

Characterization of PINK1 (PTEN-induced Putative Kinase 1) Mutations Associated with Parkinson Disease in Mammalian Cells and *Drosophila**

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Background: Mutations in PINK1 cause recessive Parkinson disease.

Results: PINK1 mutations in the kinase domain hamper Parkin translocation to mitochondria, and their analogous mutations in *Drosophila* cannot rescue *PINK1*-null phenotypes.

Conclusion: PINK1 kinase activity is essential for its function and for regulating Parkin functions in mitochondria.

Significance: Understanding the roles of PINK1 mutations will be helpful for deciphering the pathogenic mechanism of PINK1-linked Parkinson disease.

Mutations in PINK1 (PTEN-induced putative kinase 1) are tightly linked to autosomal recessive Parkinson disease (PD). Although more than 50 mutations in PINK1 have been discovered, the role of these mutations in PD pathogenesis remains poorly understood. Here, we characterized 17 representative PINK1 pathogenic mutations in both mammalian cells and *Drosophila*. These mutations did not affect the typical cleavage patterns and subcellular localization of PINK1 under both normal and damaged mitochondria conditions in mammalian cells. However, PINK1 mutations in the kinase domain failed to translocate Parkin to mitochondria and to induce mitochondrial aggregation. Consistent with the mammalian data, *Drosophila* PINK1 mutants with mutations in the kinase domain (G426D and L464P) did not genetically interact with Parkin. Furthermore, PINK1-null flies expressing the transgenic G426D mutant displayed defective phenotypes with increasing age, whereas L464P mutant-expressing flies exhibited the phenotypes at an earlier age. Collectively, these results strongly support the hypothesis that the kinase activity of PINK1 is essential for its function and for regulating downstream Parkin functions in mitochondria. We believe that this study provides the basis for understanding the molecular and physiological functions of various PINK1 mutations and provides insights into the pathogenic mechanisms of PINK1-linked PD.

Parkinson disease (PD)³ is the second most common neurodegenerative disease characterized by dopaminergic (DA) neuron degeneration in the substantia nigra pars compacta, accompanied with locomotor defects (1). In most cases, PD occurs sporadically as a result of many different environmental risk factors; mutations in a number of genes can also cause familial forms of PD. Identification of the genes associated with parkinsonism has had a major impact on PD research, facilitating the dissection of the molecular mechanisms implicated in the pathogenesis of PD.

Since the discovery of PINK1 mutations in familial parkinsonism, ~50 different PD-linked PINK1 mutations have been identified in diverse populations (2–8). The human PINK1 is a ubiquitously expressed 581-amino acid protein with an N-terminal mitochondrial targeting sequence (MTS), a transmembrane domain (TM), and a highly conserved Ser/Thr kinase domain with homology to the Ca²⁺/calmodulin-dependent protein kinase family (2, 9, 10).

Three forms of PINK1 protein are detected in normal cell culture conditions: the full-length form (~63 kDa) and two cleaved forms (~55 and ~45 kDa). Mitochondrially localized PINK1 is processed at the N terminus by mitochondrial processing peptidase (11). In addition, studies in *Drosophila* and mammalian systems indicate that human PINK1 is cleaved between amino acids Ala-103 and Phe-104 by presenilin-associated rhomboid-like protease (12–15). Interestingly, the activity of presenilin-associated rhomboid-like protease is regulated by the mitochondrial membrane potential ($\Delta\psi_m$) (12, 13, 16). In healthy mitochondria, PINK1 is guided to the mitochondrial inner membrane through the general mitochondrial import

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³ The abbreviations used are: PD, Parkinson disease; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazine; *hs*, heat shock; *gmr*, glass multiple reporter; DA, dopaminergic; TH, tyrosine hydroxylase; MTS, mitochondrial targeting sequence; TM, transmembrane domain; MEF, mouse embryonic fibroblast; SEM, scanning electron microscope; h, human; d, *Drosophila*; FL, full-length protein.

machinery, followed by proteolytic processing by presenilin-associated rhomboid-like protease and rapid degradation, resulting in low levels of endogenous PINK1. In contrast, full-length PINK1 is accumulated at the outer mitochondrial membrane upon treatment with the mitochondrial uncoupler, carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), which dissipates the $\Delta\psi_m$ (13, 15–17).

Previously, we and others showed that PINK1 functions in a common pathway with Parkin to maintain mitochondrial integrity and function. Remarkably, *PINK1* and *Parkin* mutant flies exhibit similar phenotypes, including mitochondrial defects, muscle degeneration, locomotor defects, disrupted spermatogenesis, and a reduced number of DA neurons (18–21). Moreover, in cultured cells, Parkin overexpression compensates for mitochondrial dysfunction induced by PINK1 deficiency, indicating that Parkin acts downstream of PINK1 and that this linear pathway is well conserved in both mammals and *Drosophila* (22–25).

Evidence linking mitochondria and PD has steadily grown (26–29). Mitochondria are highly dynamic organelles that can change their shape, size, and subcellular localization depending on the cellular environment. These dynamic processes are regulated by fusion, fission, and transport, all of which are also linked to the maintenance of proper mitochondrial functions. There has been controversy over whether the PINK1/Parkin pathway modulates mitochondrial fusion or fission. Cells derived from PINK1-defective patients have small fragmented mitochondria (30, 31), whereas PINK1 and Parkin overexpression promote mitochondrial aggregation in mammalian cells (32). In *Drosophila*, PINK1 loss of function results in swelling or enlargement of mitochondria, and these defective phenotypes of the *dpINK1*-null flies are strongly suppressed by the overexpression of Drp1 (dynamin-related protein 1), which promotes mitochondrial fission, or the down-regulation of Opa1 (optic atrophy 1) or Marf (mitochondrial assembly regulatory factor), which promote fusion of the inner mitochondrial or the outer mitochondrial membrane, respectively (33–36).

Recent studies have also shown that the PINK1/Parkin pathway is critical for mitophagy. Parkin is translocated to depolarized mitochondria upon treatment with CCCP, and Parkin-labeled mitochondria are subsequently eliminated by autophagy (mitophagy) (16, 17, 37–39). Parkin ubiquitinates mitochondrial proteins at the outer membrane and facilitates the recruitment of adaptor proteins, such as p62, which links ubiquitinated cargo to the autophagic machinery (40–43).

Despite this recent progress, the mechanism of PINK1-linked PD pathogenesis still remains elusive. In this study, to investigate the consequences of PINK1 mutations, we selected 17 representative missense mutations and analyzed their effects on proteolytic processing and subcellular distribution of PINK1 and on Parkin mitochondrial translocation. Unexpectedly, PINK1 mutants exhibited normal protein stability and expression patterns and did not exhibit altered subcellular distributions under normal and damaged mitochondria conditions. However, Parkin was not localized to mitochondria in cells harboring PINK1 mutations within the kinase domain. These results were further confirmed in the *Drosophila* system. Taken

together, the results of this study, in which we systemically analyzed the effect of PINK1 mutations in both *in vitro* and *in vivo* model systems, improve our understanding of how PINK1 mutations contribute to the pathogenesis of PD.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Anti-rabbit PINK1 (Novus Biologicals), anti-mouse or anti-rabbit Myc (DSHB, Cell Signaling Technology), anti-rat HA (Roche Applied Science), anti-mouse β -tubulin (DSHB), and anti-mouse tyrosine hydroxylase (Immunostar) antibodies were used for immunocytochemistry and immunoblot analyses. Anti-mouse MTC02 (Abcam) or anti-rabbit TOM20 (Santa Cruz) antibody and MitoTracker® Red CMXRos (Invitrogen) were used to visualize mitochondria. CCCP is purchased from Calbiochem. Toluidin blue (Sigma) and Alexa 488-conjugated streptavidin (Invitrogen) were used for staining of muscle fibers and mitochondria in fly tissue.

Generation of *hPINK1* Mutants—For site-directed mutagenesis, a QuikChange™ kit (Stratagene) was used. For generation of mutations, a pcDNA3.1 zeo (+) *hPINK1* WT 3×Myc construct was used as a template. Information about the primers used in the experiments is available upon request.

Mammalian Cell Culture and Transfection—HeLa, HEK293T, and PINK1 KO mouse embryonic fibroblasts (MEFs) were grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) at 37 °C in a humidified atmosphere of 5% CO₂. The transfection of expression plasmids was performed using Lipofectamine Plus reagent (Invitrogen) or PEI (Sigma) according to the manufacturer's instructions.

Preparation of Lysates and Immunoblots—Cell and fly lysates were prepared in lysis buffer A (20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 2 mM EGTA, 50 mM β -glycerophosphate, 50 mM NaF, 1 mM sodium vanadate, 2 mM DTT, 1 mM PMSF, 10 g/ml leupeptin, 1 g/ml pepstatin A, 1% Triton X-100) and subjected to immunoblots according to standard procedure. The blots were developed and visualized using LAS-4000 (Fuji Film).

Immunocytochemistry—For immunocytochemistry, HeLa and PINK1 KO MEF cells were subcultured on 12-well culture plate coated with poly-L-lysine (Sigma). Appropriately treated cells were washed one time with PBS and were fixed in 2% paraformaldehyde for 15 min, followed by permeabilization with 0.5% Triton X-100 in PBS for 5 min. Then the cells were washed with 0.1% Triton X-100 in PBS (PBS-T) and incubated in blocking solution (4% BSA and 1% normal goat serum in PBS-T) for 1 h. Primary antibodies were added to the blocking solution, and the cells were incubated for overnight at 4 °C. After washing with PBS-T three times, the cells were incubated with appropriate secondary antibodies in blocking solution for 45 min at room temperature. The antibody-labeled cells were washed with PBS-T six times and were mounted with mounting solution (100 mg/ml 1,4-diazabicyclo[2.2.2]octane in 90% glycerol). The slides were observed with a LSM710 laser scanning confocal microscope (Carl Zeiss). All of the immunostaining experiments with HeLa cells were conducted at least three times ($n = 300$).

TABLE 1**Summary of *PINK1* mutations in PD patients**

NR, not reported.

Domain	Nucleotide change	Location (Exon)	Amino acid substitution	Mean onset ages	Frequency	Inheritance	Population origin
MTS	g.155510C>T	Exon1	P52L	Unclear	One family	NR	Caucasian
	g.15558_15559GC>CT	Exon1	R68P	Unclear	One family	Nonfamilial	Caucasian
TM	g.155630G>T	Exon1	C92F	37.0y	One family	Nonfamilial	Caucasian
	g.155728T>G	Exon1	C125G	33.7y	One family	familial	Caucasian
	g.155732A>C	Exon1	Q126P	38.0y	One family	familial	Caucasian
	g.159701G>A	Exon2	R147H	51.0y	One family	familial	Caucasian
Kinase domain	g.159763G>C	Exon2	A168P	34.5y	Two families	Nonfamilial	Caucasian
	g.159848C>T	Exon2	P196L	45.0y	Two families	familial	Caucasian
	g.166446G>A	Exon4	G309D	Unclear	One family	familial	Caucasian
	g.116458C>T	Exon4	T313M	32.0y	3 families	familial	Asian
	g.167447T>C	Exon5	L347P	33.2y	3 families	familial	Asian
	g.170345G>C	Exon6	G386A	21.0y	One family	familial	Caucasian
	g.170414G>T	Exon6	G409V	32.0y	One family	Nonfamilial	Caucasian
	g.170830C>A	Exon7	A427E	Unclear	Two families	NR	Caucasian
	g.172314A>C	Exon8	N521T	31.8y	Not pathogenic ^a		Caucasian
	g.172325G>A	Exon8	D525N	Unclear	Two families	Nonfamilial	Caucasian
C-terminal domain	g.172475T>C	Exon8	C575R	62.0y	One family	Nonfamilial	Caucasian

^a The N521T mutation was reported to be a nonpathogenic type of mutation.

Fly Stocks—We have previously generated *dPINK1*^{B9}, *UAS-dPINK1*^{WT}, and *UAS-dPINK1*^{3KD} line (18, 33). *UAS-dParkin* line was previously described (44). The following fly stocks were obtained from the Bloomington Stock Center: *hs-gal4* and *gmr-gal4*. All flies were grown on standard cornmeal-yeast-agar medium at 25 °C. Details of the genotypes used in the experiments are available upon request.

Generation of *Drosophila PINK1* Transgenic Mutants—For generation of G426D and L464P *dPINK1* mutant transgenic lines, a QuikChangeTM kit (Stratagene) was used. *dPINK1* GD and LP DNAs were subcloned into HA-tagged pUAST vector and injected into *w*¹¹¹⁸ embryos.

Eye Phenotypes and SEM Analysis—Flies were crossed and maintained at 25 °C. SEM images were analyzed by SUPRA 55VP (Carl Zeiss) in a variable pressure secondary electron mode.

Muscle Section and Mitochondria Staining—The samples embedded in Spurr's resin were trimmed and sectioned from the lateral side of the thorax (at a thickness of 4 mm and between 200 and 350 mm in depth), and the serial sections were then stained with toluidine blue dye. For streptavidin staining, fly thoraces after fixation were cut in half by dissecting vertically along the bristles in the middle of the thorax. Approximately 10 thoraces of 3- or 45-day-old flies were observed in light microscopy (Leica) for each genotype.

Quantification of Wing Phenotypes of Flies—For quantification, the percentages of male flies with normal wings at 3 or 45 days were measured (*n* > 100).

ATP Assay—ATP assays were carried out as previously described (33). The quantitative levels of ATP were measured (*n* > 5).

Behavioral Assays—Flight assays were performed as previously described with 3- or 45-day-old males (*n* > 30) (33).

TH Immunostaining and Quantification of DA Neurons—45-day-old adult fly brains were fixed with 4% paraformaldehyde and stained with anti-TH antibody as described previously (18). The samples were observed and imaged by LSM 710 confocal microscope (Carl Zeiss). TH-positive neurons were counted under blinded conditions. For quantification of DA neurons, the first dorsolateral regions from 15 brains of each

genotype were observed in a blind fashion to eliminate bias (*n* = 30).

Statistical Analysis—The statistical analyses were performed using Student's *t* test for related samples. The *p* values calculated were pooled from three independent experiments.

RESULTS

Selection of 17 *PINK1* Mutations—To understand how PD-linked mutations affect the function of *PINK1* protein, we selected 17 missense mutations based on the high frequency of disease onset in patients carrying these mutations, the conservation of these amino acids in *PINK1* across species, and the importance of these conserved residues in maintaining protein structure and *PINK1* catalytic activity (Table 1) (6, 45–52).

A majority of *PINK1* mutations are observed in the Ser/Thr kinase domain, suggesting that loss of the kinase activity plays a crucial part in the pathogenesis of *PINK1*-linked PD (Fig. 1A) (53–55). The A168P, P196L, G309D, T313M, L347P, G386A, G409V, and A427E mutations were selected to represent the mutations within the kinase domain in this study. In particular, G309D and L347P have been the most highly studied to have reduced kinase activity and protein stability, respectively (54). The Thr-313 residue was previously reported to be a phosphorylation site for microtubule affinity-regulating kinase 2 (MARK2/Par-1), and the T313M mutant exhibited defects in mitochondrial transport (56). The A168P, P196L, G386A, G409V, and A427E mutations were also reported to have effects on the kinase structure (57). An artificial kinase dead mutant (3KD; K219A/D362A/D384A) was used as a control in this study (54).

Mutations outside of the kinase domain were also selected. The P52L and R68P mutations within the MTS region were included in this study to determine the function of the MTS. Also, TM region mutants, C92F, C125G, Q126P and R147H, were selected to delineate the role of the TM region in *PINK1* function. The C terminus of *PINK1* has no homology to any known proteins; however, there are several reports that mutants with a truncated C terminus are associated with reduced *PINK1* kinase activity (55, 58–60).

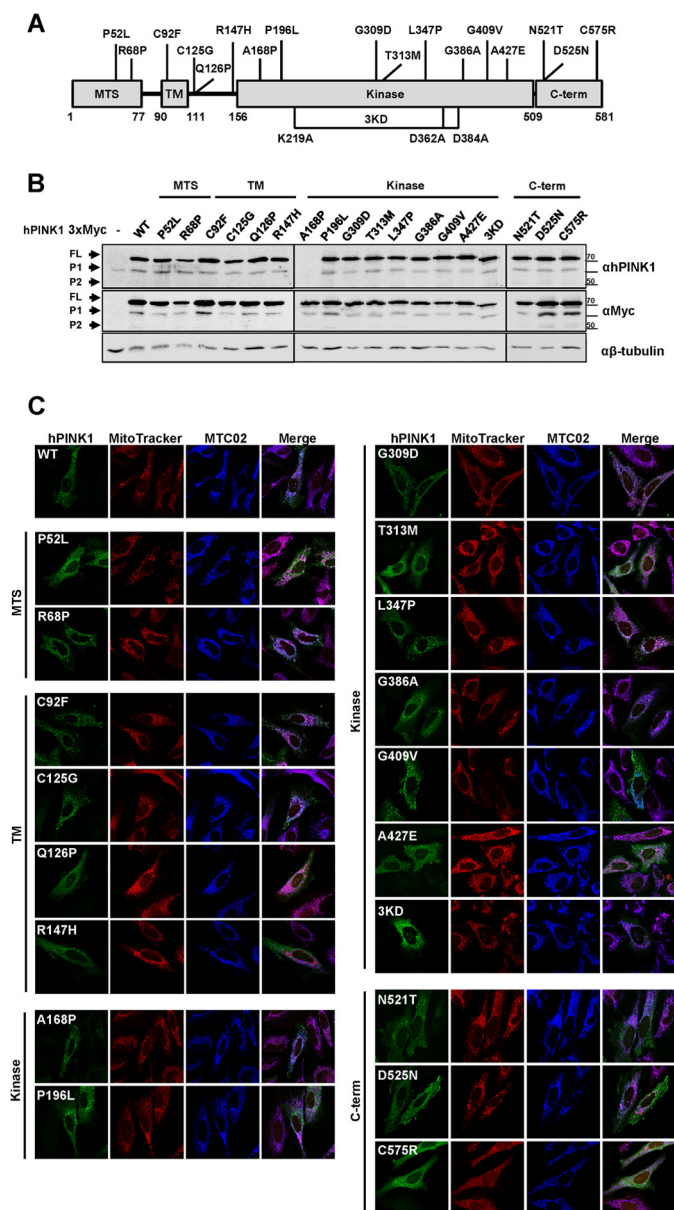


FIGURE 1. Expression and subcellular localization patterns of PINK1 patient mutants. *A*, schematic representation of the domain structure for hPINK1. The positions of patient mutations and artificial kinase dead mutations (3KD) are indicated on each domain. All of the depicted mutants were generated in the pcDNA3.1 zeo (+) hPINK1 3× Myc construct. The numbers refer to the amino acid positions of hPINK1. *B*, immunoblot analysis of the PINK1 mutations in HeLa cells. Various PINK1 constructs were expressed, and their lysates were assessed by immunoblot using anti-hPINK1 or (top panel) anti-Myc antibodies (middle panel). Three forms of PINK1 protein (FL; 72 kDa) and two processed forms (P1, 63 kDa; and P2, 54 kDa) are indicated with arrows. β-Tubulin was used as a loading control (bottom panel). Dividing lines indicate that samples were resolved on separate gels. Representative blots from three independent experiments are shown. *C*, confocal images of the subcellular localizations of hPINK1 mutants in HeLa cells. After 24 h of transfection, the cells were fixed and subjected to immunocytochemistry as described under "Experimental Procedures." Myc-tagged hPINK1 proteins were immunolabeled with Myc antibody (green), and the mitochondria were double-labeled using MitoTracker® Red CMXRos (red) and MTC02 antibody (blue). Original magnification, ×800. C-term, C-terminal.

Therefore, all of the known missense mutations in the C-terminal domain, N521T, D525N, and C575R, were also included in this study.

The cDNA for human PINK1 (hPINK1) containing 3× Myc tag at the C terminus was inserted into the pcDNA 3.1 zeo (+) vector, and all the mutations were generated in this construct using site-directed point mutagenesis method. It is well known that the C terminus-tagged PINK1 protein is detected as three protein bands in SDS-PAGE gels: the full-length protein (FL) and two processed proteins (P1 and P2) (61). Because of the triple Myc tag, FL, P1, and P2 of the WT hPINK1 can be detected as ~72, 63, and 54 kDa-sized proteins, respectively, in HeLa (Fig. 1*B*) and HEK293T cell lines (data not shown).

Expression and Subcellular Localization Patterns of PINK1 Mutants under Normal Conditions—First, we examined the proteolytic processing patterns and protein stability of PINK1 mutants (Fig. 1*B*). Each mutant construct was transfected into HeLa cells, and whole cell lysates were subjected to immunoblot analysis using anti-human PINK1 or -Myc antibodies. None of the mutations altered the typical triplet pattern of PINK1 protein bands. The A168P mutant was not detected on using the anti-human PINK1 antibody because the mutation disrupts its antigenic structure of amino acids 140–170 (Fig. 1*B*, lane 9). The presence of the typical cleavage pattern and stability in the A168P mutant was confirmed by immunoblotting using an anti-Myc antibody. Only the 3KD mutant showed a fast mobility shift, as previously shown (54). It is worth noting that the L347P mutation was reported to be unstable, because it does not bind to the Hsp90-Cdc37 chaperone complex (54, 62, 63). However, the L347P hPINK1 protein was as stable as the other mutants in our experimental conditions, even though the exogenously expressed L347P mutant interacted with the Hsp90-Cdc37 complex weakly (data not shown).

Given that PINK1 is targeted to mitochondria through its N-terminal MTS domain (64), we next assessed the subcellular distributions of hPINK1 mutants (Fig. 1*C*). Mitochondria were double labeled using MitoTracker, which accumulates in the mitochondria that have an intact $\Delta\psi_m$, and the MTC02 antibody, which recognizes a nonglycosylated protein component of mitochondria found in human cells. The WT hPINK1 protein was localized in the cytoplasm, and it showed small punctate patterns that co-localized with mitochondria. All the mutants showed a localization pattern similar to that of WT hPINK1. Thus, the PINK1 patient mutations selected in this study had no significant effects on the proteolytic processing, stability, and subcellular localization of PINK1.

PINK1 Mutants Are Targeted to Mitochondria and Stabilized by CCCP Treatment—Recent studies suggested that the reduction of $\Delta\psi_m$ by the mitochondrial uncoupler CCCP leads to the accumulation of endogenous PINK1 (FL form) on mitochondria, and eventually results in Parkin-mediated mitophagy (16, 17, 37–39, 65). Therefore, we assessed the effects of PINK1 mutations on these processes in damaged mitochondria. Each PINK1 expression construct was expressed in HeLa cells, which were then treated with CCCP (20 μ M for 2 h), and subjected to immunocytochemical analysis. When cells were treated with CCCP, fragmented mitochondria significantly increased, and MitoTracker signals decreased because of the reduction in $\Delta\psi_m$. The WT hPINK1 protein was mostly localized to punctate structures in CCCP-treated cells, compared with that in DMSO-treated cells (Fig. 2*A*). These punctate were co-local-

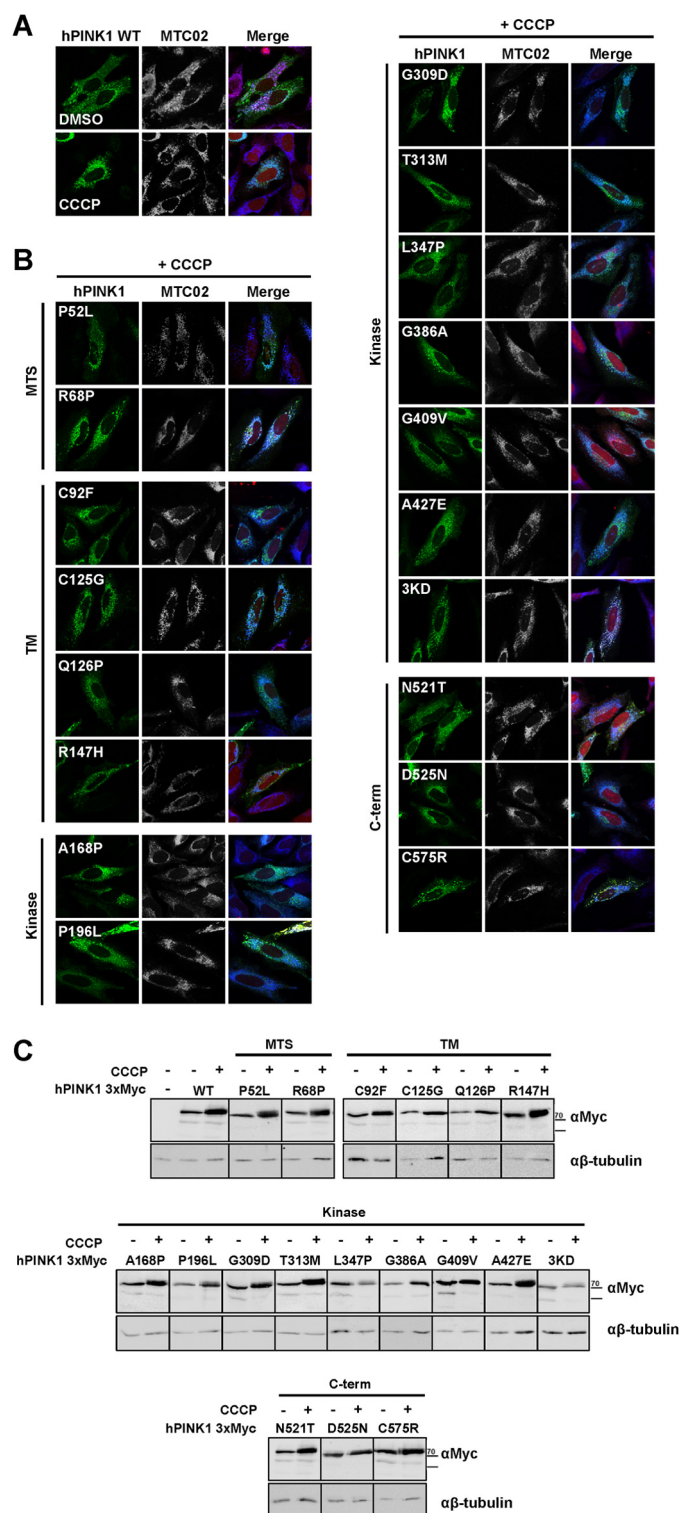


FIGURE 2. hPINK1 mutants are accumulated on mitochondria under damaged mitochondria conditions. *A*, confocal images of the subcellular localizations of WT hPINK1 in HeLa cells. After 24 h transfection, the cells were treated with DMSO (top panels) or CCCP (bottom panels, 20 μ M for 2 h), fixed, and subjected to immunocytochemistry. hPINK1 proteins were immunolabeled with Myc antibody (green), and the mitochondria were double-marked using MitoTracker[®] Red CMXRos (red) and MTC02 antibody (white, blue in merged images). Original magnification, $\times 800$. *B*, confocal images of hPINK1 mutants in HeLa cells under CCCP treatment (20 μ M for 2 h). Original magnification, $\times 800$. *C*, immunoblot analysis of PINK1 mutants under CCCP treatment. Each construct was expressed in HeLa cells, treated with CCCP (20 μ M for 4 h), and then subjected to immunoblot analysis (upper panel) with

ized with mitochondria, which were stained with an anti-MTC02 antibody. Interestingly, hPINK1 mutants showed similar distribution patterns under the low $\Delta\psi_m$ condition in HeLa (Fig. 2*B*) and PINK1-null MEFs cell lines (data not shown). In addition, as previously reported, CCCP induced the accumulation of FL-hPINK1 protein on mitochondria (17, 37–39), and all the missense mutants and 3KD mutants showed the same pattern as that of FL-PINK1 after CCCP treatment (Fig. 2*C*). In conclusion, we could not detect any significant differences between the subcellular localizations of the WT and mutant hPINK1 proteins under both normal and damaged mitochondria conditions.

Parkin Translocation to Mitochondria Is Impeded in Cells Expressing PINK1 Kinase Mutants—To investigate the effect of mutations on PINK1 activity, we used a specific downstream target of PINK1, Parkin, a product of the *PARK2* gene. Exogenously expressed Parkin was distributed evenly throughout the cytoplasm and nucleus in HeLa cells. However, co-expression of WT hPINK1 and Parkin dramatically induced Parkin translocation to mitochondria and generated highly aggregated mitochondria around the perinuclear region (Fig. 3*A*) (32). However, Parkin remained in the cytoplasm when co-expressed with the 3KD hPINK1 (Fig. 3*A*).

To examine the effect of the PINK1 mutants on Parkin translocation, we co-expressed each hPINK1 mutant with Parkin and then conducted immunocytochemical analyses (Fig. 3, *B* and *C*). Interestingly, co-expression of hPINK1 proteins carrying most of the mutations within the PINK1 kinase domain was unable to completely promote the mitochondrial localization of Parkin. In addition, the C125G and Q126P mutants within the TM region failed to recruit Parkin to mitochondria. However, when PINK1 mutants within the MTS and C terminus region were co-expressed with Parkin, they successfully induced both Parkin localization in mitochondria and mitochondrial aggregation. These experiments were also reproduced in different cell lines, including COS-1 cells and PINK1-null MEFs (data not shown). Our results suggest that PINK1 acts upstream of Parkin in regulating Parkin mitochondrial localization and consequent mitochondrial aggregation, in a process dependent on its kinase activity.

Generation of Drosophila PINK1 Kinase Mutants Analogous to PD-linked Human Mutations—We subsequently investigated whether these PD-linked PINK1 mutations have similar effects on Parkin *in vivo*, utilizing the *Drosophila* model system. hPINK1 and *Drosophila* PINK1 (dPINK1) protein structures are well conserved (Fig. 4*A*). Among the kinase mutants, transgenic flies that express G426D (analogous to human G309D) and L464P (analogous to human L347P) were generated because of the high pathogenic relevance of the corresponding mutations in humans, and 3KD (K337R/D479R/D501A) kinase dead mutant flies were included as controls (Fig. 4*B*).

anti-Myc antibody. β -Tubulin was used as a loading control (bottom panel). Dividing lines indicate that samples were resolved on separate gels. Representative blots from three independent experiments are shown. *C-term*, C-terminal.

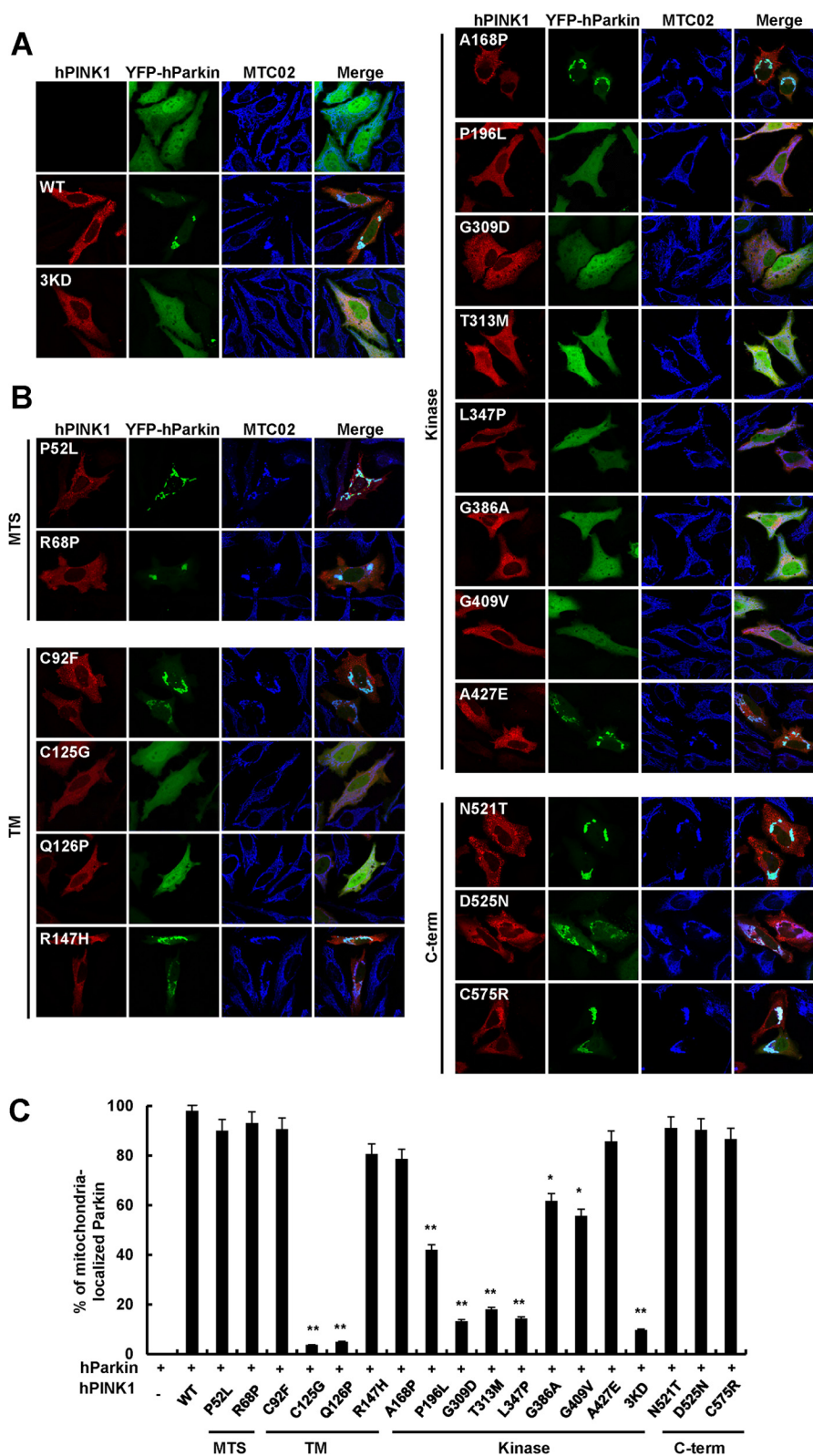


FIGURE 3. Mutations within PINK1 kinase domain mitigate the translocation of Parkin to mitochondria. *A* and *B*, confocal images of the subcellular localizations of hParkin co-expressed with indicated hPINK1 mutants. HeLa cells were co-transfected with YFP hParkin (green) and each hPINK1 mutant construct (red). Mitochondria were labeled with MTC02 antibody (blue). Original magnification, $\times 800$. *C*, quantification of cells with mitochondria-localized Parkin in the absence and presence of WT hPINK1 or mutants. *, $p < 0.005$; **, $p < 0.001$ (Student's *t* test for related samples). The error bars indicate the S.D. of three experiments ($n = 300$). *C-term*, C-terminal.

There Is No Significant Genetic Interaction between dParkin and the dPINK1 Kinase Mutants—To investigate the genetic interaction between dParkin and dPINK1 mutants, we ex-

pressed constructs encoding dParkin and dPINK1 mutants in adult fly eye tissue using the eye-specific *gmr-gal4* driver. Rough eye phenotypes and disarrayed ommatidia were

Analysis of PD-causing PINK1 Mutations

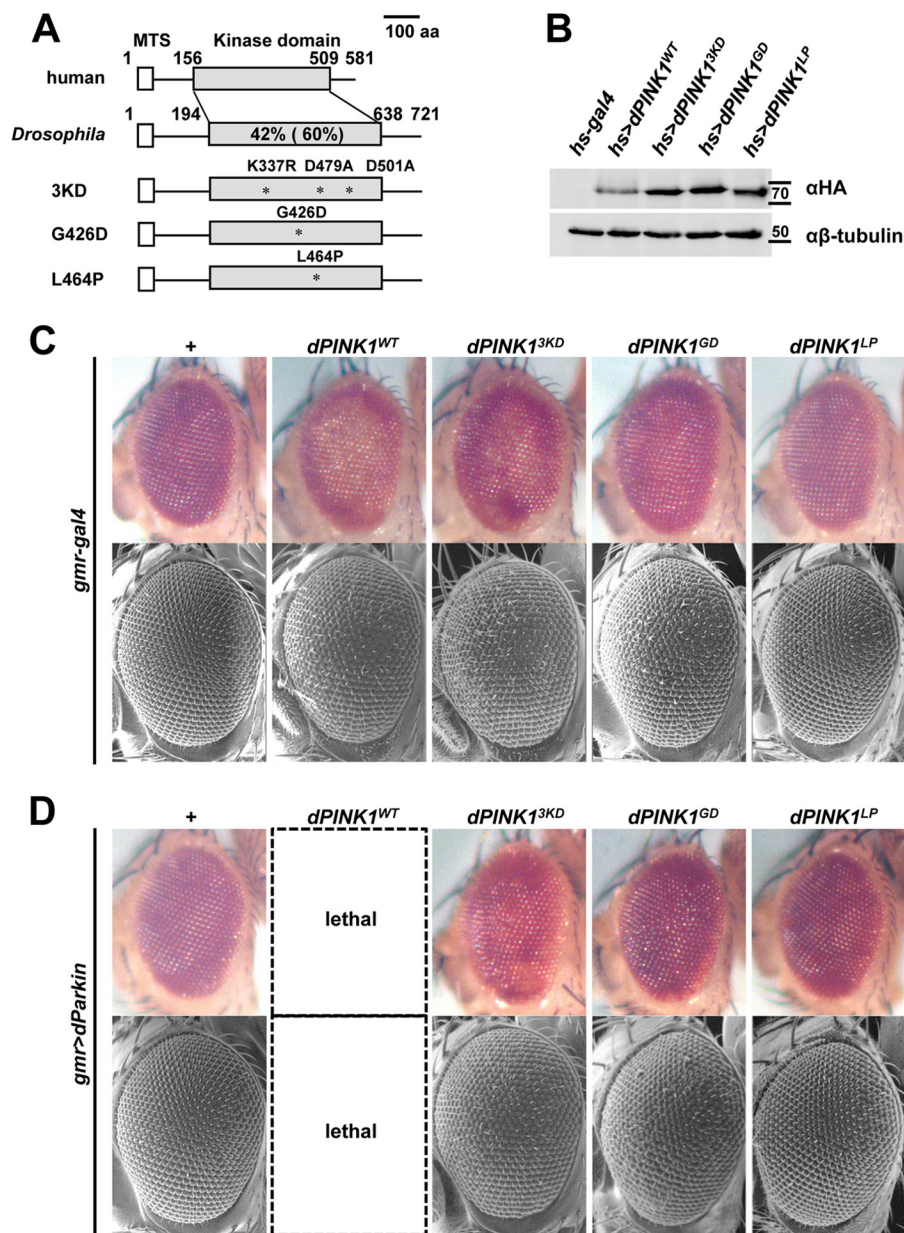


FIGURE 4. No significant genetic interactions are made between dParkin and dPINK1 kinase mutants in *Drosophila* eye. A, schematic representation of *Drosophila* PINK1 and mutants in the kinase domain (kinase dead (3KD), G426D, and L464P). MTS, mitochondria-targeting sequence. The numbers refer to the amino acid positions of dPINK1. Amino acid sequence identities (similarities) between hPINK1 and dPINK1 are denoted. B, immunoblot analysis of the expression levels of HA-tagged dPINK1 in the indicated genotypes. *hs-gal4* driver was used to express indicated dPINK1 mutant genes. Total body extraction from adult flies were subjected to immunoblot analysis using HA antibody (top panel). β-Tubulin was used as a loading control (bottom panel). C and D, representative images from light microscope (top panels) and SEM analysis (bottom panels) of *Drosophila* eyes of indicated genotypes. C, effects of WT dPINK1 (*gmr>dPINK1^{WT}*) and mutant expression (*gmr>dPINK1^{3KD}*, *gmr>dPINK1^{GD}*, and *gmr>dPINK1^{LP}*) in *Drosophila* eye. D, genetic interaction with dParkin and WT dPINK1 (*gmr>dParkin/dPINK1^{WT}*) or mutants (*gmr>dParkin/dPINK1^{3KD}*, *gmr>dParkin/dPINK1^{GD}*, and *gmr>dParkin/dPINK1^{LP}*) in *Drosophila* eye. aa, amino acids.

observed in WT dPINK1-expressing flies (*gmr>dPINK1^{WT}*) upon analysis using light microscopy and a SEM (Fig. 4C). The 3KD (*gmr>dPINK1^{3KD}*), G426D (*gmr>dPINK1^{GD}*), and L464P mutant-expressing flies (*gmr>dPINK1^{LP}*) showed rough eye phenotypes similar to the WT dPINK1 expressing flies, whereas dParkin-expressing flies (*gmr>dParkin*) had normal eyes. Next, the genetic interaction between dParkin and dPINK1 mutant flies were examined. As reported before, co-expression of WT dPINK1 and dParkin (*gmr>dParkin/dPINK1^{WT}*) led to lethality (Fig. 4D) (14), indicating that PINK1 and Parkin have a strong genetic interaction *in vivo*. However,

co-expression of dParkin and dPINK1 kinase mutants, including the 3KD (*gmr>dParkin/dPINK1^{3KD}*), G426D (*gmr>dParkin/dPINK1^{GD}*), and L464P mutants (*gmr>dParkin/dPINK1^{LP}*), did not yield lethal phenotypes or additive eye defects. These results were further confirmed by SEM analysis. In conclusion, two PINK1 kinase mutations, G426D and L464P, which were defective in translocation of Parkin to mitochondria in mammalian cells, did not interact with Parkin *in vivo*.

The Phenotypes of dPINK1-null Mutant Are Not Rescued by the LP Mutant, but Are Rescued by the GD 3 Days after Eclosion— In a previous study, we found that PINK1-null mutant flies

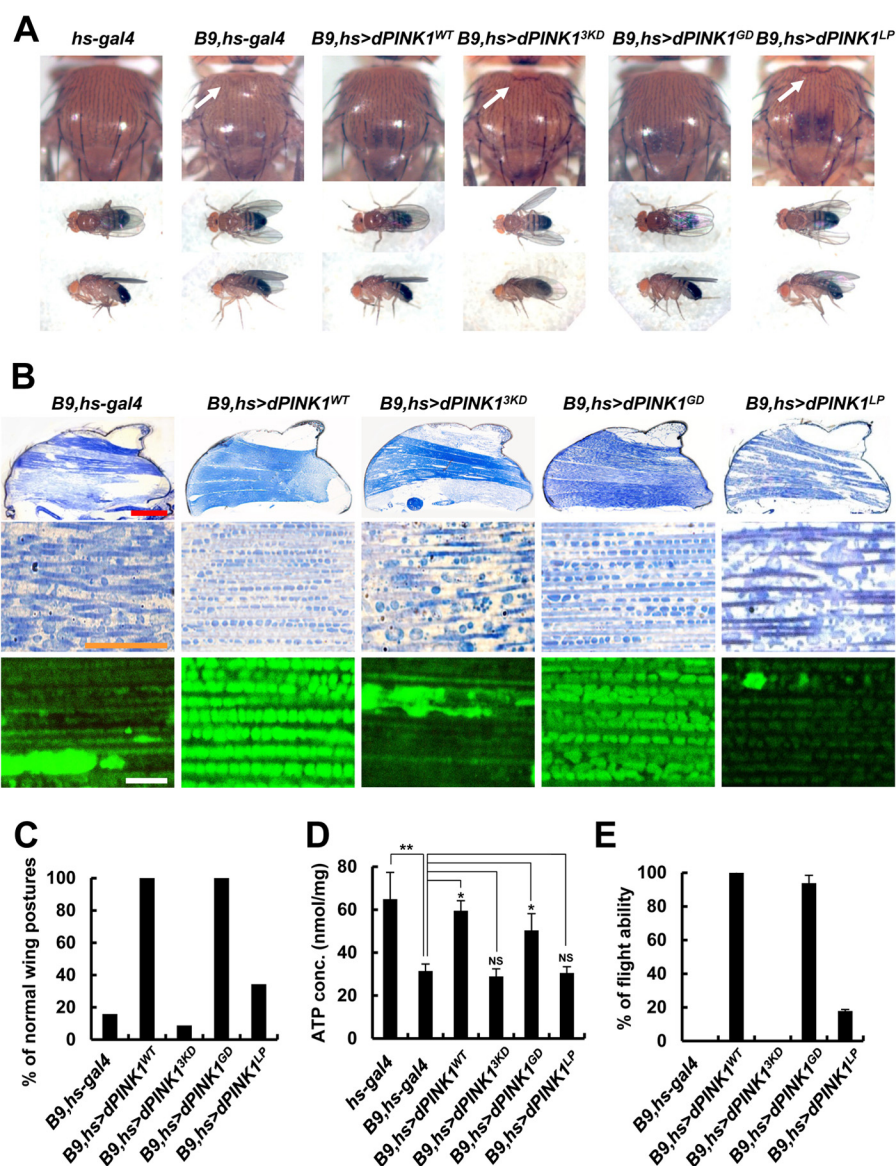


FIGURE 5. dPINK1-null mutant phenotypes are not rescued by LP mutant but are rescued by GD 3 days after eclosion. *A*, light stereo micrographs of the thoraces (top panels) and wing postures (bottom panels) of PINK1-null mutants (*B9,hs-gal4*), WT-expressing PINK1-null mutants (*B9,hs>dPINK1^{WT}*), 3KD-expressing PINK1-null mutants (*B9,hs>dPINK1^{3KD}*), G426D-expressing PINK1-null mutants (*B9,hs>dPINK1^{GD}*), and L464P-expressing PINK1-null mutants (*B9,hs>dPINK1^{LP}*). White arrows indicate crushed thorax phenotypes. *B*, toluidine blue-stained (blue, top panels) longitudinal sections of thorax muscles of indicated genotypes. Magnified images are presented in the middle panels. Mitochondria stained by Alexa 488-conjugated streptavidin (green, bottom panels). Red bar, 200 μ m; orange bar, 20 μ m; white bar, 5 μ m. *C*, the percentage of male flies with the normal wing phenotype ($n > 100$). *D*, quantitative ATP levels of indicated genotype. *, $p < 0.005$; **, $p < 0.001$; NS, not significant (Student's *t* test for related samples). The error bars indicate S.D. of three experiments ($n > 5$). *E*, the percentage of male flies with the normal flight ability. The error bars indicate S.D. of three experiments ($n > 30$). conc., concentration.

(*dPINK1^{B9}*) exhibit multiple PD-related phenotypes, including severe muscle degeneration, locomotor defects, and DA neuronal loss (18–21). Transgenic flies expressing exogenous PINK1 kinase mutants in the *dPINK1^{B9}* background were examined 3 days after eclosion. The most representative features of the *dPINK1^{B9}* flies were the crushed thoraces and downturned wing postures, and these abnormalities were rescued by the WT dPINK1 expression (*B9,hs>dPINK1^{WT}*) but not by the 3KD (*B9,hs>dPINK1^{3KD}*) (Fig. 5, *A* and *C*). When we expressed PINK1 kinase mutants in the *dPINK1^{B9}* background, the defects of the *dPINK1^{B9}* flies were rescued by the G426D dPINK1 expression (*B9,hs>dPINK1^{GD}*) but not by the L464P dPINK1 expression (*B9,hs>dPINK1^{LP}*). To further observe the effects of the PINK1 mutants on mitochondrial structure and

integrity, the thoracic muscles were dissected and then stained with toluidine blue dye and streptavidin antibodies (Fig. 5*B*). The *dPINK1^{B9}* flies had swollen or enlarged mitochondria between disorganized muscle fibers. These structural defects were completely complemented by WT, but not by 3KD. The expression of L464P dPINK1 also failed to rescue the impaired muscle structures and mitochondrial defects in *dPINK1^{B9}*. However, the expression of G426D dPINK1 rescued the defective muscles and mitochondria. In addition, the level of ATP was markedly reduced in the muscles in *dPINK1^{B9}* flies compared with control flies (*hs-gal4*), and this reduced ATP level was rescued by WT or G426D expression, but not by 3KD or L464P dPINK1 (Fig. 5*D*). Because of the structural and functional defects, *dPINK1^{B9}* flies could not fly (Fig. 5*E*). Again, the

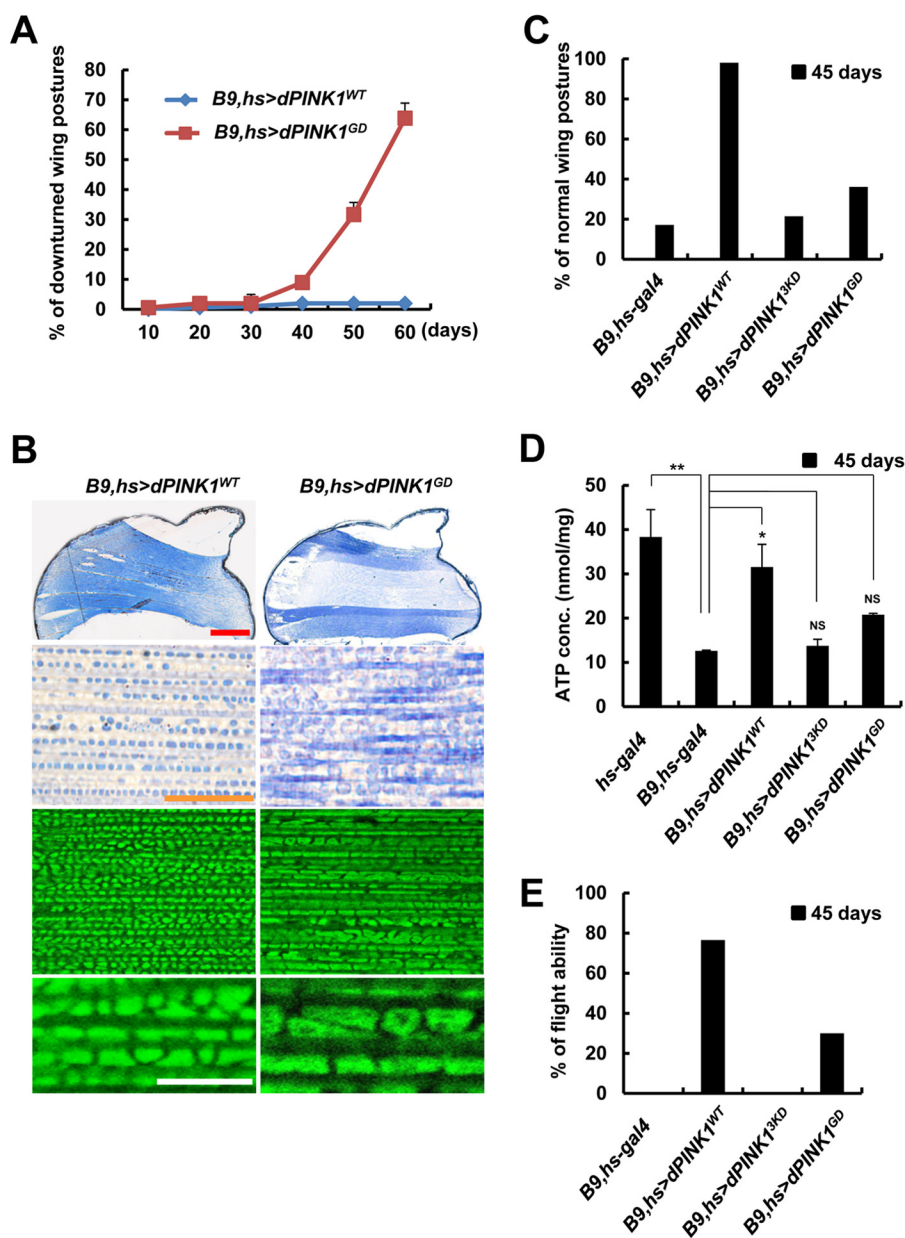


FIGURE 6. GD mutant-expressing dPINK1-null flies display defective phenotypes with aging. *A*, the percentage of male flies with downturned wing postures with aging. *B*, longitudinally sectioned thorax images of 45-day-old flies from indicated genotypes stained with toluidine blue (blue, upper panels) and Alexa 488-conjugated streptavidin (green, bottom panels). Red bar, 200 μ m; orange bar, 20 μ m; white bar, 5 μ m. *C*, the percentage of male flies with the normal wing phenotype at 45 days ($n > 100$). *D*, quantitative ATP levels of indicated genotypes at 45 days. *, $p < 0.005$; **, $p < 0.001$; NS, not significant (Student's *t* test for related samples). The error bars indicate S.D. of three experiments ($n > 5$). *E*, the percentage of flies with normal flight ability at 45 days ($n > 30$).

expression of WT or G426D dPINK1 rescued the flight ability of *dPINK1^{B9}* flies, but the expression of 3KD or L464P dPINK1 did not. Collectively, these data showed that the expression of L464P and 3KD dPINK1 did not rescue the defective phenotypes of dPINK1-null mutant flies, but the expression of G426D dPINK1 rescued many of the defective phenotypes at 3 days after eclosion.

dPINK1-null Flies Expressing Exogenous GD Mutant Display Defective Phenotypes with Aging—The ability of G426D dPINK1 to rescue phenotype defects in the *dPINK1^{B9}* flies was an unexpected result; therefore, we decided to observe this rescue further over a period of time. Notably, at 45 days of age, the G426D dPINK1-expressing flies (*B9,hs>dPINK1^{GD}*) showed

downturned wing postures in contrast to the WT dPINK1-expressing *dPINK1^{B9}* flies (*B9,hs>dPINK1^{WT}*) (Fig. 6, *A* and *C*). Moreover, abnormally swollen mitochondria between sparse muscle fibers were observed (Fig. 6*B*). Other age-dependent defects were also observed in the G426D dPINK1-expressing flies, such as reduced ATP levels (Fig. 6*D*) and flight abilities (Fig. 6*E*), at 45 days. Overall, we found that G426D dPINK1 expression, which rescued the defective phenotypes of young *dPINK1*-null mutants (Fig. 5), failed to sustain the rescue effects as the flies got older.

DA Neuronal Loss of dPINK1-null Mutant Is Not Rescued by Kinase Mutants—Because the loss of DA neurons is a hallmark of PD pathology, we examined DA neurons in the brains of

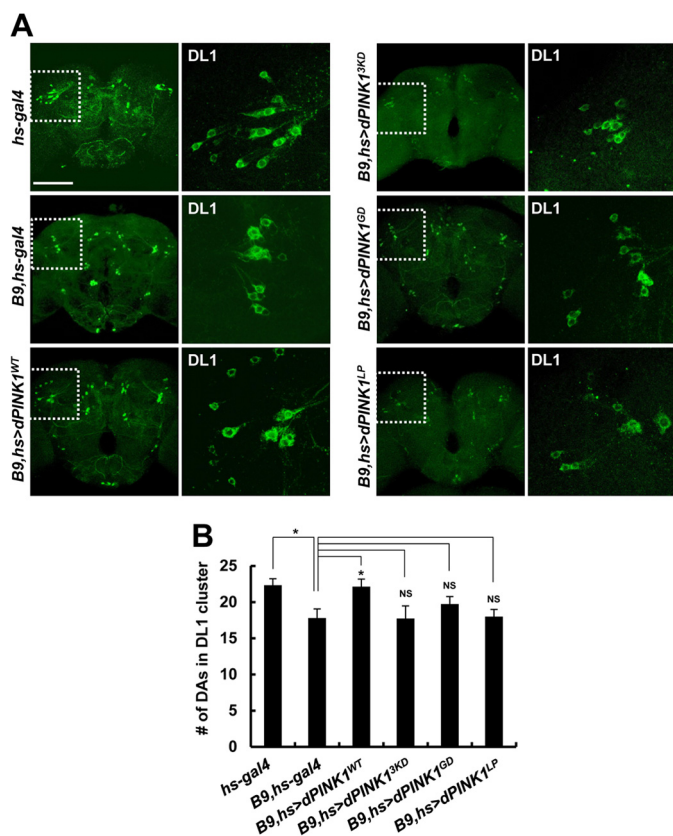


FIGURE 7. DA neuronal loss of PINK1-null flies are not complemented by kinase mutants. A, whole mount adult male brains (45 days old) showing DA neuronal clusters were marked by anti-TH antibody (green). Images of the DA neurons within the dorsolateral region (DL1) were magnified. Scale bar, 100 μ m. B, graph showing the average number of DA neurons in the dorsolateral region 1 clusters. *, $p < 0.001$; NS, not significant (Student's t test for related samples). The error bars indicate S.D. ($n > 30$).

adult *dPINK1^{B9}* flies using anti-TH staining. Consistent with our previous reports (18), *dPINK1^{B9}* flies displayed weak TH staining signals and reduced numbers of DA neurons in dorsolateral region 1 at 45 days (Fig. 7A). This loss of DA neurons was almost fully rescued by expression of WT (*B9, hs>dPINK1^{WT}*), but not 3KD (*B9, hs>dPINK1^{3KD}*), G426D (*B9, hs>dPINK1^{G426D}*), or L464P *dPINK1* mutant (*B9, hs>dPINK1^{L464P}*) expression (Fig. 7). These results strongly suggest that the kinase activity is critical for the protective roles of PINK1 in DA neurons.

DISCUSSION

Because mutations in PINK1 are inherited primarily in a recessive manner, the loss of its function is thought to cause early onset PD. In this study, we dissected the underlying mechanism by which PINK1 missense mutations lead to PD pathogenesis using mammalian cells and an *in vivo Drosophila* model system.

Expression and Subcellular Localization Patterns of PINK1 Are Not Altered by Missense Mutations under Both Normal and Damaged Mitochondria Conditions—We first examined the proteolytic patterns of hPINK1 mutants in mammalian cells. All of the mutants selected in the study generated typical triple bands in SDS-PAGE, similar to the WT hPINK1 protein (Fig. 1). In addition, they showed similar patterns of subcellular localization, both in the cytoplasm and in mitochondria. Also,

their expression did not change mitochondrial morphology nor the $\Delta\psi_m$.

We also investigated the role of hPINK1 mutations in response to damaged mitochondria. When treated with CCCP, cells expressing any of the 17 PINK1 mutants accumulated on mitochondria and the mutant proteins were stabilized just like the WT hPINK1 protein (Fig. 2). These results strongly suggest that the proteolysis, stability, and subcellular localization of PINK1 are not critical for the mechanism of PD pathogenesis associated with these missense mutations.

The Kinase Activity of PINK1 Is Indispensable for Its Function—The PINK1/Parkin pathway regulates mitochondrial integrity and function by modifying mitochondrial morphology and dynamics (32). In this study, Parkin was translocated to mitochondria when PINK1 was co-expressed with Parkin, and the mitochondria were highly aggregated. Surprisingly, in the cells expressing the hPINK1 with patient mutations in the kinase domain, Parkin translocation to mitochondria or mitochondrial aggregation did not occur (Fig. 3). Meanwhile, the subcellular localizations of PINK1 protein were not dramatically changed by Parkin co-expression. We anticipate that the catalytic activity of the overexpressed PINK1 protein localized in mitochondria, although its concentration may be much lower than the total PINK1 protein in the cell, is sufficient for mobilizing Parkin to mitochondria. The two PINK1 kinase mutants, human G309D (*Drosophila* G426D) and L347P (*Drosophila* L464P), which failed to mobilize Parkin to mitochondria in mammalian cells, also failed to genetically interact with Parkin in adult fly eye tissue (Fig. 4). These data consistently support the hypothesis that the kinase activity of PINK1 is essential for its function.

Other groups have studied the kinase activity of PINK1 *in vivo* or *in vitro* (32, 58–60). PD-associated mutations in the kinase domain, including G309D, L347P, G386A, and G409V mutation, markedly inhibit PINK1 kinase activity (59, 60). Previous biochemical studies confirmed that the PINK1 kinase domain mutants used in the present study have reduced kinase activity.

Notably, the C125G mutation within TM region also moderately suppresses PINK1 kinase activity, by 40% (59). Interestingly, co-expression of the C125G mutant or of PINK1 harboring the neighboring mutation, Q126P, failed to localize Parkin to mitochondria in our study, indicating that the TM region affects PINK1 kinase activity and subsequently regulates Parkin mobilization to mitochondria (Fig. 3). These results again emphasize the importance of PINK1 kinase activity in PD and also further substantiate that the role of PINK1 in regulating Parkin in a kinase activity-dependent manner.

Next, we investigated the mechanism by which PINK1 regulates Parkin. A growing body of evidence suggests that Parkin might be a direct substrate of PINK1 (32, 58, 66). A recent study clearly showed that Parkin is phosphorylated at Ser-65 in the ubiquitin-like domain by PINK1, when PINK1 is activated by CCCP (58). However, it is interesting to note that in our study, the Parkin S65A mutant was still recruited to mitochondria and induced mitochondrial aggregation, when PINK1 was co-expressed. Moreover, phosphomimetic Ser-65 mutants, S65D and S65E, were not translocated to mitochondria, nor did they

induce mitochondrial aggregation (data not shown), suggesting that Ser-65 phosphorylation by PINK1 may not be critical for the functional regulation of Parkin. Previous work from our group also showed that PINK1 can directly phosphorylate Parkin at Thr-175 in the linker region (32). Mutational analysis confirmed that mitochondrial translocation of the Parkin T175A mutant was markedly reduced even when PINK1 was co-expressed. Currently we are conducting mapping analyses for Parkin phosphorylation in the presence and absence of PINK1 co-expression and CCCP treatment to delineate the regulation of their biochemical interactions more thoroughly.

Differential in Vivo Effects Were Shown Depending on the Levels of PINK1 Kinase Activity with Aging—Previous studies showed that hPINK1 L347P mutation completely abolished its kinase activity, whereas the G309D mutant still retained a modest level of kinase activity (54, 59, 60). Our *Drosophila* studies also showed differences in the activities of G426D (human G309D) and L464P dPINK1 (human L347P); although the L464P mutant, as well as the 3KD mutant, exhibited defective phenotypes at a young age, the G426D mutant exhibited defects only at older ages (Figs. 5–7).

As the prevalence of PD increases with aging, it is interesting that the G426D mutant flies showed age-dependent defective phenotypes in accordance with the level of kinase activity. Because both Parkin E3 ligase activity (60) and its localization to mitochondria are dependent on PINK1 kinase activity (Fig. 3), the mitochondrial substrate(s) of Parkin would be accumulated or deposited in the cell when the kinase activity of PINK1 is disrupted. For example, if the kinase activity of PINK1 is not sufficient to properly phosphorylate and regulate Parkin (e.g., the G426D mutant), its substrate(s) would be gradually accumulated in mitochondria and eventually cause progressive deterioration in mitochondrial and cellular functions. This may explain the mechanism underlying the age-dependent defects seen in our fly models and possibly the different disease onset ages of PD patients.

PINK1 May Regulate Various Cellular Responses through Different Effector Molecules—Meanwhile, we may need to consider that there are other substrates or effector molecules of PINK1 that are involved in regulating cellular responses, because not all of the PINK1 mutants were defective in Parkin translocation to mitochondria or in mitochondrial morphology (Figs. 1–3). Excitingly, recent proteomic analyses and genetic approaches have identified novel interaction partners for PINK1 (67–73), including TRAP1 (tumor necrosis factor receptor-associated protein 1), Omi/HtrA2 (a product of the *PARK13* gene), Miro (an atypical GTPase that regulates mitochondrial trafficking), and FOXO (Forkhead box O transcription factor). Moreover, recent data suggested that PINK1 functions are not limited to mitochondria alone. For example, there was a study that addressed the role of PINK1 in the cytoplasm, showing that PINK1 exerts a cytoprotective function by activating Akt (71). Based on these data, we are currently investigating the possible connections between the PD-linked PINK1 mutations and the downstream targets of PINK1 in addition to Parkin.

In conclusion, we confirm that the kinase activity of PINK1 is critical for its function and the early onset of PD. We validated

these data in the *Drosophila* model, proving the pathophysiological relevance of PD. Further studies addressing the modulation of PINK1 kinase activity or identifying novel targets of PINK1 are necessary to enhance our understanding of PINK1 mutation-associated PD pathogenesis.

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