The Loss of PGAM5 Suppresses the Mitochondrial Degeneration Caused by Inactivation of PINK1 in Drosophila

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Abstract

PTEN-induced kinase 1 (PINK1), which is required for mitochondrial homeostasis, is a gene product responsible for early-onset Parkinson’s disease (PD). Another early onset PD gene product, Parkin, has been suggested to function downstream of the PINK1 signalling pathway based on genetic studies in Drosophila. PINK1 is a serine/threonine kinase with a predicted mitochondrial target sequence and a probable transmembrane domain at the N-terminus, while Parkin is a RING-finger protein with ubiquitin-ligase (E3) activity. However, how PINK1 and Parkin regulate mitochondrial activity is largely unknown. To explore the molecular mechanism underlying the interaction between PINK1 and Parkin, we biochemically purified PINK1-binding proteins from human cultured cells and screened the genes encoding these binding proteins using Drosophila PINK1 (dPINK1) models to isolate a molecule(s) involved in the PINK1 pathway. Here we report that a PINK1-binding mitochondrial protein, PGAM5, modulates the PINK1 pathway. Loss of Drosophila PGAM5 (dpGAM5) can suppress the muscle degeneration, motor defects, and shorter lifespan that result from dPINK1 inactivation and that can be attributed to mitochondrial degeneration. However, dpGAM5 inactivation fails to modulate the phenotypes of parkin mutant flies. Conversely, ectopic expression of dpGAM5 exacerbated the dpINK1 and Drosophila parkin (dparkin) phenotypes. These results suggest that PGAM5 negatively regulates the PINK1 pathway related to maintenance of the mitochondria and, furthermore, that PGAM5 acts between PINK1 and Parkin, or functions independently of Parkin downstream of PINK1.

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Introduction

Parkinson’s disease (PD (OMIM #168600)) is a neurodegenerative disease that affects the maintenance of dopaminergic (DA) neurons. PD prevalence is estimated at ~1% among people over the age of 65 and increases with age. Clinical features of PD include motor abnormalities (tremor, rigidity, akinesia), autonomic disturbances, psychiatric disability and cognitive impairment. The recent identification of PD-associated genes has advanced our understanding of the molecular mechanisms underlying PD. Two of these genes, PINK1 (PARK6, OMIM #605909, Gene ID: 65018) and parkin (PARK2, OMIM #600116, Gene ID: 5071), are associated with early-onset autosomal recessive PD, in which loss-of-function (LOF) of a single gene product results in the clinical manifestation of Parkinsonism [1,2]. The PINK1 gene encodes a serine/threonine kinase with a predicted mitochondrial target sequence and a probable transmembrane domain at the N-terminus [3]. The gene product of the parkin gene encodes a protein with an E3 activity [4-6]. Recent genetic studies in Drosophila have reported that dPINK1 (Gene ID: 31607) acts as an upstream regulator of dparkin (Gene ID: 40336) in a common pathway that influences mitochondrial maintenance in a subset of tissues, including the flight muscle and DA neurons [7-9]. LOF of the dPINK1 or the dparkin genes results in enlarged or swollen mitochondria, a phenotype that can be partially rescued by heterozygosity for LOF mutations of the mitochondrial fusion-promoting components optic atrophy 1 (OPA1) and Mitofusin (Mfn), or by increased mitochondrial fission activity via increased dosage of the dynamin-related protein 1 (dnap1) gene [10-12]. Studies in mammalian or Drosophila cultured cells report that PINK1 is required to recruit Parkin to damaged depolarized mitochondria, and promotes their degradation through an autophagic event called mitophagy [13–16]. Thus, there is strong evidence to support an important role for PINK1 and Parkin in regulating...
Isolation of PGAM5 as a PINK1-Binding Protein

We and others have previously demonstrated that PINK1 is genetically upstream of parkin [7–9]. To further investigate the relationship between PINK1 and Parkin, we searched for PINK1-binding proteins using a combination of biochemical purification and mass spectrometric analysis. We affinity-purified human PINK1 with a FLAG tag at its C-terminus (hPINK1-FLAG) from lysate of human embryonic kidney (HEK) 293 cells stably expressing hPINK1-FLAG using an anti-FLAG column, and elution fractions, which include cytoskeleton-related proteins (MAP1B (GeneID: 4131), KIF11 (GeneID: 3833), Tubulin (GeneID: 602530, 191130)), proteasome subunits (PSMD1 (GeneID: 5707), PSMD2 (GeneID: 5708), PSMC6 (GeneID: 5706)), PRKDC (GeneID: 5591), Hsp70 (1A, GeneID: 3303; 1B, GeneID: 3304), Hsp90 (GeneID: 3320), Cdc37 (GeneID: 11140), Insulin substrate-4 (IRS-4, GeneID: 8471) and PGAM5 (Figure 1A). PRKDC is one of proteins non-specifically associated with FLAG-tagged proteins in our proteomic analyses (data not shown). The roles of Hsp90, Cdc37 and the proteasome for PINK1 have been characterized previously [15,19–22]. We therefore chose IRS-4 and PGAM5 and tested whether these proteins modulate the dPINK1 LOF phenotypes by Drosophila genetics. Our initial in vivo tests revealed that a mutant allele for dpGAM5 (CG14816, GeneID: 31145), PGAM5NP0568 significantly suppressed the abnormal wing postures observed in dPINK1 knockdown flies [9] (Figure 1B), while it failed to improve the viability (Figure 1C). Reducing the dose of chico (GeneID: 64890), which encodes a Drosophila orthologue of IRS-4, significantly suppresses the short lifespan phenotype caused by dPINK1 knockdown, without affecting wing posture (Figure 1D and 1E). Inhibition of chico activity has previously been reported to extend the lifespan of Drosophila, such that we reasoned that the effect on lifespan we observed might reflect a general phenomenon rather than reflecting a specific interaction with dPINK1 [23]. Thus for subsequent studies, we chose to focus on PGAM5.

The results of co-immunoprecipitation confirmed that C-terminally Myc-tagged human PGAM5 (hPGAM5-Myc) specifically binds to hPINK1-FLAG in transfected HEK293 cells (Figure 2A). Moreover, we found that hPGAM5 and hPINK1 immunoreactivity co-localizes with mitochondria in transfected HeLa cells, consistent with the previous finding that PGAM5 is localized to the mitochondria (Figure 2B and 2C) [24]. To test if endogenous hPGAM5 interacts with hPINK1, we first generated an anti-hPGAM5 antibody (Figure 2D). Next, we used a previously established anti-PINK1 antibody to immunoprecipitate PINK1 from HEK293 cell lysate, then probed with anti-PGAM5 to detect endogenous hPGAM5. As shown in Figure 2E, endogenous hPGAM5 was detectable in the fraction immunoprecipitated using anti-hPINK1 antibody but not a control antibody, confirming the results of mass spectrometric analysis. Physical association of dPINK1 with dpGAM5 was also observed in Drosophila S2 cells (Figure 2F), suggesting that their functional interaction is conserved between human and Drosophila.

Previous findings that PINK1 and PGAM5 possess kinase and phosphatase activities, respectively [18,25,26], prompted us to test the possibility of their enzyme-substrate relationships. A mobility shift assay to monitor the status of PINK1 phosphorylation suggested that overexpression of hPGAM5 has little effect on hPINK1 phosphorylation (Figure 2G). On the other hand, an in vitro kinase assay using recombinant dPINK1 failed to show a possibility that PGAM5 is a substrate for PINK1, or that PGAM5 modifies hPINK1 kinase activities (Figure 2H).

dPGAM5 Alters Mitochondrial Morphology in Drosophila

The Drosophila genome appears to have two orthologs of mammalian PGAM5, one on the X (CG14816) and the other on the second chromosome (CG15874, GeneID: 37899). We have renamed CG14816 and CG15874 as dPGAM5 and dPGAM5-2, respectively. Our initial in vivo genetic study and most subsequent analyses were performed using dPGAM5 mutant and transgenic animals because dPGAM5 is more similar to hPGAM5 than is dPGAM5-2 (dPGAM5 vs. hPGAM5, 44% amino acid identity, and dPGAM5-2 vs. hPGAM5, 38% identity, as determined using ClustalW v1.4 to align the sequences), and because the results of high-throughput analysis of transcript abundance suggest that the dPGAM5-2 transcript is expressed at very low levels at the adult stage, if at all (see http://flybase.org/reports/FBgn0035004.html). We determined the P-element insertion allele PGAM5NP0568 as a hypomorph allele, which showed a reduction of dPGAM5 transcript levels to about 25% of normal levels (Figure S1). We then generated a dPGAM5 null allele PGAM55, in which the expression of dPGAM5 completely disappeared at both the transcript and protein levels (Figure S1B and S1C). The PGAM55 homozygous animal is viable, fertile and grossly normal. However, it displayed longer lifespan (Figure 3A). By contrast, overexpression of dPGAM5 or dPGAM5-2 resulted in shorter longevity (Figure 3B). Since a previous report described that overexpression of human PGAM5 affects the mitochondrial morphology or
mobility in the cultured cells [24], we observed the mitochondria in the dPGAM5 null and transgenic flies. Although inactivation of dPGAM5 gene function did not cause mitochondrial degeneration, the morphology of the mitochondria appears to be moderately altered (Figure 3D and 3H compared to Figure 3C and 3G). The mitochondria in the indirect flight muscles of the PGAM5 mutant flies were longer in the long-axis direction compared to control animals (Figure 3K). A similar tendency was seen in DA neurons of the adult brain although the difference did not reach statistical significance (Figure 3L and 3M). In addition, we frequently observed constrictions in the mitochondria (see broken lines in Figure 3D). In contrast, transgenic expression of dPGAM5 or dPGAM5-2 in Drosophila leads to fragmentation of mitochondria, with cristae well-preserved in the indirect flight muscles (Figure 3E, 3F, 3I, and 3J) and in the tyrosine-hydroxylase (TH)-positive neurons of the adult fly brain (Figure 3N and 3O). These results suggested that dPGAM5 is likely to promote the mitochondrial fission process in Drosophila.

Figure 1. Identification of PINK1-binding proteins that modulate the phenotypes of dPINK1 knockdown fly. (A) Silver-stained polyacrylamide gel to visualize hPINK1-binding proteins. FLAG elution fractions purified from cells stably expressing hPINK1-FLAG (PINK1-FLAG lane) and parental cells (Control lane) are separated on a gel (For details of the procedure, see Materials and Methods). Bands corresponding to hPINK1 (dots) and representative co-purified proteins are indicated. (B, C) The wing phenotype typical of 10- and 20-day-old dPINK1 RNAi flies [9] (B) was suppressed by the PGAM5np0568 mutant allele, whereas viability of 10-, 20- and 30-day-old adult flies was not improved (C). *, p<0.05; **, p<0.01 vs. age-matched dPINK1 RNAi group in Student's t-test. The genotypes are as follows: MHC-GAL4:: dPINK1RNAi (+/+), PGAM5np0568/Y; MHC-GAL4:: dPINK1RNAi (PGAM5np0568/Y); MHC-GAL4:: dPINK1RNAi (PGAM5np0568/+); MHC-GAL4:: dPINK1RNAi (PGAM5np0568/+). MHC-GAL4, a muscle-specific driver. Flies were raised at 29°C. doi:10.1371/journal.pgen.1001229.g001
Figure 2. PGAM5 associates with PINK1 at mitochondria. (A) hPGAM5 binds to hPINK1 in HEK293 cells. Lysate expressing C-terminally Myc-tagged hPGAM5 (hPGAM5-Myc) and FLAG-tagged hPINK1 (hPINK1-FLAG) was subjected to immunoprecipitation with anti-FLAG antibody (FLAG-IP), and analyzed by immunoblotting with anti-tag antibodies. (B) hPGAM5 is localized to the mitochondria. HeLa cells transfected with hPGAM5-Myc were visualized with anti-Myc (green). Mitochondria were visualized with MitoTracker (red) and nuclei with DAPI (blue). Regions of co-localization of hPGAM5 with mitochondria appear in yellow in the merged image. (C) hPGAM5 and hPINK1 co-localize at mitochondria. HeLa cells co-transfected with hPINK1 and hPGAM5-Myc were stained with anti-PINK1 (green) and anti-Myc (red). (D) Anti-hPGAM5 antibody specifically recognizes 30 kDa bands in extract from HEK293 cells, which were reduced in lysates from cells treated with siRNAs directed against hPGAM5. Lysate expressing hPGAM5-Myc and anti-tubulin signals served as a positive control and a loading control, respectively. (E) Endogenous hPGAM5 is associated with hPINK1. An anti-PINK1 (PINK1-IP) or an antibody against the unrelated protein Delta (Control-IP) was used for immunoprecipitation of proteins in HEK293 cells. Cell lysate in which hPINK1 was knocked down by RNAi (PINK1 RNAi) and lysate from cells that overexpressed hPINK1-FLAG (PINK1-FLAG) served as additional controls. The PINK1-FLAG lysate was diluted eight-fold with loading buffer to reduce the strong signal present in that sample. Asterisk, bands attributable to detection of the antibodies themselves, which may mask lower molecular weight hPINK1 bands (~52 kDa). (F) dPGAM5 is associated with dPINK1 in Drosophila S2 cells. S2 cell lysate expressing dPINK1-Myc and dPGAM5-FLAG was subjected to immunoprecipitation with anti-Myc antibody (Myc-IP), and analyzed by immunoblotting with anti-tag or anti-dPGAM5 antibodies. Asterisks, a putative processed form of dPGAM5. (G) HEK 293 cell lysate expressing hPINK1-FLAG together with hPGAM5-Myc was subjected to Phos-tag immunoblotting [43]. hPINK1-FLAG lysate treated with alkaline phosphatase (CIP) was used as a positive control. A phospho-protein FoxO1 was efficiently dephosphorylated by the CIP treatment. (H) An in vitro kinase assay was performed using 2x GST-dPINK1 and GST-hPGAM5. Recombinant
The Relationship between PGAM5 and the Mitochondrial Fission/Fusion Machinery

Evolutionarily-conserved GTPases Mfn and OPA1 promote the mitochondrial fusion event while another GTPase Drp1 regulates the mitochondrial fission [27,28]. To determine the role of PGAM5 in the mitochondrial fission pathway, we manipulated the activities of the genes that are involved in mitochondrial fission/fusion in dPGAM5 null flies. Decreased Mfn activity resulted in fragmented mitochondria in the indirect muscle tissues, which was not affected by removal of the dPGAM5 gene (Figure 4A–4D and 4G, Figure S2). Conversely, an increased mitochondrial fission activity by introducing an extra copy of the dpl1 gene was not suppressed in the dPGAM5 null genetic background (Figure 4E–4G). These results suggested that dPGAM5 may function upstream of Mfn or Drp1, or that the mechanism of the mitochondrial morphological changes by dPGAM5 is independent of that of the known fission/fusion components.

dPGAM5 Modulates Phenotypes Caused by dPINK1 Inactivation in Drosophila

We next confirmed that the results of the genetic tests in Figure 1B and 1C using a LOF allele for dPINK1, PINK1B9 to exclude off-target effects due to RNAi (Figure 5). Adult PINK1B9 flies often have abnormal thoraces with dents in the mid-anterior region, which is likely due to degeneration of the muscle tissues lining the inside of the thorax (Figure 5B) [8]. This thorax phenotype seen in PINK1B9 flies can be suppressed by introduction of the PGAM5NP0568 or the PGAM5 allele (Figure 5A and 5D). We then examined the effects of dPGAM5 inactivation on dPINK1 mutant phenotypes that progressively increase over time. As described above, loss of dPINK1 activity leads to the appearance of abnormal wing postures, which is indicative of flight muscle degeneration, and the percent of affected flies increases with advancing age (Figure 5C and 5F) [8]. Introduction of the dPGAM5 mutant alleles dramatically suppresses this phenotype (Figure 5E and 5F), whereas ectopic expression of dPGAM5 enhances the phenotype (Figure 5G). Progressive loss of climbing ability and the shorter lifespan of PINK1B9 flies are additional prominent phenotypes that may represent dysfunction of DA neurons of the central nervous system and muscle degeneration. The dPGAM5 mutant alleles also significantly improved these phenotypes (Figure 5H and 5J). Conversely, overexpression of dPGAM5 worsened the phenotypes (Figure 5I and 5K).

Transmission electron microscopy (TEM) sections from one day-old adult PINK1B9 mutant flies reveal that mitochondria in the indirect flight muscles are abnormally fused with one another and that the structures of the mitochondrial cristae are unclear (i.e. the cristae have lost the normal electron density seen by TEM) as compared to those of a dPINK1 revertant line (Figure 6A and 6D compared to Figure 3C and 3G). Importantly, the mitochondrial hyperfusion and loss of cristae usually observed in dPINK1 mutant animals can be partly suppressed by introduction of the PGAM5NP0568 or the PGAM5 allele (Figure 6B, 6E, and 6G). In sharp contrast, transgenic expression of dPGAM5 further promoted mitochondrial degeneration (Figure 6C and 6F). Similar results were obtained when mitochondria in DA neurons of the adult brain (Figure 6H, 6I, and 6J) and in the indirect muscle tissues (Figure S3) were visualized using a version of GFP with a mitochondrial targeting signal (mitoGFP). Mitochondrial morphology in DA neurons in wild-type flies showed a long tubular network in the cytoplasm (Figure 3L). As previously reported, DA neurons in PINK1B9 flies form spherical aggregates of mitochondria (Figure 6L). Removal of dPGAM5 from PINK1B9 flies led to an increase in the number of small fragmented or tubular mitochondria (Figure 6J). These results suggest that excessive mitochondrial aggregation, which is modulated by dPGAM5 inactivation, is indicative of a functional failure of mitochondria in DA neurons.

Consistent with the beneficial effects of dPGAM5 inactivation on the mitochondrial degeneration seen in PINK1B9 flies, we observed that dPGAM5 inactivation suppresses the loss of DA neurons in the protocerebral posterior lateral 1 (PPL1) and protocerebral posterior medial 1 and 2 (PPM1/2) clusters of aged flies (Figure 6K–6M).

Removal of dPGAM5 Fails to Suppress Phenotypes Resulting from dparkin Inactivation

Previous studies in Drosophila suggested that dPINK1 is genetically associated with dparkin and furthermore, that dPINK1 functions upstream of dparkin [7–9]. In addition, dparkin null mutations cause mitochondrial degeneration of a subset of tissues in Drosophila, which phenocopies dPINK1 inactivation [29,30]. Given the evidence that PGAM5 is involved in the PINK1 pathway, we next asked if dPGAM5 also affects the in vivo mitochondrial phenotypes associated with mutations in dparkin. Introduction of PGAM5NP0568 in the parkin hypomorphic genetic background (parkinP21) had little effect on abnormal wing postures (Figure 7A–7C) [29,30]. Consistent with the result in the wing phenotype, loss of dPGAM5 activity failed to rescue the age-dependent motor defects and shorter lifespan observed in parkinP21 flies (Figure 7D and 7F). In the same settings, overexpression of dPGAM5 further enhanced both motor defect and reduced lifespan phenotype (Figure 7E and 7G). Loss of dparkin activity results in an elongated morphology in mitochondria of the adult indirect flight muscle tissues, a phenotype that was suppressed by loss of the dPGAM5 gene (Figure 7H, 7I, and 7L). However, the cristae structures of the mitochondria were not restored by inactivation of the dPGAM5 gene (Figure 7J and 7K).

Taken together, these data suggest that dPGAM5 lies genetically upstream of dparkin, or functions independently of dparkin downstream of dPINK1 in Drosophila.

Activation of a Redox Control Pathway Improves Viability of dPINK1 Mutant Flies

PGAM5 was previously reported to interact with Keap1 (Gene ID: 9817), a substrate adaptor protein for a Cullin-3-dependent E3
complex [17]. In a normal redox state, the Keap1 complex suppresses activity of a bZIP transcription factor, Nrf2, through ubiquitin/proteasome-dependent protein degradation [31]. Oxidative stress impairs inhibition of Nrf2 by Keap1 [31]. Nrf2 thus becomes stabilized and activates oxidative stress protective genes, restoring cellular redox homeostasis. Although we confirmed the association of PGAM5 with Keap1 in human cultured cells, the proposed Keap1-binding motif in PGAM5, NXXESGE, was not conserved in dPGAM5 (Figure S1). On the other hand, Keap1/Nrf2 signaling does appear to be conserved in Drosophila [32].

Figure 3. dPGAM5 is dispensable for normal development, but affects lifespan in Drosophila. (A) Loss of dPGAM5 genes extends the lifespan. Adult male wild-type (yw); n = 125) vs. dPGAM5 null (yw, PGAM51/Y; n = 125) flies, p<0.001 by log rank test. (B) Overexpression of dPGAM5 or dPGAM5-2 in Drosophila causes shorter lifespan. Overexpression of the transgenes was induced using the ubiquitous daughterless (Da)-GAL4 driver. Lifespan of adult male EGFP (n = 130), dPGAM5 (n = 76) and dPGAM5-2 (n = 117) flies. EGFP vs. dPGAM5, p<0.001; EGFP vs. dPGAM5-2, p<0.001; by log rank test. (C–J) Transmission electron microscopy (TEM) analysis of the indirect flight muscle and morphology of mitochondria in 2-day-old adult flies with the indicated genotypes. In C–F, we outlined some mitochondria with broken lines to highlight morphology. The insets in G–J show representative mitochondria matrixes. A revertant, PINK1RV, was used as a wild-type comparison [8]. The genotypes are: PINK1RV/Y (C, G), PGAM5NP0568/Y (D, H), Da-GAL4> UAS-dPGAM5 (E, I), Da-GAL4> UAS-dPGAM5-2 (F, J). Scale bars = 1 μm in C–F and 200 nm in G–J. (K) Quantification of the percentage of mitochondrial size distribution in the indirect flight muscle tissue from wild-type (n = 136 from 5 adult flies), PINK1RV/Y (n = 155 from 5), PGAM51 (n = 87 from 5), dPGAM5 Tg (n = 143 from 5) and dPGAM5-2 Tg flies (n = 147 from 5) as shown in (C–J). The length of the mitochondria in the direction of the myofibrils was measured. Data are shown as means ± SE (* p<0.05, ** p<0.01 vs. wild-type). (L–O) Brain tissues of 5-day-old adult flies were stained with anti-TH antibody (red). Mitochondria labeled with mitoGFP (green) were observed in the PPL1 TH-positive neurons of the indicated genotypes. The genotypes are as follows: TH-GAL4> mitoGFP (wild-type), PGAM51/Y; TH-GAL4> UAS-mitoGFP (PGAM511), UAS-dPGAM5/TH-GAL4> UAS-mitoGFP (dPGAM5 Tg), UAS-dPGAM5-2/TH-GAL4> UAS-mitoGFP (dPGAM5-2 Tg). TH-GAL4, a DA neuron-specific driver. Scale bar = 5 μm.

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Data are shown as means ± SE (**p<0.01; N.S., not significant). The genotypes are as follows: +/Y; MHC-GAL4/+; UAS-mitoGFP; UAS-dPGAM5/Y; MHC-GAL4/+; UAS-drp1; UAS-mitoGFP; UAS-mfn RNAi (VDRC40478) (C, mfn RNAi), PGAM5/Y; MHC-GAL4/+; UAS-drp1; UAS-mitoGFP; UAS-mfn RNAi (VDRC40478) (D, mfn RNAi; PGAM5/+; MHC-GAL4/+; UAS-drp1; UAS-mitoGFP; drp1/+ (E, drp1/+), PGAM5/Y; MHC-GAL4/+; UAS-drp1; UAS-mitoGFP; PGAM5. doi:10.1371/journal.pgen.1001229.g004

Discussion

The event of fusion/fission is required for maintenance of a healthy mitochondrial population. Mitochondrial fusion is believed to require the interchange of a set of internal components, including copies of the mitochondrial genome, respiratory proteins and metabolic products. Mitochondrial fission has been proposed to play a role in disposal of damaged mitochondria, such as those with a reduced mitochondrial membrane potential, via mitophagy [35]. A role for PINK1 in the regulation of mitochondrial fission/fusion dynamics has recently been demonstrated in Drosophila [10–12]. The PINK1/Parkin pathway appears to promote fission and/or inhibits fusion, likely through an indirect mechanism. Indeed, loss of dPINK1 or dParkin produces swollen or enlarged mitochondria in tissues with high-energy demands, such as the muscles, which is suppressed by reduced fusion activity or increased fission activity after genetic manipulation of the mitochondrial fission/fusion machinery. Namely, either reducing the activity of the mitochondrial fusion proteins OPA1 and Mfn, or increasing the activity of a mitochondrial fission protein, Drp1, can partially rescue PINK1 and parkin mutant phenotypes.

We identified PGAM5 as a PINK1-binding protein and went on to show that dPGAM5 can modulate dPINK1 mutant phenotypes. Loss of dPGAM5 activity had little effect on the lifespan of a dPINK1 RNAi fly strain in our initial in vivo test (Figure 1C). However, we found that loss of dPGAM5 does significantly extend lifespan of PINK1 mutant flies (Figure 5J). We speculate that continuous expression of the short hairpin RNA in the RNAi-based test confers additional toxicity, leading to a shorter lifespan in a sequence-independent manner, such that the suppressive effect of dPGAM5 mutations cannot be detected in the PINK1 RNAi flies.

dPGAM5 appears to be dispensable for mitochondrial homeostasis in Drosophila, as overall, flies homozygous for a null allele of dPGAM5, PGAM5<sup>−/−</sup>, appear to be normal. It has previously been reported that ectopic expression of PGAM5 leads to perinuclear aggregation or small fragmentation of mitochondria in mammalian cultured cells, which suggested that PGAM5 has a role in regulation of mitochondrial fission/fusion process or mobility [24].

Our study also observed alteration of mitochondrial morphology in Drosophila with different dPGAM5 activities. Transgenic expression of dPGAM5 or dPGAM5<sup>−/−</sup> leads to fragmentation of mitochondria both in the TH<sup>+</sup> neurons and indirect flight muscles (Figure 3E, 3F, 3I and 3J). By contrast, dPGAM5 LOF moderately increases mitochondria with a longer tubular or a swollen morphology (Figure 3D and 3K). Our genetic tests with the
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Figure 5. Loss of dPGAM5 suppresses dPINK1 mutant phenotypes in Drosophila. A thorax defect (B, arrowheads) and abnormal wing posture (C) caused by loss of dPINK1 activity are suppressed in dPGAM5 mutant genetic backgrounds (A, D and E). (F) Percentage of 10-, 20- and 30-day-old male flies showing abnormal wing postures. Error bars show S.E. from three experiments. (G) Percentage of 10-day-old male flies ubiquitously overexpressing dPGAM5 showing abnormal wing postures. Error bars show S.E. from three experiments. (H, I) Percentage of locomotor activity. Error bars show S.E. from three repeated experiments. (J) Lifespan of adult male flies. Loss of dPGAM5 partially improved the reduced lifespan seen in PINK1B9; dPGAM5 Tg vs. PINK1B9; PGAM5NP0568 or PINK1B9; PGAM51, p<0.01; wild-type vs. PINK1B9; PGAM5NP0568 or PINK1B9; PGAM51, p<0.001 by the log rank test). (K) Lifespan of adult male flies ubiquitously overexpressing dPGAM5. Overexpression of dPGAM5 further reduced the lifespan (PINK1B9; PGAM5Tg vs. PINK1B9; PGAM51, p<0.001). The same files were used in (A–F, H and J), and in (G, I and K).

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Figure 6. Loss of dPGAM5 improves degeneration of the mitochondria and DA neurons caused by dPINK1 inactivation in Drosophila. (A–F) TEM analysis of the indirect flight muscle and morphology of mitochondria in 2-day-old adult flies with the indicated genotypes. In A and B, some mitochondria are outlined with broken lines. The insets in D–F show representative mitochondria matrixes. Scale bars = 1 μm in A–C and 200 nm in D–F. (G) Quantification of the percentage of mitochondrial size distribution in the indirect muscle tissue from wild-type (n = 136 from 5 adult flies), PINK1B9 (n = 96 from 5), PINK1B9, PGAM5NP0568 (n = 116 from 5), PINK1B9, PGAM51 (n = 111 from 5) as shown in Figure 3K. Data are shown as means ± SE (* p < 0.05, ** p < 0.01). (H–J) Quantification of the percentage of cytoplasmic mitochondrial aggregates with diameter of 0.5–1.0, 1.0–1.5 or ≥1.5 μm in each PPL1 TH+ neuron from wild-type (n = 373 from 18 adult flies), PGAM51 (n = 356 from 18), PINK1B9 (n = 231 from 11), PINK1B9, PGAM51.
known mitochondrial fusion/fission machinery suggested that PGAM5 acts upstream of them or in an independent function (Figure 4). Given that PGAM5 is involved in mitochondrial fission, loss of PGAM5 would be expected to enhance the PINK1 mutant phenotype in Drosophila, similar to the interaction between PINK1 and the mitochondrial fusion/fission machinery [10–12]. Interestingly, the number of large aggregated mitochondria, which are frequently seen in PINK1PGAM5 flies (Figure 9A–J). Moreover, loss of dPGAM5 also modulated the mitochondrial morphology of dParkin mutant flies without suppressing the mitochondrial degeneration (Figure 7H–7L). Based on these observations, it could be speculated that PGAM5 does not directly regulate mitochondrial fission but instead, modulates the PINK1 pathway in a different way. Recent studies have proposed two different mechanisms for the mechanism of mitochondrial morphological changes through the PINK1/Parkin pathway in Drosophila and mammals. Ziviani et al. and Poole et al. have demonstrated that dParkin promotes degradation of Mfn in a dPINK1-dependent manner, which leads to mitochondrial fragmentation in Drosophila [16,36]. Our current results demonstrated that the loss of dPGAM5 activity does not affect mitochondrial fragmentation caused by reduction of Mfn activity, suggesting that dPGAM5 might not contribute to the proposed PINK1/Parkin pathway (Figure 4). Sandreling et al. have proposed that accumulation of damaged mitochondria by PINK1 inactivation results in mitochondrial calcium efflux, which activates Drp1 through Calciumin-mediated dephosphorylation of Drp1 in human cells [37]. This model well explains the observation that loss of PINK1 indirectly promotes mitochondrial fragmentation in mammalian cells and the indication that PINK1 is not a core component of the fusion/fission machinery in a Drosophila study [11]. However, most of Drosophila studies do not support a result that loss of PINK1 leads to mitochondrial fragmentation in mammals. Thus, it still remains a question for further investigation how PGAM5 modulates the mitochondrial dynamics.

Although the property of PGAM5 to physically interact with PINK1 appears to be conserved between human and Drosophila, the functional significance of this binding remains to be established. PINK1 and PGAM5 have kinase and phosphatase activities, respectively. However, there is no evidence to suggest that PINK1 directly phosphorylates PGAM5, or that PGAM5 dephosphorylates PINK1. Therefore, it seems possible that PGAM5 modulates the PINK1 pathway in a different way. Because the loss of PINK1 activity is strongly linked to the accumulation of oxidatively damaged mitochondria, or that PGAM5 may regulate PINK1 stabilization. Our molecular analysis, however, did not support the idea that PGAM5 stabilizes PINK1 (data not shown). In addition, by the loss of dPGAM5 partially suppresses dPINK1 null phenotypes, it seems likely that PINK1 negatively regulates PGAM5 function (Figure 9). PGAM5 was originally identified as a Bcl-xL-binding protein, and in itself can be toxic to cells, promoting mitochondrial fragmentation, when expressed at high levels (Figure 3B). Therefore, it seems possible that PGAM5 modulates a cell protective or a mitochondrial morphogenetic activity of the Bcl-2 family member Bcl-xL downstream of PINK1 but in a pathway that is independent from Parkin (Figure 9A) [39]. In this context, PINK1 may suppress the cell toxic action of PGAM5 through an indirect mechanism where an unidentified substrate of PINK1 inactivates PGAM5. Interestingly, a recent report suggests that the dPINK1 phenotype can be partially suppressed by transgenic expression of Drosophila Bcl-2 protein Bffy [8]. Alternatively, PGAM5 may be one of components of a negative regulator complex against Parkin E3, downstream of PINK1 (Figure 9B). Matsuda et al. have reported that Parkin E3 activity is activated only at the depolarized mitochondria, suggesting the existence of its negative regulator(s) [38]. E3 activity of Parkin may be released when PINK1 associates with the negative regulator complex via PGAM5 and suppresses its function by phosphorylation of another complex component(s). This idea might be partly supported by our observation that loss of dPGAM5 had little effect on dParkin mutant flies.

lost of PGAM5 Rescues PINK1 Phenotypes
Materials and Methods

Purification of PINK1-Binding Proteins

HEK293 cells stably expressing hPINK1-FLAG or parent cells were grown in suspension culture (Joklik-modified Eagle’s minimum essential medium with 5% fetal bovine serum). The cell pellet (2.4 x 10^8 cells) was homogenized in lysis buffer (50 mM Tris pH 7.4, 120 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton-X100) supplemented with Complete Protease Inhibitors (Roche Diagnostics). The soluble fraction of the suspension was...
Kyoto Drosophila GAL4 pUAST vector. Introduction of transgenes into RT-PCR from adult transgenic lines, cDNA for digestion. The digest extracted from the gel was subjected to specific bands detected by silver staining were excised for in-gel digestion. For quantitative RT-PCR analysis, reverse transcription and PCR reactions with total RNA extracted from fly heads were performed using a Superscript VILO cDNA synthesis kit (Invitrogen) and SYBR GreenER qPCR SuperMix (Invitrogen), performed using a Superscript VILO cDNA synthesis kit [9,20].

RT-PCR and Plasmids

For quantitative RT-PCR analysis, reverse transcription and PCR reactions with total RNA extracted from fly heads were performed using a Superscript VILO cDNA synthesis kit (Invitrogen) and SYBR GreenER qPCR SuperMix (Invitrogen), respectively. Full-length cDNAs corresponding to hPGAM5 (GenBank NP_0117054.1) and a short isoform of hPGAM5 (GenBank NP_001170543) were amplified by RT-PCR from total RNA purified from HEK293 cells or a cDNA clone (RIKEN clone ID: IRAK003D15), and was cloned in pGEX4T-1 (hPGAM5-S, GenBank_NM_138575) were amplified by RT-PCR from total RNA purified from HEK293 cells or a cDNA clone (RIKEN clone ID: IRAK003D15), and was cloned in pGEX3-Myc, pGEX6P-1 and pGEX-F1 vectors. Expression plasmids for hPINK1-FLAG and pUAST-dPINK1-Myc have been reported elsewhere [9,20].

Antibodies

Rabbit anti-human PGAM5 polyclonal antibody was raised against recombinant GST-tagged PGAM5 domain (89–289 aa) produced in the E. coli strain BL21(DE3)pLyS8 (Novagen), and was affinity-purified against the antigen. Rabbit anti-dPGAM5 polyclonal antibody was raised against the peptide ELTSNRRPRDVKNNV. Anti-hPINK1 antibody (BC100-494), anti-Myc (4A6) and anti-FLAG (M2) antibodies were purchased from Novus, Millipore and Sigma-Aldrich, respectively. Mouse anti-Th monoclonal antibody was purchased from ImmunoStar, and rabbit anti-Drosophila TH polyclonal antibody was described previously [9].
Cell Culture, Immunoprecipitation, and Immunoblot Analysis

Transfection of mammalian cultured cell, immunopurification of FLAG-protein from transfected cell lysate and immunoblot analysis was performed as described previously [4,41]. For the hPGAM5 RNAi experiment, HEK293 cell lysate transfected with 20 μM stealth RNAi reagent against hPGAM5 or a control RNAi reagent (Invitrogen), was analyzed 72 hrs after transfection. To detect an endogenous interaction between PINK1 and PGAM5, we treated HEK293 cells (2×10^6 cells) with 20 μM carbonyl cyanide 3-chlorophenylhydrazone for 24 hrs to induce a sufficient level of human PINK1 protein for the study. The treated cells were subjected to immunoprecipitation using a Rabbit TrueBlot kit combined with rabbit anti-human PINK1 or rabbit anti-Delta (Santa Cruz) as a species-matched control. For the preparation of fly samples for immunoblot analysis, fly heads were directly homogenized in 20 μl/head of SDS sample buffer using a motor-driven pestle. After centrifugation at 16,000 g for 10 min, the supernatant was used in SDS-PAGE.

In Vitro Phosphorylation Assay

Recombinant 2x GST-dPINK1 (153–709 aa), which has an N-terminal GST-tag and a C-terminal GST/6x His tag, was produced in the E. coli strain pG-KJE8/BL21 (TAKARA) and purified by a sequential purification with Ni-NTA TrueBlue kit combined with rabbit anti-human PINK1 or rabbit anti-Delta (Santa Cruz) as a species-matched control. For the preparation of fly samples for immunoblot analysis, fly heads were directly homogenized in 20 μl/head of SDS sample buffer using a motor-driven pestle. After centrifugation at 16,000 g for 10 min, the supernatant was used in SDS-PAGE.

Whole-Mount Immunostaining and Transmission Electron Microscopic (TEM) Analysis

Counting of TH-positive neurons was performed by whole-mount immunostaining of brain samples as described previously [9]. TEM images were obtained at the Biomedical Research Core of Tohoku University Graduate School of Medicine. All histochemical analyses were performed using DeltaVision microscope system (Applied Precision) or LSM5 PASCAL Laser scanning microscope system (Carl Zeiss). The images obtained by DeltaVision system were deconvolved through 10 iterations using the DeltaVision deconvolution software (Applied Precision). Area calculation of the mitochondria was performed following established criteria for classification [12] using softWoRx (Applied Precision) or ImageJ software from the US National Institute of Health (http://rsb.info.nih.gov/ij/).

Lifespan Assay and Quantification of Wing Phenotypes and Climbing Ability

For lifespan assays, twenty female adult flies per vial were maintained at 25°C, transferred to fresh fly food, and scored for survival every 4 or 5 days. To control for isogeny, the PGAM5NP0568 PINK1B9 and PGAM51 PINK1B9 alleles were backcrossed to PINK1B9 for six generations, and parkin^{Δ21} and PGAM5NP0568 parkin^{Δ21} were backcrossed to w− wild-type background for six generations. UAS-dPGAM5 transgenic flies were generated in the w− genetic background and thus have matched genetic backgrounds. The lifespan of PGAM51 was compared in the w− genetic background. The number of flies exhibiting defective abnormal wing posture (held-up or drooped) was determined for each genotype [9]. A climbing assay was performed as described previously [42].

Statistical Analysis

One-way repeated measures ANOVA was performed to determine significant differences among multiple groups unless otherwise indicated. If a significant result was achieved (p<0.05), the mean of the control and the specific test groups was analyzed using the Tukey-Kramer test. For lifespan assays, the Kaplan-Meier analysis with log-rank test was performed.

Supporting Information

Figure S1 DPAG5 mutant alleles. (A) PGAM5NP0568 and PGAM51 mutant alleles are depicted. Boxes, exons of the PGAM5 gene; triangles, the positions of the transposon NP0568 and K09727 insertions. Coding regions and the transcript are depicted by black and yellow boxes, respectively. (B) Quantitative RT-PCR of the dPGAM5 transcript in homozygous dPGAM5 mutant and RNAi lines. Expression of the dPINK1 RNAi was induced via the Da-GAL4 driver. Primer-binding sites for PCR are shown as arrows in (A). (C) Immunoblot analysis of dPGAM5 in the homozygous dPGAM5 mutant, RNAi and transgenic lines. LE, longer exposure. (D) Alignment of the amino acid sequences of PGAM5 orthologues. Putative transmembrane domains are underlined in green for mammalian PGAM5 and blue for Drosophila PGAM5. A red arrowhead indicates the point of insertion of the transposon NP0568. Red underlining, sequences corresponding to the reported keap1-binding motif in human PGAM5. Found at: doi:10.1371/journal.pgen.1001229.s001 (0.69 MB TIF)

Figure S2 Quantitative RT-PCR of the mfn transcript in the mfn RNAi lines. Expression of the mfn RNAi was induced via the Da-GAL4 driver, and total RNA was purified from 3rd instar larvae because mfn RNAi flies exhibited a pupation-defect phenotype. Found at: doi:10.1371/journal.pgen.1001229.s002 (0.18 MB TIF)

Figure S3 Loss of dPGAM5 improved mitochondrial degeneration of the indirect flight muscles caused by dPINK1 inactivation. To visualize the mitochondria under a fluorescence microscopy, we used the MHC-GAL4 driver to induce expression of a mitoGFP (green) transgene in 5-day-old adult flies with the indicated genotypes. Muscle tissue was counterstained with phalloidin (red). Integrity of the mitochondria in PINK1B9 flies was partially restored by removal of dPGAM5 as shown by recovery of the mitoGFP signal (green) in PINK1B9/dPGAM5 flies. The genotypes are as follows: MHC-GAL4>MitoGFP (wild-type); PGAM51/Y; MHC-GAL4>UAS-mitoGFP [PGAM51], PINK1B9/Y; MHC-GAL4>UAS-mitoGFP [PINK1B9], PINK1B9; PGAM51/Y; MHC-GAL4>UAS-mitoGFP [PINK1B9], PGAM51. Found at: doi:10.1371/journal.pgen.1001229.s003 (1.40 MB TIF)

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Author Contributions

Conceived and designed the experiments: Y Imai, T Kanoa. Performed the experiments: Y Imai, T Kanoa, T Sawada, Y Kobayashi, Y Moriwaki. Analyzed the data: Y Imai, T Kanoa, T Sawada. Contributed reagents/materials/analysis tools: Y Moriwaki, Y Ishida, K Takeda, H Ichijo, B Lu, R Takahashi. Wrote the paper: Y Imai.
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