounts for normal cardiac uptake of MIBG in PARK2. Mov Disord 2005;10:1350–1353.
23. Verstraeten A, Wauters E, Crosiers D, et al. Contribution of VPS35 genetic variability to LBD in the Flanders-Belgian population. Neurobiol Aging 2012;33:e11–e13.
24. Caudle WM, Colebrooke RE, Emson PC, et al. Altered vesicular dopamine storage in Parkinson's disease: a premature demise. Trends Neurosci 2008;31:303–308.
25. Berwick DC, Harvey K. LRRK2 signaling pathways: the key to unlocking neurodegeneration? Trends Cell Biol 2011;21:257–265.

and the father of patient CII-3 also developed PD (Fig. 1B, C). Patient D, who developed upper limb rest tremor at age 42, had no family history of PD. She underwent subthalamic nucleus deep brain stimulation (STN-DBS) at age 60 because of disabling motor fluctuation and dyskinesia refractory to pharmacological treatment. All affected patients were born to nonconsanguineous parents.

Discussion

VPS35 has been reported as the pathogenic gene for ADPD, and only 1 mutation, p.D620N, has been reported in several unrelated white families. To our knowledge, there have been no reports of Asian PD patients with *VPS35* mutations.[3,8] Based on this background, we set out in this study to determine the incidence of *VPS35* mutations in Japanese patients with PD. We detected the heterozygous p.D620N mutation of *VPS35* in 3 ADPD families and 1 SPD patient with East Asian ancestry. On the other hand, we could not conclude the pathogenicity of 6 other variants that had been reported as potentially pathogenic for PD because none of the variants was detected in our patients with PD.

The frequency of the p.D620N mutation in Japanese patients was 1.0% in ADPD and 0.23% in SPD. Although the exact frequency among whites is undetermined, the frequency is relatively higher in Japanese patients compared with that reported in previous studies (0%–1.22%).[3,4,6,7,17] Moreover, the frequency in Japanese patients also differs greatly from those of other Asian populations such as Taiwanese patients and mainland Chinese patients (0%).[3,8] Although the mutation frequency was expected to be lower than that of other pathogenic genes for ADPD, such as multiplication of *SNCA*[9,18] and point mutation of *LRRK2*,[19–21] *VPS35* may be one of the most important genes in Japanese PD. Because we screened for only 7 reported variants, we cannot determine the exact frequency of *VPS35* mutations in ADPD; we would need to analyze all 17 exons of *VPS35* in ADPD patients to screen for other variants and to assess the incidence of all disease-associated *VPS35* mutations.[3,4] Furthermore, we would need to perform mutational analysis for SPD patients, in addition to ADPD, to identify Asian population-specific variants, such as *LRRK2* p.G2385R, associated with susceptibility for PD.[19]

Based on haplotype analysis reported in previous studies, the substitution of *VPS35* c.1858G>A (p.D620N) occurs from independent mutational events.[3] We were able to determine the chromosomal phase only in patient AII-11 (family A). The p.D620N mutation possibly shared a common founder between Japanese ADPD families A and B; however, it was inconclusive because the phase of patient BIII-8 was undetermined. On the other hand, the same p.D620N mutation probably occurred independently in patient CII-3 (family C) and patient D. By genotyping of D16S3105, which is located approximately 1.5 kb centromeric of *VPS35*, there were at least 3 different haplotypes in Japanese because families A and C and patient D (SPD) did not have the same alleles for this microsatellite. To determine the chromosomal phase of families B and C, detailed genetic analyses of other family members are needed in future studies. These results suggest the existence of 3 or more founders in Japanese patients, in addition to the reported white patients with the p.D620N mutation or de novo mutations, indicating that the p.D620N mutation site is a mutational hot spot in *VPS35* across different ethnic populations.

According to previous reports, the average AAO of patients with the *VPS35* mutation was 50–60 years (50.6 ± 7.3 years),[3] with a distinctive feature of a slightly younger AAO compared with patients with idiopathic PD. In our study, the AAO was nonspecific with a wide range between 30–70 years. Because the family history of patient D was unknown, she was categorized as SPD. With regard to *VPS35* mutation penetrance, it is incomplete from the results of a previous report.[3] Therefore, although the frequency is low, patients with p.D620N mutation could be found among SPD patients.

The clinical symptoms of our patients with *VPS35* mutation closely resembled the idiopathic PD form, with tremor-dominant dopa-responsive parkinsonism. Psychiatric problems were inconspicuous; however, dementia may occur in patients with a long disease course, similar to patient AII-11, who had PD for 15 years. Our patients with *VPS35* mutations had normal brain MRI and cardiac MIBG scintigraphy. There have been no definite pathological mutations of *VPS35* in the spectrum of LB disorders. On the basis of these results, patients with *VPS35* mutation could show comparatively benign disease course without widespread LBs pathology.[22,23]

VPS35 assembles into the retromer cargo-recognition complex that associates with the cytosolic face of the endosomes. The retromer mediates the retrograde transport of transmembrane cargo from the endosomes to the trans-Golgi network.[5] The p.D620N mutation of *VPS35* might cause impairment of interaction with other components of the retromer complex and impaired retrograde trafficking of recycling proteins,[4] similar to α-synuclein and LRRK2, which are involved in vesicle trafficking.[24,25] Mutations in familial PD genes, including *VPS35*, may cause disruption of intracellular trafficking and lead to neurodegeneration. These findings suggest that impairment of intracellular trafficking systems is associated with the pathogenesis of PD. Although the association between the p.D620N mutation of *VPS35* and PD remains unknown, further functional studies might shed light on the pathogenesis

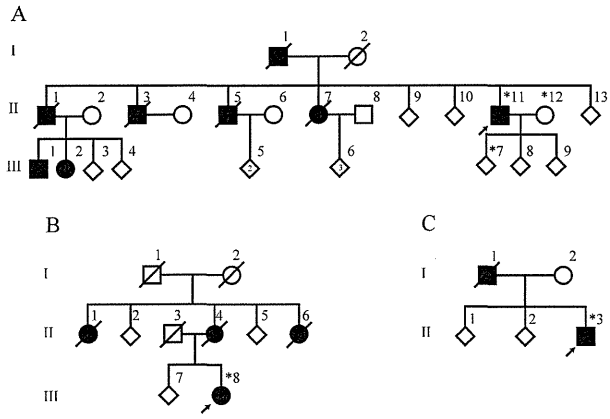

FIG. 1. Pedigrees of families with the *VPS35* p.D620N mutation (open symbol, unaffected family member; closed symbol, affected member; arrow, proband; asterisk, individual analyzed for the p.D620N mutation and/or haplotype; forward slash through symbol, deceased individuals; square, male; circle, female; diamond, unspecified sex).

patient with SPD (Fig. 1). The p.D620N has been reported previously as a pathogenic mutation for familial PD.[3,4,6] This mutation was not found in 1158 control chromosomes. Patients carrying the p.D620N mutation did not have any other variants in all exons of *VPS35*. In our population, the incidence of the p.D620N mutation was 1.0% (3 of 300) in ADPD and 0.23% (1 of 433) in SPD. The remaining variants analyzed in this study were not identified in any patients.

Haplotype analysis demonstrated that the Japanese patients with the p.D620N mutation had different genotypes from those of white patients with the same mutation.[3] One disease allele was detected by analyzing patient AII-11 and his relatives. Patients AII-11 and BIII-8 in this study carried at least the same single allele of microsatellites in the flanking region of *VPS35* (Table 1). On the other hand, patients CII-3 and D had a different genotype of D16S3105, with a locus mapped very close to *VPS35*, compared with the disease allele of AII-11 (Table 1, boldface).

TABLE 1. Haplotype analysis of VPS35 p.D620N mutation carriers

	Patient ID			
Microsatellite	AII-11	BIII-8	CII-3	D
D16S401	170	166/170	166/172	166/170
D16S3068	143	141/145	145/147	145/145
D16S753	272	272/268	268/276	264/268
VPS35 p.D620N	A	A/G	A/G	A/G
Chr16_45.333M	294	294/298	294/300	294/304
D16S3105	191	191/189	189/193	187/187
Chr16_45.615M	147	147/147	147/145	147/145
Chr16_45.806M	246	246/238	246/244	246/244
Chr16_45.835M	237	237/237	237/237	237/237
Chr16_45.855M	212	212/210	210/210	210/216
D16S3044	195	195/195	195/197	197/197

Both alleles are shown when markers of phase could not be determined.

TABLE 2. Clinical features of patients with p.D620N mutation

	Patient ID			
	AII-11	BIII-8	CII-3	D
Age at disease onset (y)	62	55	34	42
Disease duration (y)	15	2	7	21
Resting tremor	+	+	+	+
Bradykinesia	+	+	+	+
Rigidity	+	+	+	+
Gait disturbance	+	−	−	+
Postural instability	+	−	−	+
Clinical response to levodopa	+	+	+	+
Wearing off	+	−	+	+
Asymmetry at onset	+	+	+	+
Orthostatic hypotension	+	−	−	−
Incontinence	+	−	−	−
Urinary urgency	−	−	−	−
Levodopa-induced dyskinesia	+	−	+	+
Sleep benefit	+	−	+	Unknown
Dystonia at onset	−	−	−	−
Hyperreflexia	−	−	−	−
Hallucination	−	−	−	−
Other psychosis	−	−	−	−
Dementia	+	−	−	−
Gaze palsy	−	−	−	−
Brain MRI	WNL	WNL	WNL	WNL
Cardiac MIBG scintigraphy	H/M ratio (E/L), 2.38/2.68; washout ratio, 4.15%[a]	Not performed	Not performed	Not performed

[a]MIBG scintigraphy was performed when AII-11 was 76 years old.
WNL, within normal limit; H/M ratio, heart-to-mediastinum ratio; (E/L), early/late stage.

Clinical Presentation

Table 2 summarizes the clinical features of the 4 *VPS35* mutation-positive patients. Patient AII-11 was a 77-year-old man who developed right upper limb rest tremor at age 62. At age 75, he underwent gastrostomy for progressive dysphagia, then developed cognitive dysfunction without hallucination. Single-photon emission computed tomography of cerebral blood flow showed no reduction in blood flow in the basal ganglia. His father and 4 of 8 siblings were diagnosed with PD (Fig. 1A) and presented levodopa-responsive typical parkinsonism: upper limb tremor and small-step gait. His nephew and niece were also diagnosed with PD, and the nephew developed parkinsonism in his early fifties. Patients BIII-8 and CII-3 both developed upper limb rest tremor at ages 34 and 55, respectively. The mother and aunts of patient BIII-8

Parkinson's disease (PD) is a neurodegenerative disorder characterized by progressive motor disturbances manifested by tremor, rigidity, akinesia, and postural instability. Neuropathologically, PD is characterized by selective loss of dopaminergic neurons in the substantia nigra and the presence of cytosolic inclusions called Lewy bodies (LBs) in the remaining neurons. The pathogenesis of PD is multifactorial, including genetic–environmental interaction. PD is a common disease in the elderly, with an incidence of about 1%–2% in individuals older than 60 years.[1] Among PD patients, approximately 5%–10% have a positive family history of PD,[2] and among these, the Mendelian forms of PD can contribute to the elucidation of the molecular pathways that lead to the degeneration and death of dopaminergic neurons.

Mutations in the vacuolar protein sorting 35 (*VPS35*) gene have recently been identified in families with autosomal dominant late-onset PD (MIM 601501).[3,4] Patients with *VPS35* mutations present with tremor-predominant dopa-responsive parkinsonism.[3,4] VPS35, a key component of the retromer cargo-recognition complex, is thought to associate with sorting cargos into the tubular endosomal network for retrieval to the trans-Golgi network.[5] Therefore, pathogenic mutations of *VPS35* may cause disruption of the retrograde transport system and contribute to dopaminergic neuronal cell death in PD. One missense mutation has been reported to be pathogenic for PD.[3,4] Mutation of c.1858G>A (p.D620N) was identified in 3 Austrian families and 1 family each in Switzerland, the United States, Tunisia, and the United Kingdom, as well as 1 family and 1 patient with sporadic PD (SPD) among Yemenite Jews from Israel.[3,4,6] In addition, several variants, such as p.M57I, p.I241M, p.P316S, and p.R524W, have been reported in Europe and the United States as potentially pathogenic for PD.[3,4]

Although multipopulation screenings for *VPS35* mutations were preformed in recent reports, there is still no report of PD patients with *VPS35* mutations of Asian ancestry.[3,4,6–8] In the present study, we screened Japanese patients with autosomal-dominant PD (ADPD), Japanese patients with SPD, and control subjects for mutations of *VPS35*, with a special focus on 7 reported nonsynonymous variants that were found in patients with PD, including the p.D620N. Here, we report 3 families and 1 SPD patient with the p.D620N mutation in *VPS35* and describe their clinical features.

Patients and Methods

Subjects

The study was approved by the ethics committee of Juntendo University, and all subjects gave written informed consent to participate in the genetic research. The study subjects were 308 Japanese patients (300 index patients) with ADPD (age at disease onset [AAO; mean ± SD], 51.1 ± 11.7 years; range, 8–83 years; female/male [F/M] ratio, 1.35) and 433 Japanese SPD patients (AAO, 47.2 ± 12.9 years; range, 5–88 years; F/M ratio, 1.09) selected from the gene bank of Juntendo University. Some of the selected subjects had been confirmed negative for *SNCA*, *PARK2*, *PINK1*, *PARK7*, *LRRK2*, and *PLA2G6* mutations.[9–14] From the same gene bank, we also selected 579 healthy Japanese subjects without a family history of parkinsonism (age at sampling, 58.0 ± 9.3 years; range, 23–89 years; F/M ratio, 1.54). The criteria for the diagnosis of PD were adopted by the participating neurologists and were established based on the United Kingdom Parkinson's Disease Society Brain Bank.[15]

Genetic Analysis

Genomic DNA was extracted from peripheral blood using a standard protocol. Patients with ADPD and SPD were examined for the following 7 variants: p.M57I (exon 3), p.I241M (exon 7), p.P316S (exon 9), p.R524W (exon 13), p.D620N (exon 15), p.A737V (exon 16), and p.L774M (exon 17) of *VPS35* (RefSeq accession number NM_018206.4). PCR direct sequencing was performed using a BigDye Terminator v1.1 Cycle Sequencing kit and 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) or 3730 DNA Analyzer (Applied Biosystems). In addition, SPD patients and control subjects were also genotyped for c.1858G>A (p.D620N) mutation by high-resolution melting (HRM) analysis using LightScanner and LCGreen plus (Idaho Technology, Salt Lake City, UT). HRM analysis was performed using a previously described protocol[16] and the following primers: forward, GAGGATGGTTGGTCCTTGAA; reverse, TGCCAATGATCAAGGTGATG. All exons of *VPS35* were also analyzed in patients with the p.D620N mutation using the method described previously.[3]

Haplotype analysis of the *VPS35* flanking region was performed using 3130 Genetic analyzer and GeneMapper software (Applied Biosystems, Foster City, CA). To adjust the size of PCR products, we also genotyped Centre d'Étude du Polymorphisme Humain (CEPH) control samples (1331-01 and 1331-02) for comparison of haplotypes with previously reported patients carrying the p.D620N mutation. The sequences of the PCR primers were reported previously.[3]

Results

Detection of p.D620N Mutation

We detected the heterozygous missense p.D620N mutation in 3 unrelated patients with ADPD and 1

RESEARCH ARTICLE

VPS35 Mutation in Japanese Patients with Typical Parkinson's Disease

Maya Ando, MD,[1] Manabu Funayama, PhD,[1,2*] Yuanzhe Li, MD, PhD,[2] Kenichi Kashihara, MD, PhD,[3] Yoshitake Murakami, MD,[4] Nobutaka Ishizu, MD,[5] Chizuko Toyoda, MD,[6] Katsuhiko Noguchi, MD,[7] Takashi Hashimoto, MD,[8] Naoki Nakano, MD,[9] Ryogen Sasaki, MD, PhD,[10] Yasumasa Kokubo, MD, PhD,[10] Shigeki Kuzuhara, MD, PhD,[11] Kotaro Ogaki, MD,[1] Chikara Yamashita, MD,[1] Hiroyo Yoshino, PhD,[2] Taku Hatano, MD, PhD,[1] Hiroyuki Tomiyama, MD, PhD,[1,12] and Nobutaka Hattori, MD, PhD[1,2,12*]

[1]*Department of Neurology, Juntendo University School of Medicine, Tokyo, Japan*
[2]*Research Institute for Diseases of Old Age, Graduate School of Medicine, Juntendo University, Tokyo, Japan*
[3]*Department of Neurology, Okayama Kyokuto Hospital, Okayama, Japan*
[4]*Department of Neurology, Saiseikai Kurihashi Hospital, Saitama, Japan*
[5]*Department of Neurology, Saitama National Hospital, Saitama, Japan*
[6]*Department of Neurology, Jikei Daisan Hospital, Tokyo, Japan*
[7]*Department of Neurology, Kakio Kinen Hospital, Tokyo, Japan*
[8]*Hashimoto Clinic, Osaka, Japan*
[9]*Department of Neurosurgery, Kinki University Hospital, Osaka, Japan*
[10]*Department of Neurology, Mie University Graduate School of Medicine, Tsu, Mie, Japan*
[11]*Department of Medical Welfare, Faculty of Health Science, Suzuka University of Medical Science, Suzuka, Mie, Japan*
[12]*Department of Neuroscience for Neurodegenerative Disorders, Juntendo University School of Medicine, Tokyo, Japan*

ABSTRACT: Vacuolar protein sorting 35 *(VPS35)* was recently reported to be a pathogenic gene for late-onset autosomal dominant Parkinson's disease (PD), using exome sequencing. To date, *VPS35* mutations have been detected only in whites with PD. The aim of the present study was to determine the incidence and clinical features of Asian PD patients with *VPS35* mutations. We screened 7 reported nonsynonymous missense variants of *VPS35*, including p.D620N, known as potentially disease-associated variants of PD, in 300 Japanese index patients with autosomal dominant PD and 433 patients with sporadic PD (SPD) by direct sequencing or high-resolution melting (HRM) analysis. In addition, we screened 579 controls for the p.D620N mutation by HRM analysis. The p.D620N mutation was detected in 3 patients with autosomal dominant PD (1.0%), in 1 patient with SPD (0.23%), and in no controls. None of the other reported variants of *VPS35* were detected. Haplotype analysis suggested at least 3 independent founders for Japanese patients with p.D620N mutation. Patients with the *VPS35* mutation showed typical tremor-predominant PD. We report Asian PD patients with the *VPS35* mutation. Although *VPS35* mutations are uncommon in PD, the frequency of such mutation is relatively higher in Japanese than reported in other populations. In *VPS35*, p.D620N substitution may be a mutational hot spot across different ethnic populations. Based on the clinical features, *VPS35* should be analyzed in patients with PD, especially autosomal dominant PD or tremor-predominant PD.

Key Words: Parkinson's disease; *VPS35*; autosomal dominant; hotspot; mutation.

***Correspondence to:** Dr. Manabu Funayama or Prof. Dr. Nobutaka Hattori, Research Institute for Diseases of Old Age, Graduate School of Medicine, Juntendo University, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan; funayama@juntendo.ac.jp or nhattori@juntendo.ac.jp

Funding agencies: This work was supported by Strategic Research Foundation Grant-in-Aid Project for Private Universities, Grants-in-Aid for Scientific Research (80218510 [to N.H.] and 21591098 [to H.T.]), Grant-in-Aid for Young Scientists (22790829 [to M.F.] and 23791003 [to Y.L.]), Grant-in-Aid for Scientific Research on Innovative Areas (23111003 [to N.H.] and 23129506 [to M.F.]) from the Japanese Ministry of Education, Culture, Sports, Science and Technology, and Grant-in-Aid from the Research Committee on Muro Disease (Kii ALS/PDC; 21210301 [to Y.K.]), the Ministry of Health, Labor, and Welfare, and JST, CREST.

Relevant conflicts of interest/financial disclosures: Nothing to report.
Full financial disclosures and author roles may be found in the online version of this article.

Received: 5 March 2012; **Revised:** 11 July 2012; **Accepted:** 17 July 2012
Published online in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/mds.25145

Author contributions

K.O., T.O., K.T. and N.M. designed the research and analysed the data. K.O., T.O., M.K., F.K., Y.F., K.S-F. and S.S. carried out immunoblotting experiments. K.O., M.I., K.I., M.K. and E.G. performed immunocytochemistry. M.F. carried out statistical analysis. H.K., N.T. and H.T. performed mass spectrometric analysis. H.S. did structural modelling. N.M. wrote the manuscript with help and supervision from M.K., N.H., K.M. and K.T.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Okatsu, K. *et al.* PINK1 autophosphorylation upon membrane potential dissipation is essential for Parkin recruitment to damaged mitochondria. *Nat. Commun.* 3:1016 doi: 10.1038/ncomms2016 (2012).

References

1. Kitada, T. *et al.* Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* **392,** 605–608 (1998).
2. Valente, E. M. *et al.* Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science* **304,** 1158–1160 (2004).
3. Corti, O., Lesage, S. & Brice, A. What genetics tells us about the causes and mechanisms of Parkinson's disease. *Physiol. Rev.* **91,** 1161–1218 (2011).
4. Schapira, A. H. Mitochondria in the aetiology and pathogenesis of Parkinson's disease. *Lancet Neurol.* **7,** 97–109 (2008).
5. Narendra, D., Tanaka, A., Suen, D. F. & Youle, R. J. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *J. Cell Biol.* **183,** 795–803 (2008).
6. Matsuda, N. *et al.* PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. *J. Cell Biol.* **189,** 211–221 (2010).
7. Narendra, D. P. *et al.* PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. *PLoS Biol.* **8,** e1000298 (2010).
8. Rakovic, A. *et al.* Effect of endogenous mutant and wild-type PINK1 on Parkin in fibroblasts from Parkinson disease patients. *Hum. Mol. Genet.* **19,** 3124–3137 (2010).
9. Geisler, S. *et al.* PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nat. Cell Biol.* **12,** 119–131 (2010).
10. Ziviani, E., Tao, R. N. & Whitworth, A. J. Drosophila parkin requires PINK1 for mitochondrial translocation and ubiquitinates mitofusin. *Proc. Natl Acad. Sci. USA* **107,** 5018–5023 (2010).
11. Lin, W. & Kang, U. J. Characterization of PINK1 processing, stability, and subcellular localization. *J. Neurochem.* **106,** 464–474 (2008).
12. Vives-Bauza, C. *et al.* PINK1-dependent recruitment of Parkin to mitochondria in mitophagy. *Proc. Natl Acad. Sci. USA* **107,** 378–383 (2010).
13. Chaugule, V. K. *et al.* Autoregulation of Parkin activity through its ubiquitin-like domain. *EMBO J.* **30,** 2853–2867 (2011).
14. Ding, W. X. *et al.* Nix is critical to two distinct phases of mitophagy, reactive oxygen species-mediated autophagy induction and Parkin-ubiquitin-p62-mediated mitochondrial priming. *J. Biol. Chem.* **285,** 27879–27890 (2010).
15. Gegg, M. E. *et al.* Mitofusin 1 and mitofusin 2 are ubiquitinated in a PINK1/parkin-dependent manner upon induction of mitophagy. *Hum. Mol. Genet.* **19,** 4861–4870 (2010).
16. Glauser, L., Sonnay, S., Stafa, K. & Moore, D. J. Parkin promotes the ubiquitination and degradation of the mitochondrial fusion factor mitofusin 1. *J. Neurochem.* **118,** 636–645 (2011).
17. Okatsu, K. *et al.* p62/SQSTM1 cooperates with Parkin for perinuclear clustering of depolarized mitochondria. *Genes to Cells* **15,** 887–900 (2010).
18. Poole, A. C., Thomas, R. E., Yu, S., Vincow, E. S. & Pallanck, L. The mitochondrial fusion-promoting factor mitofusin is a substrate of the PINK1/parkin pathway. *PloS One* **5,** e10054 (2010).
19. Tanaka, A. *et al.* Proteasome and p97 mediate mitophagy and degradation of mitofusins induced by Parkin. *J. Cell Biol.* **191,** 1367–1380 (2010).
20. Wang, X. *et al.* PINK1 and Parkin target Miro for phosphorylation and degradation to arrest mitochondrial motility. *Cell* **147,** 893–906 (2011).
21. Liu, S. *et al.* Parkinson's disease-associated kinase PINK1 regulates Miro protein level and axonal transport of mitochondria. *PLoS Genet.* **8,** e1002537 (2012).
22. Chan, N. C. *et al.* Broad activation of the ubiquitin-proteasome system by Parkin is critical for mitophagy. *Hum. Mol. Genet.* **20,** 1726–1737 (2011).
23. Lee, J. Y., Nagano, Y., Taylor, J. P., Lim, K. L. & Yao, T. P. Disease-causing mutations in parkin impair mitochondrial ubiquitination, aggregation, and HDAC6-dependent mitophagy. *J. Cell Biol.* **189,** 671–679 (2010).
24. Yoshii, S. R., Kishi, C., Ishihara, N. & Mizushima, N. Parkin mediates proteasome-dependent protein degradation and rupture of the outer mitochondrial membrane. *J. Biol. Chem.* **286,** 19630–19640 (2011).
25. Cai, Q., Zakaria, H. M., Simone, A. & Sheng, Z. H. Spatial parkin translocation and degradation of damaged mitochondria via mitophagy in live cortical neurons. *Curr. Biol.* **22,** 545–552 (2012).
26. Jin, S. M. *et al.* Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL. *J. Cell Biol.* **191,** 933–942 (2010).
27. Deas, E. *et al.* PINK1 cleavage at position A103 by the mitochondrial protease PARL. *Hum. Mol. Genet.* **20,** 867–879 (2011).
28. Shi, G. *et al.* Functional alteration of PARL contributes to mitochondrial dysregulation in Parkinson's disease. *Hum. Mol. Genet.* **20,** 1966–1974 (2011).
29. Meissner, C., Lorenz, H., Weihofen, A., Selkoe, D. J. & Lemberg, M. K. The mitochondrial intramembrane protease PARL cleaves human Pink1 to regulate Pink1 trafficking. *J. Neurochem.* **117,** 856–867 (2011).
30. Greene, A. W. *et al.* Mitochondrial processing peptidase regulates PINK1 processing, import and Parkin recruitment. *EMBO Rep.* **13,** 378–385 (2012).
31. Narendra, D. P. & Youle, R. J. Targeting mitochondrial dysfunction: role for PINK1 and Parkin in mitochondrial quality control. *Antioxid.. Redox Signal.* **14,** 1929–1938 (2011).
32. Pogson, J. H., Ivatt, R. M. & Whitworth, A. J. Molecular mechanisms of PINK1-related neurodegeneration. *Curr. Neurol. Neurosci. Rep.* **11,** 283–290 (2011).
33. Springer, W. & Kahle, P. J. Regulation of PINK1-Parkin-mediated mitophagy. *Autophagy* **7,** 266–278 (2011).
34. Kinoshita, E., Kinoshita-Kikuta, E., Takiyama, K. & Koike, T. Phosphate-binding tag, a new tool to visualize phosphorylated proteins. *Mol. Cell. Proteomics* **5,** 749–757 (2006).
35. Beilina, A. *et al.* Mutations in PTEN-induced putative kinase 1 associated with recessive parkinsonism have differential effects on protein stability. *Proc. Natl Acad. Sci. USA* **102,** 5703–5708 (2005).
36. Gautier, C. A., Kitada, T. & Shen, J. Loss of PINK1 causes mitochondrial functional defects and increased sensitivity to oxidative stress. *Proc. Natl Acad. Sci. USA* **105,** 11364–11369 (2008).
37. Zhou, C. *et al.* The kinase domain of mitochondrial PINK1 faces the cytoplasm. *Proc. Natl Acad. Sci. USA* **105,** 12022–12027 (2008).
38. Vives-Bauza, C., de Vries, R. L., Tocilescu, M. & Przedborski, S. PINK1/Parkin direct mitochondria to autophagy. *Autophagy* **6,** 315–316 (2010).
39. Klein, C. *et al.* PINK1, Parkin, and DJ-1 mutations in Italian patients with early-onset parkinsonism. *Eur. J. Hum. Genet.* **13,** 1086–1093 (2005).
40. Mills, R. D. *et al.* Biochemical aspects of the neuroprotective mechanism of PTEN-induced kinase-1 (PINK1). *J. Neurochem.* **105,** 18–33 (2008).
41. Jura, N. *et al.* Catalytic control in the EGF receptor and its connection to general kinase regulatory mechanisms. *Mol. Cell* **42,** 9–22 (2011).
42. Huse, M. & Kuriyan, J. The conformational plasticity of protein kinases. *Cell* **109,** 275–282 (2002).
43. Nolen, B., Taylor, S. & Ghosh, G. Regulation of protein kinases; controlling activity through activation segment conformation. *Mol. Cell* **15,** 661–675 (2004).
44. Cardona, F., Sanchez-Mut, J. V., Dopazo, H. & Perez-Tur, J. Phylogenetic and in silico structural analysis of the Parkinson disease-related kinase PINK1. *Hum. Mutat.* **32,** 369–378 (2011).
45. Kim, Y. *et al.* PINK1 controls mitochondrial localization of Parkin through direct phosphorylation. *Biochem. Biophys. Res. Commun.* **377,** 975–980 (2008).
46. Kawajiri, S. *et al.* PINK1 is recruited to mitochondria with parkin and associates with LC3 in mitophagy. *FEBS Lett.* **584,** 1073–1079 (2010).
47. Koh, H. & Chung, J. PINK1 and Parkin to control mitochondria remodeling. *Anatomy Cell Biol.* **43,** 179–184 (2010).
48. Chew, K. C. *et al.* Parkin mediates apparent E2-independent monoubiquitination *in vitro* and contains an intrinsic activity that catalyzes polyubiquitination. *PloS One* **6,** e19720 (2011).
49. Lim, K. L., Ng, X. H., Grace, L. G. & Yao, T. P. Mitochondrial dynamics and Parkinson's disease: focus on parkin. *Antioxid. Redox Signal.* **16,** 935–949 (2012).
50. Woodroof, H. I. *et al.* Discovery of catalytically active orthologues of the Parkinson's disease kinase PINK1: analysis of substrate specificity and impact of mutations. *Open Biol.* **1,** 110012 (2011).
51. Sim, C. H. *et al.* C-terminal truncation and Parkinson's disease-associated mutations down-regulate the protein serine/threonine kinase activity of PTEN-induced kinase-1. *Hum. Mol. Genet.* **15,** 3251–3262 (2006).
52. Silvestri, L. *et al.* Mitochondrial import and enzymatic activity of PINK1 mutants associated to recessive parkinsonism. *Hum. Mol. Genet.* **14,** 3477–3492 (2005).
53. Nakajima, A., Kataoka, K., Hong, M., Sakaguchi, M. & Huh, N. H. BRPK, a novel protein kinase showing increased expression in mouse cancer cell lines with higher metastatic potential. *Cancer Lett.* **201,** 195–201 (2003).
54. Sha, D., Chin, L. S. & Li, L. Phosphorylation of parkin by Parkinson disease-linked kinase PINK1 activates parkin E3 ligase function and NF-kappaB signaling. *Hum. Mol. Genet.* **19,** 352–363 (2010).
55. Lazarou, M., Jin, S. M., Kane, L. A. & Youle, R. J. Role of PINK1 binding to the TOM complex and alternate intracellular membranes in recruitment and activation of the E3 ligase Parkin. *Dev. Cell* **22,** 320–333 (2012).
56. Kitamura, T. *et al.* Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genomics. *Exp. Hematol.* **31,** 1007–1014 (2003).

Acknowledgements

We thank Dr. S. Yokoyama and Ms. M. Kanesaka for valuable suggestions, Dr. J. Shen for providing *PINK1*$^{-/-}$ MEF cells, Dr. T. Kitamura for PLAT-E cells, Dr. T. Iwatsubo for the PINK1 plasmid and Dr. N. Fujita for the mCAT1 plasmid. This work was supported by Grant-in-Aid for JSPS Fellows (to K.O., Grant Number 23-6061), Grant-in-Aid for Young Scientists (A) (to N.M., JSPS KAKENHI Grant Number 23687018), Grant-in-Aid for Scientific Research on Innovative Area 'Brain Environment' (to N.M., MEXT KAKENHI Grant Number 24111557), the Takeda Science Foundation (to H.K. and K.T.), Grant-in-Aid for Specially Promoted Research (to K.T., JSPS KAKENHI Grant Number 21000012) and Heath and Labor Science Research Grants (N.H. and K.T.).

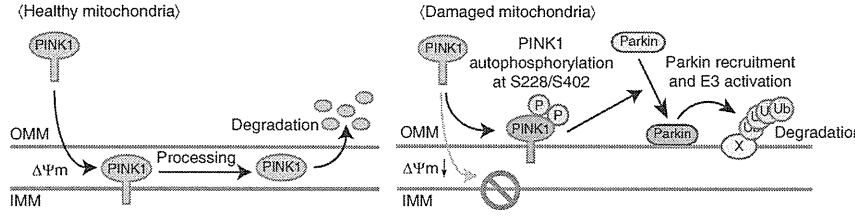

Figure 6 | A model for Parkin recruitment to the damaged mitochondria. The integrity of the mitochondria is sensed and transduced to Parkin by two sequential processes, that is the escape from membrane potential-dependent degradation and autophosphorylation of PINK1 at Ser228/Ser402. Both steps are essential for optimized Parkin recruitment and activation.

the pathogenic mutations severely compromised PINK1 autophosphorylation (Fig. 2a). Second, when the expression of exogenous PINK1 was under the control of the CMV(d1) promoter, Parkin mitochondrial localization coincided with PINK1 autophosphorylation (Fig. 5c,d). Third, a phosphorylation-deficient mutation of PINK1 (S228A/S402A) hindered the mitochondrial localization of Parkin, whereas a phosphorylation-mimic mutation of PINK1 (S228D/S402D) bypassed the necessity of PINK1 autophosphorylation for Parkin recruitment (Fig. 5c–g). Although the need for PINK1 autophosphorylation in recruiting Parkin can be suppressed by excessive over-production, we propose that PINK1 autophosphorylation under conditions such as dissipation of $\Delta\Psi$m in the presence of endogenous PINK1, or the controlled, appropriate expression of exogenous PINK1 is more physiologically relevant.

In the case of the MAP kinase extracellular signal-regulated kinase 2, its phosphorylation increases the k_{cat} by ~1,000-fold relative to the unphosphorylated form[43]. Although the physiological relevance of PINK1 autophosphorylation is unknown, we speculate that the phosphorylation of PINK1 increases the k_{cat} similar to extracellular signal-regulated kinase 2. This model is compatible with our observation described above that over-production of PINK1 suppresses the requirement of its phosphorylation.

As described in the Introduction, the basic mechanisms connecting mitochondrial damage to PINK1 accumulation have recently been elucidated[31]. The essence of the prevailing model is that PINK1 monitors mitochondrial damage via its escape from $\Delta\Psi$m-dependent import and degradation; namely, that dissipation of $\Delta\Psi$m prevents PINK1 from reaching the inner membrane, and as a consequence PINK1 remains localized to the outer mitochondrial membrane and tethered to the TOM complex[55]. However, herein we have experimentally demonstrated that while the above monitoring route is necessary for the discrimination of damaged mitochondria it might not be sufficient for fulfilling downstream events such as Parkin recruitment to mitochondria. Indeed, the low-level (but more than endogenous-level) accumulation of nonphosphorylated PINK1 on the outer membrane (for example, CMV(d1) promoter-driven PINK1(S228A/S402A) following CCCP treatment) cannot retrieve cytosolic Parkin efficiently, and autophosphorylation of PINK1 (for example, CMV(d1) promoter-driven PINK1(S228D/S402D) or endogenous PINK1 following CCCP treatment) is an essential step for optimized Parkin recruitment. We propose that the integrity of the mitochondria is sensed by the escape from $\Delta\Psi$m-dependent degradation and is transduced to Parkin via autophosphorylation of PINK1, as depicted in Fig. 6.

Methods

Plasmids. Plasmids used in this study are summarized in Supplementary Table S1. To generate the plasmid for weak PINK1 expression, the *Bgl*II–*Hin*dIII fragment within the CMV promoter was excised from pCMVTNT (Promega) and refilled with a 130-bp DNA fragment amplified using the following primers: 5′-TTGTTA GATCTCCAAAATCAACGGGACTTTCCAA-3′ and 5′-AGTGCCTCACGAC CAACTTCTG-3′ and digested with *Bgl*II and *Hin*dIII. As a result, the upstream 620 bp of the CMV promoter was deleted from pCMVTNT and the resulting plasmid was named pCMV(d1)TNT.

Cells. Various stable MEF transformants were established by infecting MEFs with recombinant retroviruses[6,56]. PINK1 and/or Parkin were cloned into a pMXs-puro vector. Retrovirus packaging cells PLAT-E[56] were transfected with the above vectors and were cultured at 37 °C for 24 h. After changing the medium, cells were further incubated at 37 °C for 24 h and the viral supernatant was collected and used for infection. MEFs were plated on 35-mm dishes at 24 h before infection, and the medium was replaced with the undiluted viral supernatant described above and 8 μg ml^{-1} polybrene (Sigma). After 2 days, transformants were selected in medium containing 5 μg ml^{-1} puromycin. HeLa cells stably expressing GFP-Parkin were established using recombinant retroviruses as well; however, as murine ecotropic retrovirus made by PLAT-E cells cannot infect HeLa cells, the retroviral receptor mCAT1 was transiently expressed in HeLa cells before viral infection. Plasmid transfection into HeLa cells and MEFs was performed using Fugene6 (Roche) and polyethylenimine (Polyscience), respectively. To depolarize the mitochondria, HeLa cells and MEFs were treated with 10 and 30 μM CCCP for 1–3 h.

Phos-tag assay and CIAP treatment. To detect phosphorylated proteins via PAGE, 7.5% polyacrylamide gels containing 50 μM phos-tag acrylamide (Wako chemicals) and 100 μM $MnCl_2$ were used. After electrophoresis, phos-tag acrylamide gels were washed with transfer buffer containing 0.01% SDS and 1 mM EDTA for 10 min with gentle shaking, and then replaced with transfer buffer containing 0.01% SDS without EDTA for 10 min according to the manufacturer's protocol. Proteins were transferred to polyvinylidene difluoride membranes and analysed by conventional immunoblotting. For CIAP treatment, a mitochondria-rich fraction (10 μg) collected by low-speed centrifugation from un-transfected or PINK1-3HA-transfected HeLa cells with CCCP treatment was incubated with 0, 10 or 30 U per reaction CIAP (Takara) for 1 h at the indicated temperature in reaction buffer (50 mM Tris–HCl pH 9.0, 1 mM $MgCl_2$, and 0.3 M trehalose), and then subjected to standard PAGE or phos-tag PAGE containing 25 μM phos-tag acrylamide (Wako Chemicals) and 50 μM $MnCl_2$ and then immunoblotted.

Immunofluorescence and immunoblotting. To detect the autoubiquitylation of GFP-Parkin by immunoblotting (IB), the cell lysate of HeLa cells or MEFs was collected in the presence of 10 mM *N*-ethylmaleimide to protect ubiquitylated Parkin from deubiquitylation. For immunofluoresecnce (IF) experiments, cells were fixed with 4% paraformaldehyde, permeabilized with 50 μg ml^{-1} digitonin and stained with the primary antibodies described below and the following secondary antibodies: mouse and/or rabbit Alexa Fluor 488, 568 and 647 (Invitrogen, 1:2,000 dilution). Cells were imaged using a laser-scanning microscope (LSM510; Carl Zeiss, Inc.). Image contrast and brightness were adjusted in Photoshop (Adobe). Antibodies used are as follows: anti-FLAG (M2 agarose; Sigma, 1:80 dilution) for immunoprecipitation, anti-actin (AC-40; Sigma, 1:1,000 dilution), anti-HA (4B2; Wako chemicals, 1:250 dilution), anti-Parkin (PRK8; Sigma-Aldrich, 1:1,500 dilution) and anti-PINK1 (BC100-494; Novus, 1:1,000 dilution) for IB, anti-GFP (3E6; Wako chemicals, 1:1,000 dilution), anti-PINK1 (BC100-494; Novus, 1:200 dilution and N4/15; NeuroMab, 1:200 dilution) and anti-Tom20 (FL-145, 1:3,000 dilution and F-10, 1:200 dilution; Santa Cruz Biotechnology) for IF. Statistical comparisons were made using analysis of variance with a Tukey–Kramer *post hoc* test in the JMP8.0.1 software (SAS Institute Inc.).

LC-MS/MS analysis of PINK1-GST. PINK1-GST from CCCP-treated and -untreated cells was subjected to SDS–PAGE and stained with CBB. PINK1-GST protein bands were excised, reduced, alkylated and digested with endoproteinase Asp-N (Roche) in 12.5 mM ammonium bicarbonate, pH 8.0, and 5 mM Tris–Cl for 16 h at 37 °C. The resultant peptides were analysed on an LTQ Orbitrap XL mass spectrometer (Thermo Scientific) with the raw data processed using Xcalibur (Thermo Scientific). The peak list files were searched against the NCBI nonredundant protein database restricted to *Homo sapiens* using the MS/MS ion search function of the Mascot search engine (Matrix Science). The 'Asp-N_ambic' enzyme parameter setting was selected for cleavage of peptide bonds N-terminally at both Asp and Glu residues.

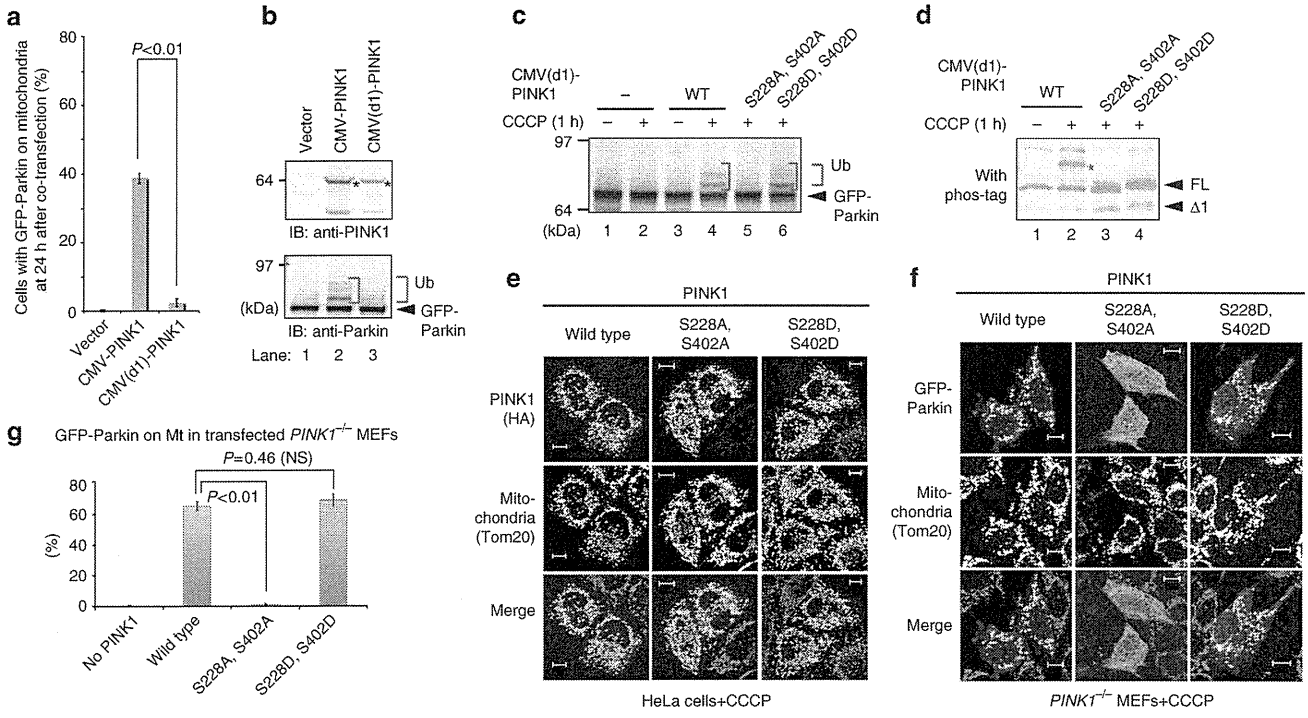

Figure 5 | PINK1 autophosphorylation of S228/S402 is essential for mitochondrial localization of Parkin. (**a**) HeLa cells were co-transfected with GFP-Parkin and CMV or CMV(d1) promoter-driven PINK1. The number of cells with Parkin-positive mitochondria was counted in >100 cells at 24 h post transfection. Error bars represent the mean±s.d. values of three experiments. Statistical significance was calculated using analysis of variance with a Tukey–Kramer *post hoc* test. (**b**) Immunoblotting with an anti-PINK1 antibody to measure the quantity of PINK1 (marked by asterisks) expressed by the CMV or CMV(d1) promoter, and autoubiquitylation activity of GFP-Parkin when the indicated PINK1 plasmids were co-transfected. The slower-migrating bands were derived from ubiquitylation (Ub). (**c**) *PINK1*$^{-/-}$ MEFs co-expressing GFP-Parkin and CMV(d1) promoter-driven PINK1 harboring the S228A/S402A or S228D/S402D mutation were subjected to immunoblotting with an anti-Parkin antibody. Ub shows autoubiquitylation of GFP-Parkin, which is taken as an indicator of its mitochondrial localization. (**d**) Both the S228A/S402A and S228D/S402D mutations hindered autophosphorylation of PINK1 in phos-tag PAGE. The asterisk indicates the phosphorylated form. FL and Δ1 mean full-length and N-terminal processed PINK1, respectively. (**e**) Subcellular localization of indicated PINK1 mutants in HeLa cells following CCCP treatment. Immunocytochemistry confirmed that both of the S228A/S402A and S228D/S402D PINK1 mutants localized on mitochondria following CCCP treatment. Bars, 10 μm. (**f**) Subcellular localization of GFP-Parkin in *PINK1*$^{-/-}$ MEFs co-expressing CMV(d1) promoter-driven PINK1 harboring the S228A/S402A or S228D/S402D mutation. Immunocytochemistry showing that the S228A/S402A PINK1 mutant disturbed the mitochondrial localization of Parkin, whereas the S228D/S402D mutant recruited Parkin to the mitochondria equivalent to WT PINK1. Bars, 10 μm. (**g**) The number of cells with Parkin-positive mitochondria was counted in >100 cells following CCCP treatment. Statistical analysis was performed as in (**a**).

that PINK1 is essential for the recruitment of Parkin to damaged mitochondria, the role of PINK1 kinase activity in Parkin recruitment remains to be fully addressed. Indeed, measurements of PINK1 kinase activity have been difficult and thus biochemical-based studies, especially *in vivo*, have not advanced enough. In this study, we addressed these problems and revealed several new findings.

The solubilization and purification of full-length PINK1 has been difficult and thus almost all studies, excluding one exception[50], of *in vitro* kinase activity have been done using a truncated PINK1 kinase domain[35,45,51–53]. Moreover, although several candidates have been reported[20,54], the physiological substrate(s) of PINK1 *in vivo* remains controversial; as a consequence the role of PINK1 kinase activity has not been fully elucidated.

In this study, we indicated that, once localized to depolarized mitochondria, PINK1 catalyses the intermolecular autophosphorylation of itself (Figs 1 and 2), although other modes of phosphorylation by another kinases cannot be ruled out completely. To study the significance of this autophosphorylation, we sought to identify the autophosphorylation site(s). Mutational analyses indicated that Ser228 and Ser402 are the PINK1 autophosphorylation sites. Moreover, direct evidence for *in vivo* phosphorylation by mass spectrometric analysis was obtained for Ser228 (Fig. 4). Although Muqit's lab[50] recently reported that insect (*Tribolium castaneum*) PINK1 catalysed autophosphorylation of Ser205 (equivalent to Ser228 in human PINK1) *in vitro*, our work provides the first direct evidence of autophosphorylation at Ser228 of PINK1 *in vivo*. Unfortunately, a peptide containing phospho-Ser402 was not identified in the MS analysis and thus the data are not as explicit as those of Ser228. However, mutational analyses suggest that both Ser228 and Ser402 are PINK1 phosphorylation sites (Figs 3a, 4e and 4f). Moreover, the PINK1 structural model predicts that these Ser residues are spatially very close and comprise a small patch on the surface, supporting the above conclusion (Fig. 4d and Supplementary Fig. S3).

It has been reported that when WT PINK1 or PINK1 continuously localized to mitochondria (OPA3-PINK1 chimera) is overexpressed in cells, Parkin localizes on the mitochondria irrespective of $\Delta\Psi m$[7,45,46]. On the other hand, autophosphorylation was not induced by over-production (Fig. 1), but did require a decrease in $\Delta\Psi m$. This result seemingly suggests that autophosphorylation of PINK1 is not important for Parkin recruitment to mitochondria. However, we propose that autophosphorylation is indeed significant for three reasons. First, based on the phosphorylation status shown in Fig. 1f,g, almost all of the endogenous PINK1 is phosphorylated in a loss of $\Delta\Psi m$-directed manner. Moreover, a majority of

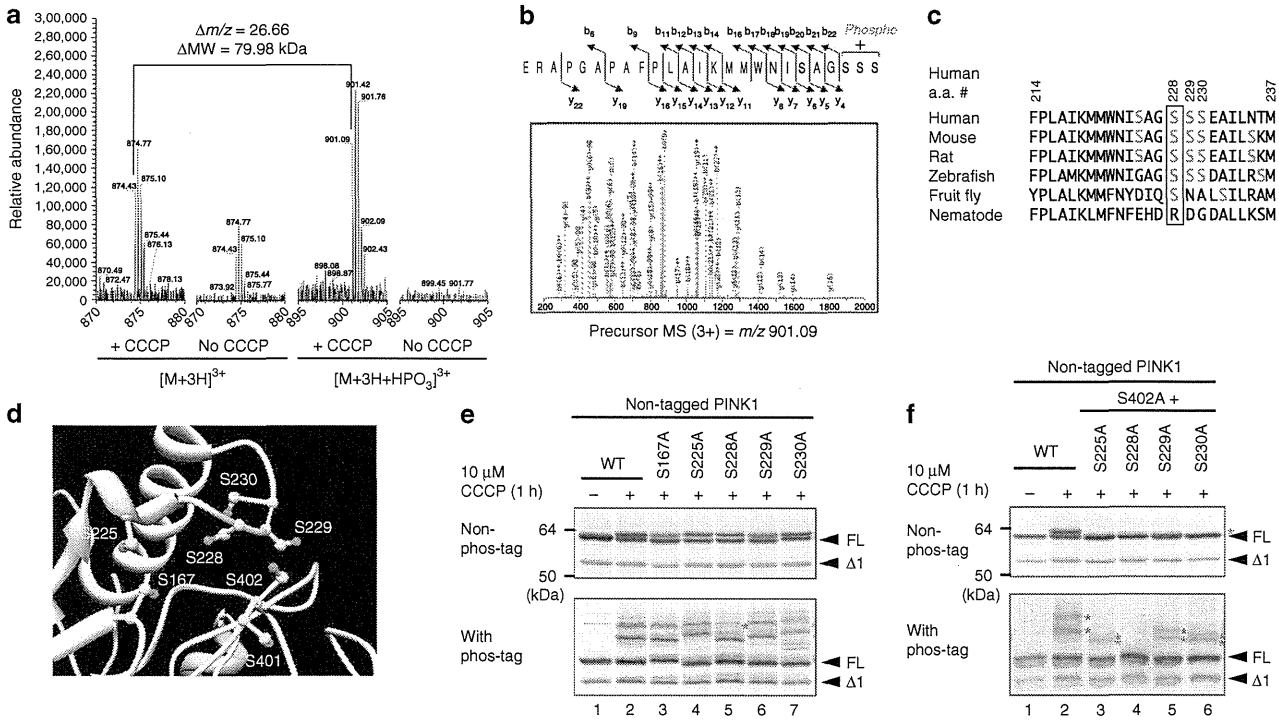

Figure 4 | S228 is another autophosphorylation site for PINK1. (**a**) Mass spectrometric analysis of the *in vivo* autophosphorylation site of PINK1. PINK1-GST purified from cells +/−CCCP treatment was subjected to LC-MS/MS analysis with a phosphorylated peptide equivalent to amino acids 206–230 detected only from CCCP-treated cells. (**b**) The MS/MS data suggested that phosphorylation occurs at Ser228, Ser229 or Ser230. (**c**) Multiple sequence alignment of PINK1 residues neighboring Ser228 from various organisms. Ser228 (boxed) has been evolutionarily conserved across most species. (**d**) Structural model (Protein Model Database ID: PM0076345) of PINK1 revealed that the possible phosphorylation sites including S228 and S402 are spatially close to one another. Ser residues are shown in yellow and hydroxyl groups are highlighted in red. (**e**) The S228A mutation changed the phosphorylation pattern of PINK1. The first band (shown by a red asterisk) in phos-tag PAGE is not observed in cell expressing the S228A mutation. (**f**) Autophosphorylation-derived signals of PINK1 in cells expressing the S228A/S402A double mutation are completely abolished. Asterisks indicate the phosphorylated form.

experimental system in which Parkin does not migrate to the healthy mitochondria even in the presence of exogenously expressed PINK1. When WT PINK1 was expressed under a strong CMV promoter, a significant amount of co-transfected GFP-Parkin was recruited to the mitochondria even without CCCP treatment (Fig. 5a, the second bar). Therefore, we modified the CMV promoter to reduce expression and found that deletion of the enhancer region in the plasmid (referred to as CMV(d1) hereafter) significantly attenuated PINK1 expression (anti-PINK1 panel of Fig. 5b, compare lane 2 with 3). With this attenuated CMV(d1) promoter, at 24 h post transfection, we observed almost no recruitment of co-expressed GFP-Parkin to mitochondria by the WT PINK1 (Fig. 5a, the third bar). We also checked the autoubiquitylation status of GFP-Parkin, a good index of its mitochondrial localization[6], and confirmed that GFP-Parkin exhibits autoubiquitylation when co-expressed with PINK1 under the control of a CMV promoter, whereas the autoubiquitylation was hardly observed with PINK1 under the control of the CMV(d1) promoter (anti-Parkin panel of Fig. 5b). Moreover, when *PINK1*$^{-/-}$ MEFs co-expressing GFP-Parkin and CMV(d1) promoter-driven PINK1 were treated with CCCP, autoubiquitylation of GFP-Parkin was clearly observed only following CCCP treatment (Fig. 5c, lane 4), suggesting that a defined expression level of exogenous PINK1 enables Parkin to localize specifically to depolarized mitochondria.

We next determined the effect of phosphorylation-deficient (Serine-to-Alanine) mutations or phosphorylation-mimic (Serine-to-Aspartic acid) mutations of Ser228 and Ser402 on Parkin recruitment. When PINK1 with either the S228A/S402A or the S228D/S402D double mutation was subjected to phos-tag PAGE, the autophosphorylation-derived PINK1 bands disappeared in both cases because the phosphorylation sites were missing (Fig. 5d). We then examined the Parkin-recruitment activity of these mutants. Importantly, the S228A/S402A mutant was unable to restore autoubiquitylation of GFP-Parkin in *PINK1*$^{-/-}$ MEFs (Fig. 5c, lane 5), whereas the S228D/S402D mutant complemented it (Fig. 5c, lane 6), although neither underwent autophosphorylation (Fig. 5d). We confirmed that the PINK1 S228A/S402A mutant localized on mitochondria following CCCP treatment as well as WT PINK1 (Fig. 5e). Immunocytochemistry of Parkin further demonstrated that the PINK1 S228D/S402D mutant promoted mitochondrial localization of Parkin equivalent to WT PINK1, whereas the PINK1 S228A/S402A mutant failed to recruit Parkin onto mitochondria (Fig. 5f,g). The results shown in Fig. 5 reveal that autophosphorylation of PINK1 is indeed important for recruiting Parkin onto damaged mitochondria, and is the first evidence that autophosphorylation of PINK1 has a crucial role in the PINK1/Parkin pathway.

Discussion

Recently, our understanding of how PINK1 and Parkin function has undergone a significant transformation that continues to evolve. We can accurately state that PINK1 and Parkin function in maintaining mitochondrial integrity by cooperatively working together to identify and label damaged mitochondria via ubiquitylation (reviewed by refs 3,31–33,47). Biochemically, Parkin functions as an E3 enzyme that catalyses the transfer of ubiquitin from E1/E2~ubiquitin to the substrate[48,49], whereas PINK1 is thought to possess Ser/Thr kinase activity. Although we and other investigators demonstrated