Molecules and Cells

Pyruvate Dehydrogenase Kinase Protects Dopaminergic Neurons from Oxidative Stress in *Drosophila DJ-1* Null Mutants

Yoonjeong Lee[1,2,6], Jaehyeon Kim[1,3,6], Hyunjin Kim[1,2], Ji Eun Han[1,3], Sohee Kim[1,3], Kyong-hwa Kang[1,4], Donghoon Kim[1,2,3,4], Jong-Min Kim[5], and Hyongjong Koh[1,2,3,4,*]

[1]Department of Pharmacology, Dong-A University College of Medicine, Busan 49201, Korea, [2]Peripheral Neuropathy Research Center, Dong-A University College of Medicine, Busan 49201, Korea, [3]Department of Translational Biomedical Sciences, Dong-A University College of Medicine, Busan 49201, Korea, [4]Neuroscience Translational Research Solution Center, Dong-A University College of Medicine, Busan 49201, Korea, [5]Department of Anatomy and Cell Biology, Dong-A University College of Medicine, Busan 49201, Korea, [6]These authors contributed equally to this work.
*Correspondence: hjkoh@dau.ac.kr
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DJ-1 is one of the causative genes of early-onset familial Parkinson's disease (PD). As a result, DJ-1 influences the pathogenesis of sporadic PD. DJ-1 has various physiological functions that converge to control the levels of intracellular reactive oxygen species (ROS). Based on genetic analyses that sought to investigate novel antioxidant DJ-1 downstream genes, pyruvate dehydrogenase (PDH) kinase (PDK) was demonstrated to increase survival rates and decrease dopaminergic (DA) neuron loss in *DJ-1* mutant flies under oxidative stress. PDK phosphorylates and inhibits the PDH complex (PDC), subsequently downregulating glucose metabolism in the mitochondria, which is a major source of intracellular ROS. A loss-of-function mutation in *PDK* was not found to have a significant effect on fly development and reproduction, but severely ameliorated oxidative stress resistance. Thus, PDK plays a critical role in the protection against oxidative stress. Loss of *PDH phosphatase* (*PDP*), which dephosphorylates and activates PDH, was also shown to protect *DJ-1* mutants from oxidative stress, ultimately supporting our findings. Further genetic analyses suggested that DJ-1 controls PDK expression through hypoxia-inducible factor 1 (HIF-1), a transcriptional regulator of the adaptive response to hypoxia and oxidative stress. Furthermore, CPI-613, an inhibitor of PDH, protected *DJ-1* null flies from oxidative stress, suggesting that the genetic and pharmacological inhibition of PDH may be a novel treatment strategy for PD associated with DJ-1 dysfunction.

Keywords: DJ-1, *Drosophila*, oxidative stress, Parkinson's disease, pyruvate dehydrogenase kinase

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease and is characterized by typical motor symptoms and selective loss of dopaminergic (DA) neurons in the substantia nigra (Lang and Lozano, 1998). Various studies have supported the link between DA neuron loss and oxidative stress originating from excess generation or insufficient elimination of reactive oxygen species (ROS) (Henchcliffe and Beal, 2008). In postmortem brain analyses, oxidative damage to macromolecules, such as DNA, proteins, and lipids, has been shown to be substantially elevated in the substantia nig-

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ra of PD patients (Bender et al., 2006; Henchcliffe and Beal, 2008). Various PD animal models have been generated using oxidative stress-inducing agents, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, paraquat, and 6-hydroxydopamine (6-OHDA) (Betarbet et al., 2000; Brooks et al., 1999; Langston et al., 1983; Ungerstedt and Arbuthnott, 1970). However, the molecular mechanisms underlying oxidative stress-induced degeneration of DA neurons have not been fully elucidated.

PD mainly develops sporadically, but can also occur due to monogenic mutations (Lesage and Brice, 2009). Among the PD-linked genes, DJ-1 is most closely associated with oxidative stress (Henchcliffe and Beal, 2008). Bonifati et al. (2003) reported that DJ-1 is associated with the development of an autosomal recessive early-onset type of familial PD. Further, subsequent studies revealed that DJ-1 plays various roles in cell biology, such as transcriptional regulation, anti-apoptotic signaling, proteostasis, and mitochondrial regulation in response to oxidative stress (Ariga et al., 2013). DJ-1 is sequentially oxidized at its cysteine residues under oxidative stress, and its activity and subcellular localization are regulated by its oxidative status (Ariga et al., 2013). Notably, excessive oxidation leads to DJ-1 inactivation, and this excessively oxidized form has been identified in the brain of patients with sporadic PD (Bandopadhyay et al., 2004; Choi et al., 2006). Such findings suggest that DJ-1 plays a role in the pathogenesis of both sporadic and familial PD. *Drosophila* exhibits two DJ-1 isoforms, DJ-1α and DJ-1β. DJ-1α is expressed mainly in the testes, whereas DJ-1β is expressed throughout the body (Meulener et al., 2005; Park et al., 2005), similar to mammalian DJ-1 (Nagakubo et al., 1997). Interestingly, *DJ-1β* mutant flies show locomotive defects and DA neuron degeneration, resembling the symptoms of PD patients (Hwang et al., 2013; Park et al., 2005; Yang et al., 2017).

In this study, the overexpression of pyruvate dehydrogenase (PDH) kinase (PDK) was demonstrated to enhance survival rates and ameliorate DA neuron loss in *DJ-1β* mutant flies under oxidative stress. PDK phosphorylates and inhibits PDH complex (PDC) (Park et al., 2018), subsequently downregulating mitochondrial glucose metabolism, which is a major source of intracellular ROS. Indeed, *Drosophila* PDK mutants showed hypersensitivity to oxidative stress. Further, a loss-of-function mutation in *PDH phosphatase* (*PDP*), which dephosphorylates and activates PDC (Park et al., 2018), was demonstrated to protect *DJ-1β* mutants from oxidative stress, supporting the idea that PDK plays a critical role in oxidative stress resistance by inhibiting PDC. Further genetic analyses revealed that DJ-1 controls PDK expression through hypoxia-inducible factor 1α (HIF-1α), a transcriptional regulator that mediates the adaptive response to hypoxia and oxidative stress. Consistent with these genetic data, CPI-613, an inhibitor of PDH (Zachar et al., 2011), has been shown to protect *DJ-1β* null flies from oxidative stress, suggesting that PDH inhibitors may be novel treatment candidates for PD associated with DJ-1 dysfunction.

MATERIALS AND METHODS

Drosophila strains

All flies were grown as described previously (Jang et al., 2020). The w^{1118}, *hs-GAL4*, and *elav-GAL4* strains were obtained from Bloomington Stock Center (USA). PDK^{P} ($PDK^{EY01879}$) and PDP^{P} (PDP^{G1628}) mutants were also obtained from Bloomington Stock Center and backcrossed with the w^{1118} controls for six generations to remove genetic background effects. The insertion site of the P-element in PDK^{P} is located at +88 bp of the PDK open reading frame, and the P-element insertion site in PDP^{P} is located at +52 bp of the PDP transcription start site. PDK cDNA was sub-cloned from the LD23669 BDGP cDNA clones, inserted into the pUAST vector with a C-terminal HA (hemagglutinin)-tag, and microinjected into w^{1118} embryos. $DJ\text{-}1\beta^{ex54}$ flies were generated as previously described (Park et al., 2005). The $dVHL^{1}$ and *UAS-sima* fly lines were kindly provided by Dr. Tien Hsu and Dr. Pablo Wappner.

Oxidative stress assays

For the oxidative stress assays, three or four groups of 30 3-day-old flies (n = 90 or 120) were starved for 6 h and transferred to a vial containing a gel consisting of phosphate-buffered saline (PBS), 5% sucrose, and an oxidative stress agent (5 mM rotenone or 1% H_2O_2). To determine the role of *PDK* in oxidative stress resistance, male and female PDK^{P} flies were used. However, for the other assays, only male flies were used. The flies were transferred to fresh vials daily, and the number of dead flies was counted at the time points indicated in the figures. All oxidative stress assays were performed at 25°C and repeated at least twice.

mtDNA PCR and ATP assay

For mitochondrial DNA (mtDNA) polymerase chain reaction (PCR), total DNA was extracted from the thoraces of five 3- and 10-day-old male flies. Quantitative real-time (RT) PCR was performed as described previously (Yang et al., 2017). Rp49 genomic DNA levels were used as an internal control. The results are expressed as fold-changes relative to the control. For the ATP assay, the thoraces of five 3-day-old male flies were dissected, and ATP concentration was measured as previously described (Yang et al., 2017). The relative ATP level was calculated as the ratio of ATP concentration to the total protein concentration. The total protein concentration was determined using the bicinchoninic acid assay (Sigma, USA). The results of the mtDNA PCR and ATP assays are expressed as mean ± SD of three independent experiments.

Drosophila DA neuron and tissue staining

To investigate the change in the number of DA neurons in *DJ-1β* mutants under oxidative stress, 30 male flies (3 days old) were starved for 6 h and incubated for 3 days in a vial containing a gel consisting of PBS, 5% sucrose, and an oxidative stress agent (0.2 mM rotenone or 1% H_2O_2). To stain DA neurons, adult brains from ten random flies were fixed with 4% paraformaldehyde and stained with an anti-TH rabbit antibody (1:50; Pel-Freez, USA), as previously described (Yang et al., 2017). The stained brain samples were then

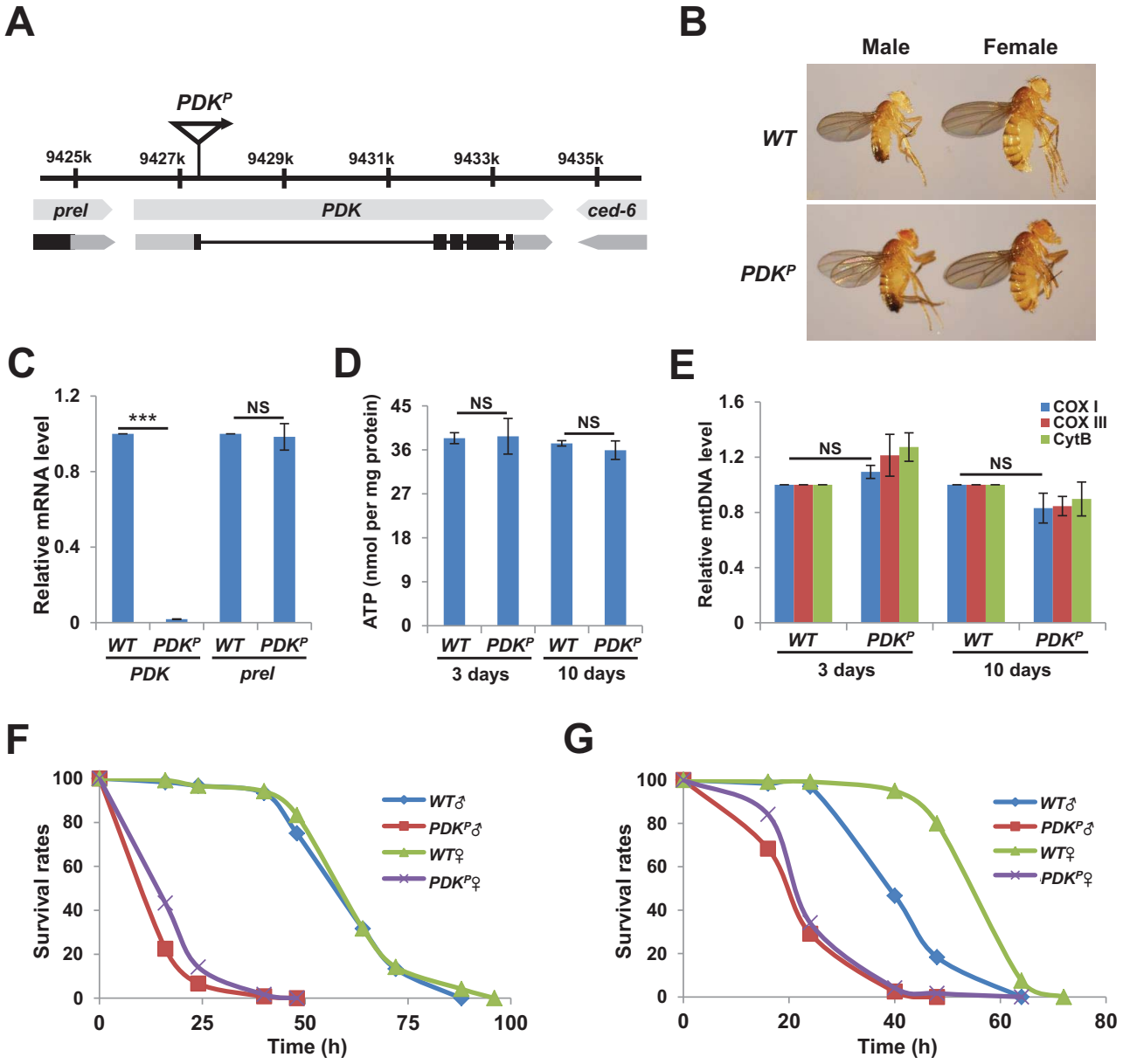

Fig. 1. Characterization of *PDK^P^* mutant flies. (A) Schematic genomic organization of the *PDK* locus. Black rectangles, coding sequences; Gray rectangles, untranslated regions. The genomic structures of the *PDK* mutants (*PDK^P^*) are described in the Materials and Methods section. (B) Light stereo micrographs of wild-type (*WT*) and *PDK^P^*. (C) Comparison of the *PDK* and *prel* mRNA levels in *WT* and *PDK^P^*. (D) Comparison of the ATP content in the analyzed thoraces. (E) Quantification of mtDNA content in the analyzed thoraces. Cox I, cytochrome c oxidase subunit I; Cox III, cytochrome c oxidase subunit III; Cyt B, cytochrome b. (F and G) Survival curves of 3-day-old *WT* and *PDK^P^* male (♂) and female (♀) flies fed 0.2 mM rotenone (F) or 1% H_2O_2 (G) containing food (log-rank test: $P < 0.001$, n = 90 for *PDK^P^* males administered H_2O_2 containing food; n = 120 for other genotypes). For detailed statistical analyses of life span data in this and other figures, please see Supplementary Table S1. Significance was determined using the Student's *t*-test. ***$P < 0.001$; NS, not significant. Error bars indicate mean ± SD.

observed and imaged using an LSM 700 confocal microscope (Zeiss, Germany) at the Neuroscience Translational Research Solution Center (Korea).

Genotypes

WT (*+/Y*); *PDK^P^* (*PDK^P^/PDK^P^*); *hs* (*hs-GAL4/+*); *hs DJ-1β^ex54^* (*hs-GAL4/+*; *DJ-1β^ex54^/DJ-1β^ex54^*); *hs>PDK DJ-1β^ex54^* (*hs-GAL4/UAS-PDK*; *DJ-1β^ex54^/DJ-1β^ex54^*); *elav* (*elav-GAL4/+*); *elav DJ-1β^ex54^* (*elav-GAL4/+*; *DJ-1β^ex54^/DJ-1β^ex54^*); *elav>PDK DJ-1β^ex54^* (*elav-GAL4/UAS-PDK*; *DJ-1β^ex54^/DJ-1β^ex54^*); *PDP^P^* (*PDP^P^/Y*); *DJ-1β^ex54^* (*DJ-1β^ex54^/DJ-1β^ex54^*); *PDP^P^ DJ-1β^ex54^* (*PDP^P^/Y*; *DJ-1β^ex54^/DJ-1β^ex54^*); *VHL^-/+^ DJ-1β^ex54^* (*VHL^1^/+*; *DJ-1β^ex54^/*

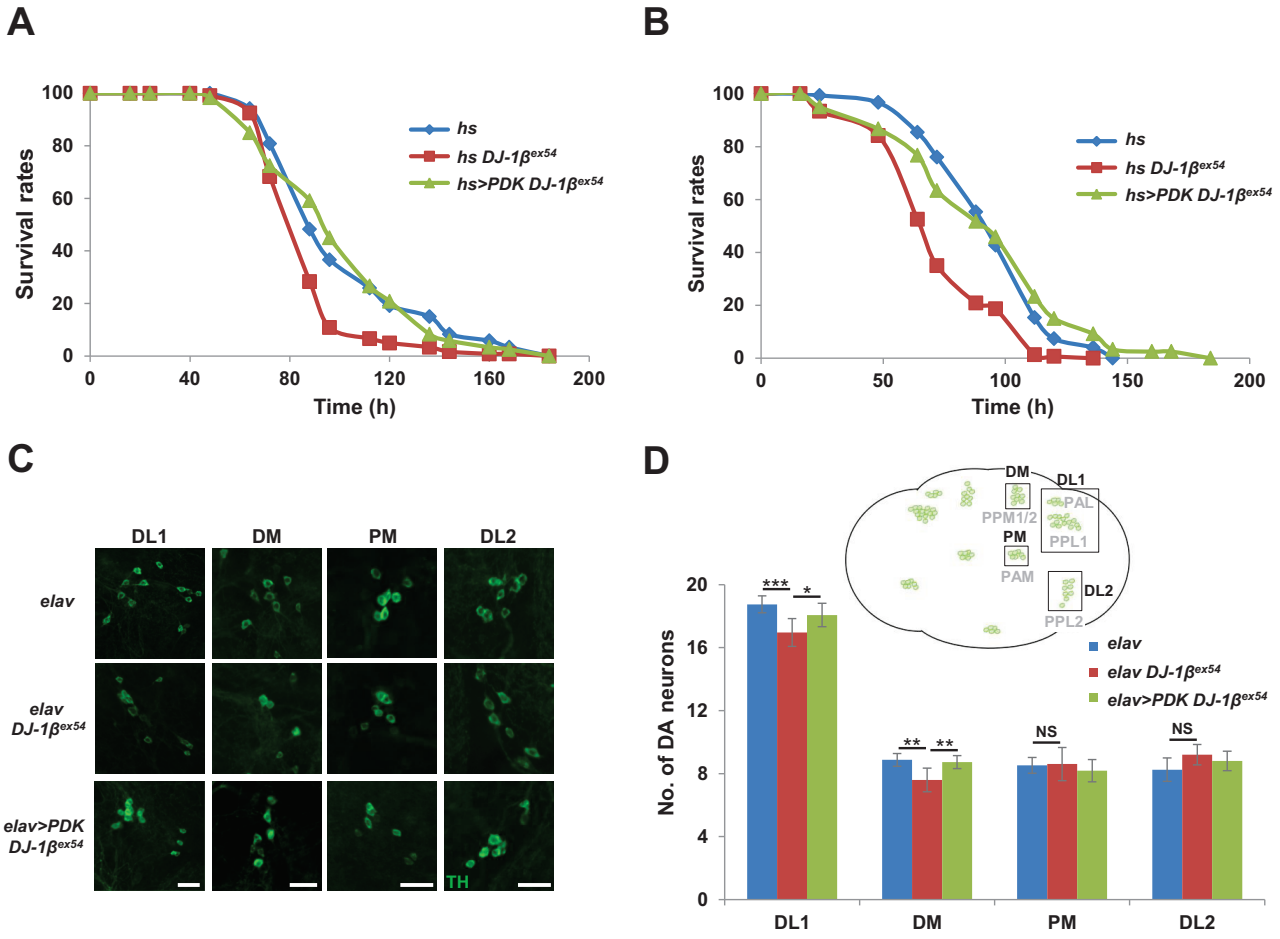

Fig. 2. PDK rescued *DJ-1β* mutant phenotypes induced by oxidative stress. (A and B) Survival curves of 3-day-old control (*hs*), *DJ-1β* null mutants (*hs DJ-1β*^{ex54}), and *PDK*-overexpressing *DJ-1β* mutants (*hs>PDK DJ-1β*^{ex54}) fed 0.2 mM rotenone (A) or 1% H_2O_2 (B) containing food (log-rank test: *hs* vs *hs DJ-1β*^{ex54} and *hs DJ-1β*^{ex54} vs *hs>PDK DJ-1β*^{ex54}: $P < 0.001$, n = 150 for *hs* flies on rotenone containing food; n = 120 for other genotypes). All oxidative stress assays were carried out at 25°C and were repeated at least twice. (C and D) Confocal images (C) and graphs (D) of the average number of DA neurons within the DL1, DM, PM, and DL2 clusters in adult brains from 6-day-old *elav-GAL4/+* (*elav*), *DJ-1β* null mutants (*elav DJ-1β*^{ex54}), and *PDK*-overexpressing *DJ-1β* null mutants (*elav>PDK DJ-1β*^{ex54}) after H_2O_2 treatment. DA neurons were stained with an anti-TH antibody (green) (n = 20 for *elav*; n = 30 for *elav DJ-1β*^{ex54} and *elav>PDK DJ-1β*^{ex54}). Inset: The DA neuron clusters in *Drosophila* adult brain. Four clusters (DL1, DM, PM, and DL2) (Park et al., 2006) and 5 clusters (PPM1/2, PAM, PPL1, PPL2, and PAL) (Coulom and Birman, 2004) are represented. Significance was determined by one-way ANOVA with Sidak correction. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; NS, not significant ($P > 0.05$). Error bars indicate mean ± SD. Scale bars = 20 μm.

DJ-1β^{ex54}); *elav>Sima DJ-1β*^{ex54} (*elav-GAL4/UAS-Sima; DJ-1β*^{ex54}/*DJ-1β*^{ex54}).

Luciferase assay

To measure the transactivation activity of Sima on the *PDK* gene, the promoter region was subcloned into the pGL3 reporter plasmid (Promega, USA) using the following primers: PDK promoter F (CGC ACG CGT CCA ACA CTT GTA GTG GTT AAA AGT GTA G) and R (CGC AGA TCT CTC ACT CTC TTC GCA GGA CGT TG). PDK reporters with HRE mutations were generated using the QuikChange™ site-directed mutagenesis kit (Agilent Technologies, USA) with the following primer pairs: HRE1 mutant F (ACA CTG CGG CTC TCC CGA TCA AAA GCG CTC GCA GC) and R (GCT GCG AGC GCT TTT GAT CGG GAG AGC CGC AGT GT); and HRE2 mutant F (CCC GCC TTT CAA CAC AAA AAC AAC AAC AAC AAA TCG GAG CAG AGC TAA CAC AA) and R (TTG TGT TAG CTC TGC TCC GAT TTG TTG TTG TGT TGA AAG GCG GG). S2 cells were transfected with the wild-type or HRE mutant PDK reporters, pUAST-Sima, pR-TK Renilla reporter, and pMT-GAL4 plasmids. Two days post-transfection, Sima expression was induced by $CuSO_4$ treatment. After 24 h, the luciferase assay was performed using the Dual-Luciferase™ Reporter Assay Kit (Promega) according to the manufacturer's instructions. The results are expressed as average luciferase activity ± SD from three independent experiments.

Quantitative RT-PCR

Total RNA was extracted from five 3-day-old flies and reverse-transcribed, as previously described (Park et al., 2006).

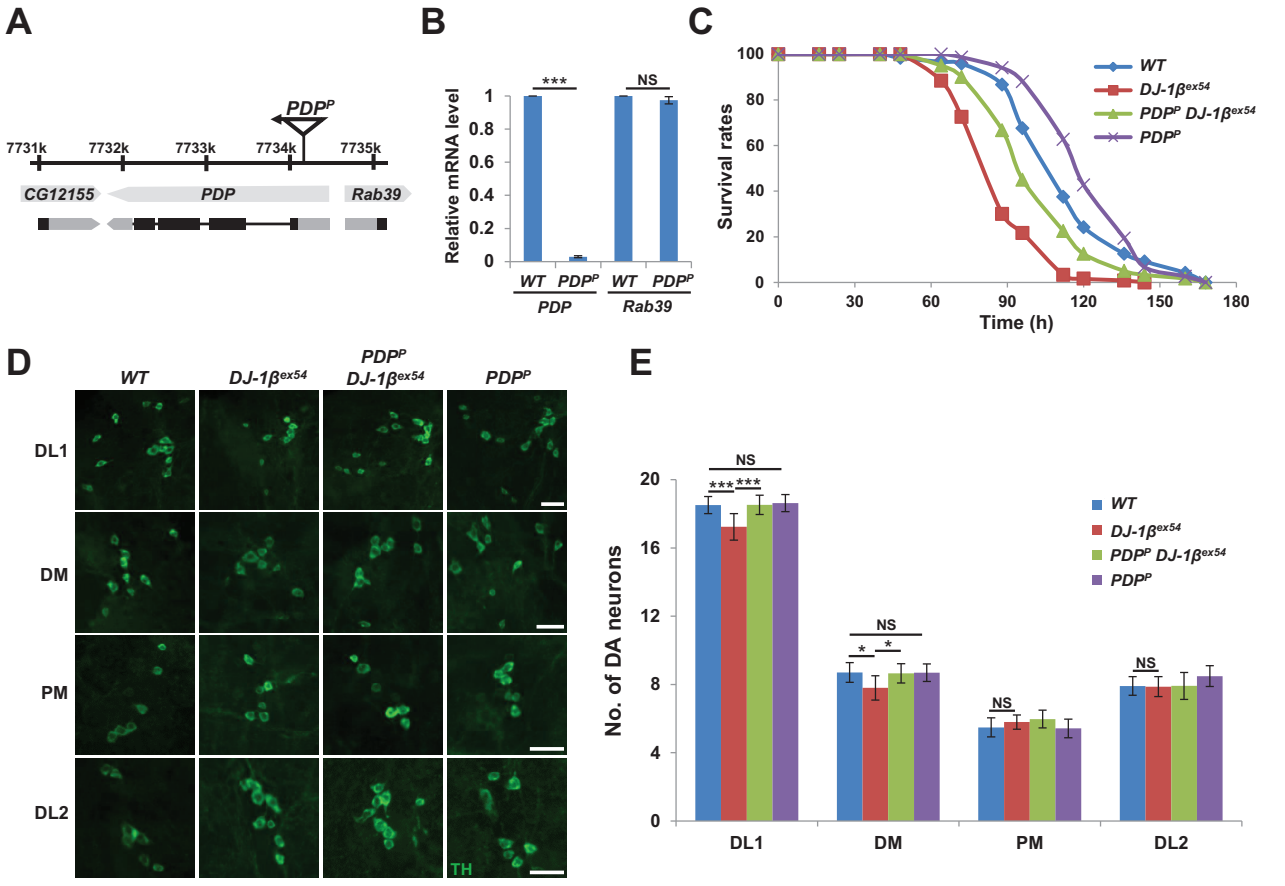

Fig. 3. *PDP*^{P} mutation prevented oxidative stress-induced defects in *DJ-1β* mutants. (A) Schematic genomic organization of the *PDP* locus. Black rectangles, coding sequences; Gray rectangles, untranslated regions. The genomic structures of *PDP*^{P} are described in the Materials and Methods section. (B) Comparison of *PDP* and *Rab39* mRNA levels in wild-type (*WT*) and *PDP* mutants (*PDP*^{P}). (C) Survival curves of *WT*, *DJ-1β* null mutants (*DJ-1β*^{ex54}), *PDP*^{P}, and *PDP*^{P} and *DJ-1β* double mutants (*PDP*^{P} *DJ-1β*^{ex54}) fed 1% H_2O_2-containing food (log-rank test: *WT* vs *DJ-1β*^{ex54}, *PDP*^{P} vs *DJ-1β*^{ex54}, and *DJ-1β*^{ex54} vs *PDP*^{P} *DJ-1β*^{ex54}: $P < 0.001$, n = 120 for each genotype). All oxidative stress assays were carried out at 25°C and were repeated at least twice. (D and E) Confocal images (D) and graphs (E) of the average number of DA neurons within the DL1, DM, PM, and DL2 clusters of adult brains from 6-day-old flies after H_2O_2 treatment. DA neurons were stained with an anti-TH antibody (green) (n = 30 for each genotype). Significance was determined by one-way ANOVA with Sidak correction. $*P < 0.05$; $***P < 0.001$; NS, not significant ($P > 0.05$). Error bars indicate mean ± SD. Scale bars = 20 μm.

Quantitative RT-PCR was performed using SYBR Premix Ex Taq (Takara, Japan) on QuantStudio 3 (Thermo Fisher Scientific, USA) at the Neuroscience Translational Research Solution Center. Rp49 levels were used as internal controls. The results are expressed as fold-changes relative to the control. The average mRNA levels ± SD were obtained from three independent experiments. The following primer pairs were used: rp49 F (GCT TCA AGA TGA CCA TCC GCC C) and R (GGT GCG CTT GTT CGA TCC GTA AC); PDK F (CTG TTT CCA GTC CGA TTC TCA G) and R (CAG AAG GGC GAT CTC CTT CAT G); prel F (CAC CAG GAA CCT GGG AAT GAC) and R (CAG CAC GTA GTT GAA TCC ATT GGA G); PDP F (CTC CAT ATA GAT GTT CAA GTT CGT G) and R (GTA AAC GAA CTC GTT CTC CCT CAG); and Rab39 F (GAC AGC ACC GTG GGC AAG AG) and R (GAC TTG GTG ATC GAA CGG AAG C).

Statistical analysis

To quantify DA neurons, four major DA neuron clusters from more than 15 brains of each genotype were observed in a blinded fashion to eliminate bias (n = 30-40). One-way ANOVA with Sidak correction was used to compare three or more groups, while Student's two-tailed *t*-test was used for two-group comparisons. The Kaplan–Meier method and Log-rank test were used to analyze survival data and determine whether the treatments had any effect on the longevity of individuals using online application survival analysis lifespan assays (http://sbi.postech.ac.kr/oasis). Significance was considered at $P < 0.05$.

RESULTS

PDK mutants are highly sensitive to oxidative stress

The central nervous system (CNS) receives most of its energy from oxygen-dependent glucose metabolism. Further, mitochondrial PDC mediates a major step in glucose oxidation, namely the irreversible decarboxylation of pyruvate to ace-

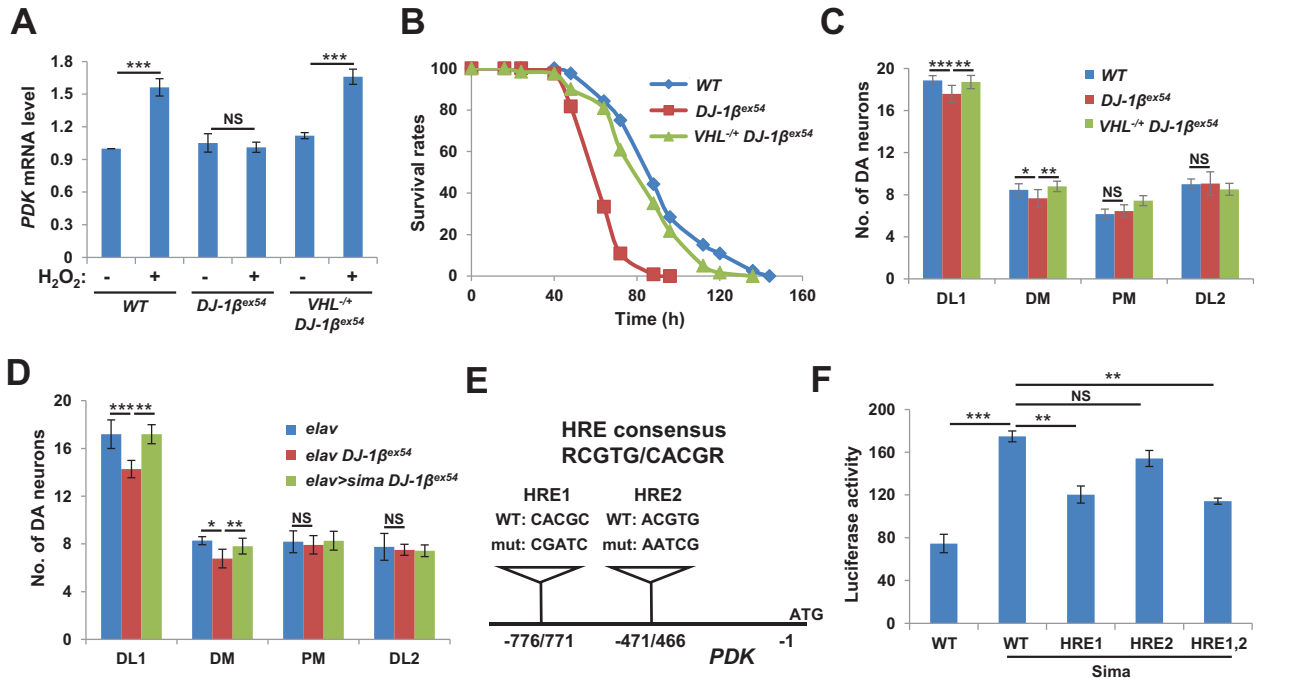

Fig. 4. DJ-1 induced PDK expression via the VHL-HIF-1α pathway. (A) Comparison of the *PDK* mRNA expression levels in wild type (*WT*), *DJ-1β* null mutants (*DJ-1β*^{ex54}), and *DJ-1β* null mutants with a heterozygous *VHL* mutation (*VHL*$^{-/+}$ *DJ-1β*^{ex54}) after treatment with control vehicle or 1% H_2O_2 (n = 3). (B) Survival curves of *WT*, *DJ-1β*^{ex54}, and *VHL*$^{-/+}$ *DJ-1β*^{ex54} fed 1% H_2O_2-containing food (log-rank test: *WT* vs *DJ-1β*^{ex54} and *DJ-1β*^{ex54} vs *VHL*$^{-/+}$ *DJ-1β*^{ex54}: $P < 0.001$, n = 120 for each genotype). All oxidative stress assays were carried out at 25°C and were repeated at least twice. (C) Graphs of the average number of DA neurons within the DL1, DM, PM, and DL2 clusters of adult brains from 6-day-old flies after H_2O_2 treatment (n = 30 for each genotype). (D) Graphs of the average number of DA neurons within the DL1, DM, PM, and DL2 clusters of adult brains from 6-day-old *elav*-GAL4 control flies (*elav*), *DJ-1β* null mutants (*elav DJ-1β*^{ex54}), and Sima-overexpressing *DJ-1β* null mutants (*elav>sima DJ-1β*^{ex54}) after H_2O_2 treatment (n = 20 for each genotype). (E) Schematic linear map of putative HREs in the *PDK* locus. The sequences of two putative HREs are presented (HRE1: CACGC; HRE2: ACGTG) (Semenza et al., 1996). The location of each HRE is indicated using the first nucleotide preceding the ATG codon as 1. The site-directed mutagenesis performed on each HRE is also represented. (F) Comparison of the luciferase activity of *PDK* reporters in Sima-transfected S2 cells (n = 3). The reporter plasmids with wild type (WT), HRE1 site-mutated (HRE1), HRE2 site-mutated (HRE2), or HRE1 & HRE2-mutated (HRE1,2) *PDK* promoters were co-transfected to quantitatively measure the activation of each promoter by the Sima transcription factor (n = 3). The construction of the *PDK* reporters is described in the Materials and Methods section. Significance was determined by one-way ANOVA with Sidak correction. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; NS, not significant ($P > 0.05$). Error bars indicate mean ± SD.

tyl-CoA (Jha et al., 2012). PDC activity is tightly regulated by PDK- and PDP-induced phosphorylation of the PDH E1 subunit, which is the first component of PDC (Park et al., 2018). Recent studies have revealed the role of PDKs in the development of various metabolic diseases and cancers (Park et al., 2018). Accumulating evidence suggests that alterations in PDKs are associated with the development of several neurological disorders (Jha et al., 2012).

Mammals exhibit four PDK isoforms with different tissue expression levels and functions that are encoded by independent genes (Park et al., 2018). The *Drosophila* PDK ortholog is encoded by a single gene, *PDK*. *PDK*^{P} is a *Drosophila* mutant line with a P-element insertion in its first exon (Fig. 1A). *PDK*^{P} flies could successfully develop into adults (Fig. 1B), but were found to exhibit a severe reduction in PDK transcript levels via quantitative RT-PCR analysis (Fig. 1C). In contrast, there was no change in *pre1* transcript levels, which is a gene adjacent to the P-element insertion site. Such findings indicate that P-element insertion specifically inhibits *PDK* expression in *PDK*^{P} mutants (Fig. 1C).

To understand the physiological functions of PDK, we analyzed ATP levels, which indicate mitochondrial function, and mtDNA content, which allowed us to quantify the number of mitochondria in the indirect flight muscles of the *PDK*^{P} mutants. ATP levels remained unchanged in both 3- and 10-day-old *PDK*^{P} mutants compared with wild-type controls of the same age (Fig. 1D). Similarly, the mtDNA content did not change significantly in 3- or 10-day-old *PDK*^{P} mutants (Fig. 1E), suggesting that general mitochondrial function and abundance were maintained in the *PDK* mutants. However, under oxidative stress induced by rotenone, a specific inhibitor of mitochondrial respiratory chain complex I, the survival rate of *PDK*^{P} flies radically declined (Fig. 1F). Moreover, the *PDK*^{P} mutants were found to be highly sensitive to H_2O_2 treatment (Fig. 1G), suggesting a protective role of PDK against oxidative stress.

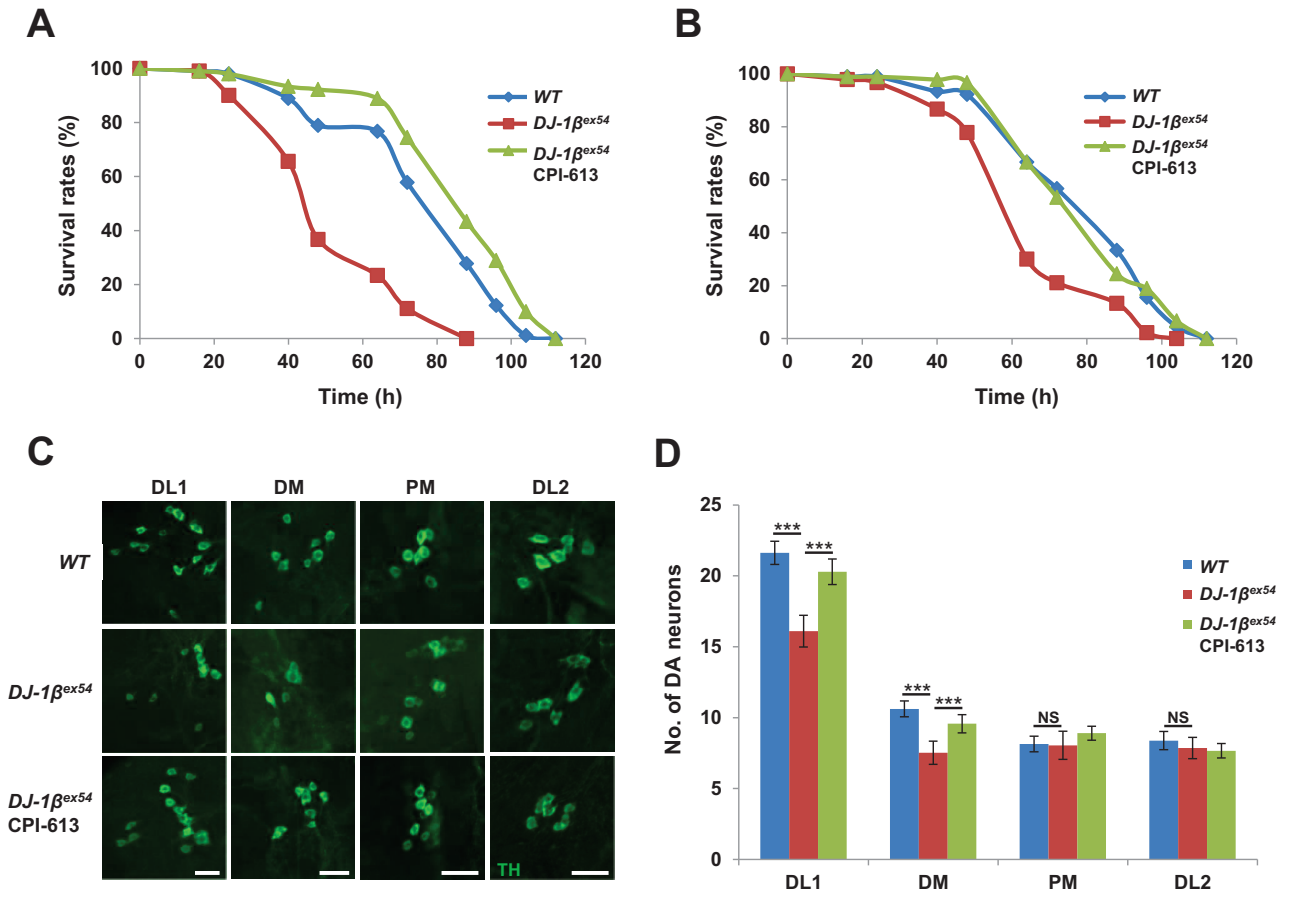

Fig. 5. The PDH inhibitor, CPI-613, protected *DJ-1β* null flies from oxidative stress. (A and B) Survival curves of wild-type (*WT*), *DJ-1β* null mutants (*DJ-1β*^{ex54}), and CPI-613-treated *DJ-1β* null mutants (*DJ-1β*^{ex54} CPI-613) fed 1% H_2O_2- (A) or 0.2 mM rotenone- (B) containing food (log-rank test: *WT* vs *DJ-1β*^{ex54} and *DJ-1β*^{ex54} vs *DJ-1β*^{ex54} CPI-613: $P < 0.001$, n = 90 for each genotype). All oxidative stress assays were carried out at 25°C and were repeated at least twice. (C and D) Confocal images (C) and graphs (D) of the average number of DA neurons within the DL1, DM, PM, and DL2 clusters of adult brains from 6-day-old flies after rotenone treatment. DA neurons were stained with an anti-TH antibody (green) (n = 21 for each genotype). Significance was determined by one-way ANOVA with Sidak correction. ***$P < 0.001$; NS, not significant ($P > 0.05$). Error bars indicate mean ± SD. Scale bars = 20 μm.

PDK restores the oxidative stress-induced phenotypes of *DJ-1β* mutant flies

Sensitivity to oxidative stress is closely related to neurodegenerative diseases, especially PD. ROS sensitivity is markedly increased in flies lacking *DJ-1β*, which is most closely linked to oxidative stress (Henchcliffe and Beal, 2008; Park et al., 2005). Under rotenone treatment, *DJ-1β* null mutants (*hs DJ-1β*^{ex54}) showed decreased survival rates compared to controls (*hs*), as previously reported (Yang et al., 2017) (Fig. 2A). Notably, PDK overexpression in *DJ-1β* null mutants was demonstrated to rescue the decreased survival rates of *DJ-1β* null mutants (Fig. 2A). In addition, PDK overexpression substantially increased the survival rate of *DJ-1β* mutants in media containing H_2O_2 (Fig. 2B). To further investigate the PDK-induced changes in the phenotype of *DJ-1β* mutants, we stained adult *Drosophila* brains with an anti-TH antibody to determine the number of DA neurons. In *Drosophila* brain, most DA neurons are found in four major DA neuron clusters: dorsolateral cluster 1 (DL1), dorsomedial cluster (DM), posteromedial cluster (PM), and dorsolateral cluster 2 (DL2) (Park et al., 2006). Under oxidative stress, *DJ-1β* mutants showed a significant decrease in the number of DA neurons in the DL1 and DM clusters (Figs. 2C and 2D), as previously reported (Yang et al., 2017). PDK overexpression also successfully inhibited DA neuron degeneration in both clusters of *DJ-1β* mutants (Figs. 2C and 2D). These results indicate that PDK can rescue oxidative stress-induced phenotypes in *DJ-1β* null mutants, confirming the protective role of PDK against oxidative stress.

Loss of *PDP* rescues the *DJ-1β* mutant phenotypes induced by oxidative stress

PDKs phosphorylate and inhibit PDH, while PDPs catalyze the reverse reaction, leading to PDH activation. There are two PDP isoforms (PDP1 and PDP2) in mammals (Park et al., 2018), whereas only one PDP isoform exists in *Drosophila*. In this study, we identified a *PDP* mutant (*PDP*^{P}) from the Genexel library that had an EP-element insertion in the first

exon (Fig. 3A). As expected, PDP^{P} mutants showed reduced *PDP* mRNA expression levels, but no significant change in the expression of *Rab39*, a gene located next to *PDP* (Fig. 3B). Notably, the induction of *PDP* mutations markedly rescued the decreased survival rates of *DJ-1β* mutants under H_2O_2 treatment (Fig. 3C). Moreover, *PDP* mutations suppressed H_2O_2-induced DA neuron loss in *DJ-1β* mutants (Figs. 3D and 3E). Such findings indicate that the loss of *PDP* can rescue oxidative stress-induced phenotypes in *DJ-1β* mutants, which were also attenuated by PDK overexpression. Accordingly, PDH downregulation can protect *DJ-1β* mutants from oxidative stress.

The HIF-1 pathway regulates PDK expression levels in a DJ-1-dependent manner

Based on its protective role against oxidative stress, we investigated PDK expression levels under oxidative stress. Surprisingly, H_2O_2 treatment increased PDK mRNA expression levels in wild-type flies but not in *DJ-1β* null mutants (Fig. 4A). Such finding suggests that oxidative stress regulates *PDK* expression levels in a DJ-1 dependent manner. Based on previous studies, DJ-1 is an important regulator of HIF-1, a transcription factor that is activated by hypoxia and oxidative stress (Vasseur et al., 2009). Under normal oxygen or ROS levels, the alpha subunit of HIF-1 (HIF-1α) is ubiquitinated by the von Hippel Lindau (VHL) protein and finally degraded by the proteasome (Kamura et al., 2000; Tanimoto et al., 2000). However, hypoxia or oxidative stress inhibits the VHL-mediated degradation of HIF-1α and induces the transcription of genes involved in adaptive responses. Interestingly, DJ-1 was demonstrated to interact directly with VHL via biochemical screening, and a subsequent study found that DJ-1 stabilizes HIF-1α by preventing its association with VHL (Parsanejad et al., 2014). Previous studies have also revealed that HIF-1 directly transactivates PDKs gene (Kim et al., 2006; Kirito et al., 2009). Thus, we hypothesized that HIF-1 may be a transcription factor that induces *PDK* expression under the control of DJ-1. *VHL* mutations in the *DJ-1β* mutants were found to recover the H_2O_2-induced increase in *PDK* mRNA expression levels (Fig. 4A). In addition, *VHL* mutations increased the survival rate of *DJ-1β* mutants (Fig. 4B) and suppressed DA neuronal death in *DJ-1β* null flies under oxidative stress (Fig. 4C). Consistently, the overexpression of Sima, a *Drosophila* ortholog of mammalian HIF-1α, protected DA neurons from oxidative stress in *DJ-1β* mutants (Fig. 4D). Two putative HIF-1 binding elements, also known as hypoxia response elements (HREs) (Semenza et al., 1996), were identified near the PDK transcription start site (Fig. 4E). Thereafter, the *PDK* promoter region was cloned into a luciferase reporter plasmid, and Sima cDNA and the HRE reporter plasmid were cotransfected into the *Drosophila* S2 cell line. Based on the results, the promoter activity was increased by Sima expression and decreased by HRE1 mutations (Fig. 4F). Therefore, DJ-1 was concluded to control *PDK* expression via the VHL-HIF-1 pathway to protect DA neurons from oxidative stress.

CPI-613 protects *DJ-1β* null mutants from oxidative stress

Owing to recent correlations between mitochondrial metabolism and cancer, small molecules that can modulate PDH activity have been developed. Zachar et al. (2011) developed CPI-613, a lipoate analog that resembles PDH catalytic intermediates and can inactivate PDH via PDK activation. As expected, CPI-613 induced PDK-dependent phosphorylation of PDH and subsequently inhibited PDC activity (Zachar et al., 2011). Thus, we investigated whether CPI-613 administration rescued oxidative stress-induced defects in *DJ-1β* mutant flies. Briefly, 3-day-old flies were treated with CPI-613 (10 mM) or vehicle alone, and their survival under oxidative stress was determined. Without CPI-613 treatment, *DJ-1β* null mutants showed decreased survival rates compared with wild-type controls after incubation in H_2O_2- (Fig. 5A) or rotenone-containing media (Fig. 5B). However, CPI-613 treatment markedly rescued the decreased survival rates of *DJ-1β* mutants in both media (Figs. 5A and 5B). Moreover, CPI-613 suppressed oxidative stress-induced DA neuronal death in the DL1 and DM clusters of *DJ-1β* null flies (Figs. 5C and 5D). Overall, these findings confirm that PDH inhibition can rescue oxidative stress-induced *DJ-1β* mutant phenotypes, suggesting that CPI-613 may suppress DJ-1-associated pathogenesis.

DISCUSSION

In this study, we isolated and characterized the *Drosophila PDK* mutants. Although mammals have four PDK isoforms encoded by four independent genes, *Drosophila* has only one PDK isoform. Thus, *PDK* expression could easily be silenced to investigate its *in vivo* functions in *Drosophila*. Notably, our *PDK* mutants successfully developed into adults (Fig. 1B) and showed no significant defects in ATP levels or mtDNA content (Figs. 1D and 1E). However, *PDK* mutant flies were identified to be sensitive to oxidative stress induced by rotenone and H_2O_2 (Figs. 1F and 1G), suggesting that PDK plays a critical role in response to external stress factors, including oxidative stress. To further test the role of PDK in the response to oxidative stress, we introduced the PDK transgene into PD model flies with the deletion of *DJ-1β*, as *DJ-1* is the PD gene most closely associated with oxidative stress. The overexpression of PDK in *DJ-1β* mutants successfully rescued oxidative stress-induced phenotypes, such as the decreased survival rates and DA neuron loss (Fig. 2). In addition, *PDP* mutations suppressed the loss of DA neurons in *DJ-1β* mutants under oxidative stress (Fig. 3), further confirming the neuroprotective role of PDK against oxidative stress.

Consistent with our results, PDCs and their regulators are critically involved in various neurological disorders. PDC deficiency in fetus causes congenital lactic acidosis and CNS malfunction, which ranges from minimal motor impairment to profound intellectual disability with motor deficits (Pliss et al., 2016). A missense mutation in *PDK3* is linked to Charcot–Marie–Tooth (CMT) disease, a hereditary motor and sensory disorder of the peripheral nerve (Kennerson et al., 2013). Interestingly, Halim et al. (2010) found that PDC activity in different cell types, including the nervous system and astrocytes, a type of glial cell, is strongly inhibited via phosphorylation due to higher PDK expression compared to neurons. This specific inhibition of PDC in astrocytes increases the production of lactate, which is metabolized by the TCA cycle in neurons. Such findings suggest that PDK is the molecular mediator of

the metabolic interaction proposed in the astrocyte-neuron lactate shuttle hypothesis (Jha et al., 2012). In contrast, neuronal PDCs showed markedly lower levels of phosphorylation (Halim et al., 2010), indicating that in neurons, PDCs operate at almost maximal levels to perform the energy-consuming transport of neurotransmitters under normal conditions. However, a recent head trauma model study suggested a neuroprotective role for PDC inhibition under oxidative stress (Egge et al., 2021). In this study, genetic preconditioning to reduce cytochrome C oxidase function prevented trauma-induced ROS production and the subsequent loss of DA neurons in the impacted brain. Further analyses also revealed the induction of PDK expression and PDH phosphorylation in the preconditioned brain. Consistently, dichloroacetate, a specific inhibitor of PDK, nullified neuroprotection in preconditioned mice. Moreover, without preconditioning, *PDP* knockdown suppressed the degeneration of DA neurons after trauma, suggesting that modulating PDC activity is sufficient to suppress trauma-induced neurodegeneration.

According to previous studies, the expression of PDK isoforms is regulated by various stress factors, such as starvation, hypoxia, and oxidative stress (Jha et al., 2012). Interestingly, H_2O_2 treatment induced *PDK* mRNA expression in *Drosophila*, whereas *DJ-1β* deletion nullified this effect, suggesting that oxidative stress induces *PDK* expression in a DJ-1-dependent manner (Fig. 4A). To protect cells from ROS, DJ-1 plays multifaceted roles in the antioxidant defense system to provide sufficient reducing power for the antioxidant enzyme network. DJ-1 also promotes glutathione synthesis and glutathione reductase expression (Meiser et al., 2016; Zhou and Freed, 2005), and facilitates the nuclear translocation of the transcription factor, Nrf2 (Clements et al., 2006), thereby inducing the expression of antioxidant enzymes, such as heme oxygenase-1 (HO-1), thioredoxin 1 (Trx1) (Im et al., 2012), and isocitrate dehydrogenase (IDH) (Yang et al., 2017). DJ-1 activates the expression of uncoupling protein 4 (UCP4) via the NF-kB pathway (Xu et al., 2018), and subsequently induces "mild uncoupling," which prevents a superfluous proton gradient and ROS formation (Ramsden et al., 2012). The molecular link between DJ-1 and PDK in response to oxidative stress indicates that DJ-1 protects DA neurons from ROS via PDK expression. When the relationship between DJ-1 and PDK was assessed, the overexpression of HIF-1α, a well-known transcription factor that plays a role in the response to hypoxia and oxidative stress, was shown to suppress oxidative stress-induced DA neuron loss in *DJ-1β* mutants (Fig. 4D). Further genetic analyses demonstrated that a *VHL* loss-of-function mutation restored *PDK* induction and the survival rates of *DJ-1β* mutants under oxidative stress (Figs. 4B and 4C). In addition, HIF-1α directly induced *PDK* expression in *Drosophila* S2 cells (Fig. 4F), supporting HIF-1α as a molecular mediator that links DJ-1 and PDK under oxidative stress. Consistent with our findings, Parsanejad et al. (2014) reported that DJ-1 stabilizes HIF-1α by inhibiting the HIF-1α-VHL interaction. Other studies have also shown that HIF-1 transactivates the mammalian PDK isoform, PDK1, which inhibits PDC and subsequently drives the metabolic transition from mitochondrial respiration to glycolysis to prevent toxic ROS production from mitochondria under cellular stress (Kim et al., 2006; Kirito et al., 2009). Thus, we suggest that DJ-1 transactivates PDK, a metabolic switch to aerobic glycolysis, to reduce toxic ROS generation via the VHL-HIF-1 pathway and protect DA neurons in response to oxidative stress.

Our genetic data suggest that PDC and its regulators may be putative molecular targets for the treatment of *DJ-1* deficiency-associated diseases. As a result, we treated *DJ-1β* mutant flies with CPI-613, a tricarboxylic acid (TCA) cycle inhibitor that targets PDH (Zachar et al., 2011). As expected, CPI-613 treatment markedly rescued the decreased survival rate of *DJ-1β* mutants induced by oxidative stress (Figs. 5A and 5B). CPI-613 treatment also successfully ameliorated oxidative stress-induced DA neuron loss in *DJ-1β* mutants (Figs. 5C and 5D). Collectively, these results suggest that PDH inhibitors developed for the treatment of various cancers or metabolic diseases should be further evaluated as treatments for human pathologies induced by *DJ-1* deficiency, including PD.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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AUTHOR CONTRIBUTIONS

Y.L., J.K., H.K. (Hyunjin Kim), and J.E.H. designed and performed experiments. Y.L., J.K., and H.K. (Hyongjong Koh) wrote the manuscript. S.K. and K.K. analyzed the data. D.K., J.M.K., and H.K. (Hyongjong Koh) supervised this study.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

ORCID

Yoonjeong Lee https://orcid.org/0000-0003-2359-2427
Jaehyeon Kim https://orcid.org/0000-0001-7576-7992
Hyunjin Kim https://orcid.org/0000-0002-0936-456X
Ji Eun Han https://orcid.org/0000-0001-7939-4142
Sohee Kim https://orcid.org/0000-0002-7457-4029
Kyong-hwa Kang https://orcid.org/0000-0003-3124-2988
Donghoon Kim https://orcid.org/0000-0002-7493-0935
Jong-Min Kim https://orcid.org/0000-0002-6201-1739
Hyongjong Koh https://orcid.org/0000-0003-2594-4799

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