Pantothenate kinase 2 interacts with PINK1 to regulate mitochondrial quality control via acetyl-CoA metabolism

Yunpeng Huang1,10,13, Zhihui Wan1113, Yinglu Tang2,13, Junxuan Xu1, Bretton Laboret2, Sree Nallamothu2, Chenyu Yang3, Boxiang Liu4, Rongze Olivia Lu5,12, Bingwei Lu6, Juan Feng7, Jing Cao3, Susan Hayflick8, Zhihao Wu2 & Bing Zhou1,9

Human neurodegenerative disorders often exhibit similar pathologies, suggesting a shared aetiology. Key pathological features of Parkinson’s disease (PD) are also observed in other neurodegenerative diseases. Pantothenate Kinase-Associated Neurodegeneration (PKAN) is caused by mutations in the human PANK2 gene, which catalyzes the initial step of de novo CoA synthesis. Here, we show that fumble (fbl), the human PANK2 homolog in Drosophila, interacts with PINK1 genetically. fbl and PINK1 mutants display similar mitochondrial abnormalities, and overexpression of mitochondrial Fbl rescues PINK1 loss-of-function (LOF) defects. Dietary vitamin B5 derivatives effectively rescue CoA/acetyl-CoA levels and mitochondrial function, reversing the PINK1 deficiency phenotype. Mechanistically, Fbl regulates Ref(2)P (p62/SQSTM1 homolog) by acetylation to promote mitophagy, whereas PINK1 regulates fbl translation by anchoring mRNA molecules to the outer mitochondrial membrane. In conclusion, Fbl (or PANK2) acts downstream of PINK1, regulating CoA/acetyl-CoA metabolism to promote mitophagy, uncovering a potential therapeutic intervention strategy in PD treatment.
Degenerative nerve disease, or often called neurodegenerative disease, is an umbrella term for a spectrum of disorders that primarily affect the neurons in different regions of human central nervous system (CNS)\(^1\). Human neurodegenerative diseases usually develop two major symptoms during disease progression, dementia, and movement disorder. While both features often appear in multiple human neurodegenerative diseases, one symptom is usually more dominant\(^2\). The commonalities in disease symptoms indicate a potential connection in at least some of their etiologies. For example, Parkinson’s disease (PD) is the second most prevalent progressive neurodegenerative disease characterized by a set of symptoms including tremor, bradykinesia, rigid muscles, impaired posture and balance, loss of automatic movements, and alterations in writing/speech and sleep\(^3\). However, Parkinsonism is not unique in Parkinson disease (PD)26,27; and proteins they encode are characteristic. For example, the Pantothenate Kinase-Associated Neurodegeneration (PKAN)\(^4\). Unfortunately, little effort has been conducted to understand the underlying mechanistic connection so far.

People have been striving for decades to clarify the pathogenesis of PD. Postmortem examinations indicated PD as a movement disorder caused by selective degeneration of dopamine (DA) neurons in the substantia nigra pars compacta\(^3\). Up to now PD treatment strategy remains limited and few biomarkers exist for PD19. Biochemical studies have revealed that PINK1 can be stabilized positions upstream of Parkin in regulating the MQC pathway.\(^{20}\)

The first observation leading to this connection was made from people exposed to MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a mitochondrial toxin, which can be metabolized to the mitochondrial complex I (C-I) inhibitor MPP+ and causes acute-onset Parkinsonism\(^4\). Other supporting evidences include the continually and consistently identified respiratory complex dysfunction in PD animal models\(^15,16\) and PD patient postmortem studies\(^7,18\), as well as the observation that rotenone, a complex I inhibitor, is strongly associated with PD risk in epidemiological studies\(^19\).

In spite of these, overwhelming evidence has been accumulated and indicates a central role of mitochondrial dysfunction in PD pathophysiology\(^13\). The first observation leading to this connection was made from people exposed to MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a mitochondrial toxin, which can be metabolized to the mitochondrial complex I (C-I) inhibitor MPP+ and causes acute-onset Parkinsonism\(^14\). Other supporting evidences include the continually and consistently identified respiratory complex dysfunction in PD animal models\(^15,16\) and PD patient postmortem studies\(^7,18\), as well as the observation that rotenone, a complex I inhibitor, is strongly associated with PD risk in epidemiological studies\(^19\).

Under normal circumstances, dysfunctional mitochondria are usually monitored and eliminated by a surveillance system termed Mitochondrial Quality Control (MQC)\(^20\). The MQC collectively includes all levels of recovery mechanisms: stimulation of local synthesis and import of nucleus-encoded mitochondrial proteins\(^21\); AAt protease for aberrant protein degradation in both mitochondrial matrix and intermembrane space\(^22\); ubiquitination/proteasome machinery for the outer mitochondrial membrane (OMM) protein removal; mitochondrial derived vesicles (MDVs) for clearing localized damages\(^23\); fission/fusion regulation\(^24\); and mitophagy to eliminate the entire dysfunctional mitochondrion\(^25\).

Among the heterogenous PD-associated genes that have been identified from the familial PD research, two candidate genes PINK1 (encoding PINK1, PTEN-induced serine/threonine kinase 1) and PRKN (encoding Parkin, E3 ubiquitin ligase), associated with early onset PD\(^26,27\); and proteins their encode are characterized to play an essential role in maintaining mitochondrial function, monitoring mitochondrial damage and initiating mitophagy\(^10,11,28–30\). PINK1\(^26\) and Parkin\(^31\), were found acting in a linear pathway in genetic epistasis analyses, first from Drosophila\(^28,29,32\) and later from mammalian cell\(^33\). PINK1 positions upstream of Parkin in regulating the MQC pathway. Biochemical studies have revealed that PINK1 can be stabilized on the outer membrane of damaged mitochondria, and this activates Parkin by directly phosphorylating both ubiquitin and Parkin; activated Parkin enacts ubiquitination on multiple targeted mitochondrial proteins and provides the poly-ubiquitination signals for recruiting autophagy receptors\(^34\). In addition, under physiological conditions or mild mitochondrial damage, the PINK1/Parkin pathway also directs the mitochondrion-localized translation of certain nucleus-encoded respiratory chain complex (nRCC) mRNAs to help maintaining the normal mitochondrial oxidative phosphorylation (OXPHOS)\(^15\).

As one of cellular metabolic hubs, mitochondria also play essential roles in synthesizing and regulating critical metabolites, including Coenzyme A (CoA) and acetyl-Coenzyme A (acetyl-CoA). Not surprisingly, a previous study has reported that both CoA and acetyl-CoA levels are significantly dropped in PINK1 mutants\(^36\). Similar extents of CoA and acetyl-CoA reduction were found in PKAN, formerly called Hallervorden-Spatz syndrome\(^37,38\), which is defective in pantothenate kinase 2 (PANK2), an enzyme catalyzing the first step of CoA synthesis. In human, there are four PANKs, with only PANK2 being mitochondrial. In Drosophila, the homolog of human PANK2 is fumble (flb) (encoding Fbl, Fumble or dPANK), and inhibiting Fbl activity also causes severe neurological defects and reproduces some of the Parkinsonism symptoms\(^38,39\). Conversely, D-pantethine, a homologue of pantethine and an active moiety of CoA\(^15\), can significantly benefit flb LOF flies\(^39\) and likely MPTP-induced PD rodents\(^15\).

Thus, emerging evidence implicates a possible link between PD and PKAN, while the key mechanistic work establishing the regulation and function of CoA metabolism in PD and PD-associated genes in PKAN is still absent.

In this study, we uncovered a sophisticated and conserved interaction among PINK1, Parkin, and Fbl/PANK2, and their roles in the control of CoA/acetyl-CoA synthesis and mitophagy regulation. Fbl/PANK2 is subject to the mitochondria-localized translational regulation by PINK1 and Parkin, and Fbl/PANK2 overexpression can significantly suppress defects of PINK1 LOF. This rescue is implemented by CoA and acetyl-CoA elevation and subsequently Ref(2)P (refractory to sigma P)/p62 (Sequestosome-1) acetylation. Our results help better understand the communality of the two diseases, PD and PKAN, and may facilitate the development of new treatment strategies in the future.

**Results**

**Knocking-down Drosophila flb results mitochondrial abnormalities resembling defects of PINK1 LOF.** The Drosophila flb mutant has been characterized to associate with multiple defects, including a significant drop of eclosion rate, abnormal wing morphology (wing inflation), progressive locomotor dysfunction, lifespan shortening, infertility, and broad neurodegeneration\(^38,40,41\). Among all flb transcripts, Fbl-L (the longest isoform) encodes a mitochondria-targeted and sole active PANK. We thus suspected that Fbl might regulate mitochondrial functions, and consequently, we systematically analyzed possible mitochondrial defects under Fbl loss.

We first looked at the mitochondria morphology in dopaminergic (DA) neurons in flb overexpression and knockdown adult flies. Prominent mitochondrial aggregations (labeled by mitoGFP) were observed in DA neurons of flb RNAi flies, reminiscent of that in PINK1 mutant (Fig. 1a, b), while flb-L OE did not generate any notable abnormality (Fig. 1a, b). In addition, loss of DA neurons in the protocerebral posterior lateral (PPL1) cluster (in the posterior inferior lateral protocerebrum near the posterior face of the brain) was seen in flb RNAi flies, which also recapTURED the PINK1 mutant phenotype (Fig. 1c). Here, the
Fig. 1 Fbl Regulates Mitochondrial Functions in Drosophila. a Immunostaining showing effects of fbl on mitochondrial morphology in TH-positive dopaminergic (DA) neurons. WT (w–/Y) and PINK1 mutant (PINK1–/Y) serves as the negative and positive controls. Scale bar, 10 μm. In this and all subsequent immunostaining figures of DA neurons, mitochondrial morphology is monitored by the mitoGFP reporter. b Violin plots showing the quantification of (a). In this and all subsequent mitochondrial quantifications in neurons, the significance was calculated by using two-proportion Z test, and the threshold was set to 3%. n = 3 biologically independent samples. c Effects of fbl on DA neuron loss in fly PPL1 cluster. In this and all subsequent DA neurons counts. n = 10 (w–, fbl-L OE) and 12 (other genotypes) biologically independent animals. d Effects of fbl on ATP level in fly indirect flight muscle. n = 3 biologically independent samples. e–g Effects of fbl on complex-I, -II and -III dependent respirations. h Effects of fbl on complex-I activity. For assays in (d–h), n = 3 biologically independent samples. For all assays in (b–h), w– or UAS-GFP was used as control, and the significance was calculated by using one way ANOVA followed by post hoc Dunnett’s multiple comparison test. Data are presented as mean values ± SEM; n.s. not statistically significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Source data are provided as a Source Data file.

Fbl genetically interacts with PINK1 in regulating mitochondrial homeostasis. To investigate the genetic interaction between fbl and PINK1 we tuned the fbl expression in PINK1 LOF flies. We first found that OE of the mitochondrial isoform – Fbl-L, but not that of the cytosolic isoforms – Fbl-S1 or Fbl-S2, in indirect flight muscles using MHC-Gal429,42, dramatically rescued the abnormal wing posture (Fig. 2a) as well as the jumping/flight defect (Supplementary Fig. 2a) in PINK1 RNAi flies. Conversely, fbl RNAi significantly aggravated the pre-existing PINK1 RNAi wing abnormality (Fig. 2a and Supplementary Fig. 2a). While in wild type (WT) background, neither fbl short forms OE nor fbl RNAi would cause wing posture or movement defects (Supplementary Fig. 1f, g). Next, we measured the ATP production in fly muscles. Consistent with phenotypical changes, the ATP measurement also indicated that fbl-L OE partially restored the mitochondrial function in PINK1 RNAi animals, whereas fbl RNAi exacerbated it (Fig. 2b). The effects of Fbl on tissue integrity (Fig. 2g) correlated well with effects on mitochondrial morphology, with fbl-L OE rescuing the mitochondrial aggregation phenotype in neuromuscular tissues caused by PINK1 LOF, whereas fbl-S isoforms had no effect and fbl knockdown had the opposite effect (Fig. 2c–f and Supplementary Fig. 2b, c).

efficiencies of fbl OE and RNAi lines were confirmed by immunoblotting (Supplementary Fig. 1a, b) and RT-PCR (Supplementary Fig. 1c, d). Notably, neuronal tissue seemed more sensitive to Fbl deprivation, since mitochondria in the indirect flight muscle did not display any severe defect in fbl RNAi flies, unlike those in the PINK1 mutant (Supplementary Fig. 1e–g). We next analyzed the mitochondrial physiology in fbl OE and RNAi flies. Thoracic ATP levels in fbl-L OE and fbl RNAi flies were both reduced, indicating the existence of an essential and sophisticated regulation of mitochondrial function by Fbl (Fig. 1d). To address the underlying basis of ATP reduction in fbl RNAi flies, we next purified the mitochondria from neuromuscular tissue of each genotype and measured their respiratory abilities of individual complex by oxygen microelectrode. Moderate decrease of complex-I, -II, and -III dependent respiration was observed in fbl RNAi flies (Fig. 1e–g), also similar to that in the PINK1 RNAi fly. Differing from PINK1 RNAi, fbl RNAi did not obviously change the activity of complex-I (Fig. 1h), suggesting the existence of certain dissimilitude between the two. Altogether, fbl LOF recapitulates many mitochondrial defects previously found in the PINK1 LOF model, prompting us to investigate the possible functional connection between fbl and PINK1.
The kinase activity of Fbl was necessary in the rescue, since a kinase-dead form of Fbl-L (Fbl-L K221A-2 and K221A-7) showed no rescue on wing posture abnormality (Supplementary Fig. 2d). In the PINK1 knockdown but not WT flies, fbl OE increased the CoA and acetyl-CoA levels, indicating a global improvement of mitochondrial health in mutants (Fig. 2h, i)\(^36\). By contrast, fbl RNAi decreased the CoA and acetyl-CoA levels in WT flies (Supplementary Fig. 2f, g), and further in PINK1 RNAi flies (Fig. 2j, k). Essentiality of CoA metabolism in the PINK1 related pathology was further confirmed by the observation that knocking down fly Ppdc, a downstream enzyme of CoA synthesis pathway, could largely reverse the rescuing effect of fbl-L OE (Supplementary Fig. 2e). Consistent with genetic interactions, reduced levels of CoA and acetyl-CoA were detected in fbl RNAi, PINK1 RNAi and Ppdc RNAi larvae, while Ppdc knockdown resulted in more severe reduction and pupal lethality (Supplementary Fig. 2f, g).

Previous studies indicated that PINK1 LOF animals displayed impaired respiratory chain complex (RCC) assembly and mitochondrial respiration deficits\(^{43,44}\). In our assays, we found a restoration of the complex assembly of multiple RCCs – complex-II, -III, -IV, -V and super complexes by fbl-L OE in the PINK1 mutant (Supplementary Fig. 2h), which was concordant with recovery of individual complex dependent respirations (Supplementary Fig. 2i–k). Worthy of mention, fbl-L OE did not increase the activity of complex-I in the PINK1 mutant (Supplementary Fig. 2i) or dramatically restore complex-I activity (Supplementary Fig. 2h), which echoed well the previous observation that fbl knockdown did not affect the complex-I activity (Fig. 1h).

In contrast to the rescuing effect of Fbl on PINK1 loss, PINK1 OE failed to alleviate fbl LOF defects, e.g. wing inflation (Supplementary Fig. 2m) and survival (Supplementary Fig. 2n), suggesting Fbl acting downstream to PINK1. Hence, it appears that Fbl epistatically interacts with PINK1 to sustain mitochondrial health and maintain the tissue integrity.

D-pantetheine rescues PINK1 LOF defects. Besides genetic manipulations, pharmacological treatments also lent strong support to the connection between PD pathogenesis and CoA synthesis. Previous studies revealed D-pantetheine (D-pan), a close relative of vitamin B5 (pantothenate) and a metabolic substrate for CoA synthesis\(^39\), could bypass PANK in CoA synthesis in a currently unclear biochemical pathway, and nicely rescue multiple animal PKAN models\(^39,45,46\). We administered D-pan through diet to WT and PINK1 LOF flies. Similar to the genetic studies, D-pan treatment significantly rescued PINK1 LOF phenotypes, including abnormal wing posture (Fig. 3a and Supplementary Fig. 3b), flight activity drop (Supplementary Fig. 3c) and mitochondrial health in mutants (Fig. 2h, i)\(^36\). By contrast, UAS-GFP as control, and the significance was calculated by using one way ANOVA followed by post hoc Dunnett’s multiple comparison test.
Fig. 3 D-pantethine Rescues PINK1 LOF Defects. a Rescue of D-pantethine (D-pan) alimentation on wing posture defect in PINK1 RNAi flies. 25 flies per genotype per group were tested, n = 10 biologically independent groups. In this and following figures in Fig. 3, NF, as normal food. b Effects of D-pan alimentation on ATP levels in WT and PINK1 RNAi flies. UAS-GFP serves as control. n = 3 biologically independent samples. c, e Immunostaining showing effects of D-pan alimentation on mitochondrial aggregations in flight muscle (c) and DA neurons (d) caused by PINK1 LOF. Scale bars: (c) 25 μm; (d) 10 μm. d, f Violin plots showing the quantifications corresponding to (c) and (e). The significance was calculated by using two-proportion Z test, and thresholds were set at 8 μm² (d) and 3 μm² (e); n = 3 biologically independent samples. g Rescue of D-pan alimentation on DA neurons loss in PPL 1 cluster of PINK1 mutants. n = 9 (w-/NF, PINK1/D-pan), n = 10 (w-/D-pan, PINK1/NF) biologically independent animals. h, i Effects of D-pan alimentation on CoA and acetyl-CoA levels in WT (w-/y) and PINK1 mutant. n = 4 biologically independent samples in assay (h); n = 3 biologically independent samples in assay (i). For assays in (a, b, g-i), the significance was calculated by using two tailed unpaired t-test. Data are presented as mean values ± SEM; n.s. not statistically significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Source data are provided as a Source Data file.

Fig. 3c–e), thoracic ATP level reduction (Fig. 3b), mitochondrial aggregation (Fig. 3c–f) and DA neuron loss (Fig. 3g). We also observed elevated CoA and acetyl-CoA levels (Fig. 3h, i), which was consistent with the previous report and correlated well with the improved mitochondrial respirations (Supplementary Fig. 3f-3h). Again, we did not find any evidence of promoting complex-I activity (Supplementary Fig. 3i) or assembly (Supplementary Fig. 3j) by D-pan alimentation. Furthermore, 4′-phosphopantetheine (4′-PPT), known to be the direct precursor of CoA, but not CoA itself (probably due to its poor bioavailability), significantly rescued the abnormal wing posture and extended lifespan in PINK1 mutant (Supplementary Fig. 3k, l). In addition, fosmetpantotenate (RE-024), another precursor of CoA, moderately but significantly rescued the abnormal wing posture and large mitochondrial aggregations in PINK1 LOF flies (Supplementary Fig. 3m–o). Similar effects of D-pan (D-pantethine), 4′-PPT (4′-phosphopantetheine) and RE-024 (fosmetpantotenate) strongly indicate that the rescue observed is via the CoA synthesis pathway. Combining the genetic and pharmacological rescue data, we believe that the benefit of β-L-OE and D-pantethine diet supplement in PINK1 LOF come from their ability of enhancing the CoA synthesis pathway.

Fbl’s rescue is in parallel with parkin, downstream of PINK1. Parkin has been reported to act downstream of PINK1 and play a critical role in MQC and maintenance of tissue integrity. Parkin mutant shows defects akin to the PINK1 mutant. We thus evaluated effects of β-L OE and D-pan in the parkin mutant. Surprisingly, neither β-L OE nor D-pan alimentation could significantly rescue the abnormal wing posture (Fig. 4a, b), mitochondrial aggregation (Fig. 4c–f) and depressed ATP production (Fig. 4g, h) in the parkin mutant. No increment of CoA or acetyl-CoA level was observed either after increasing β-L expression (Fig. 4i, j). Surprisingly, unlike in PINK1 LOF (Fig. 2h, i), the parkin mutant exhibited an elevated CoA level but a declined acetyl-CoA level (Fig. 4i, j).

It has been reported that PRKN knockdown in human H460 cells impairs the activity of PDH (pyruvate dehydrogenase) complex, which could be the reason for CoA accumulation and acetyl-CoA decline in fly parkin mutant. In order to test this hypothesis, we knocked down (RNAi) and overexpressed Pdha (encoding Pdha, Pyruvate dehydrogenase E1 alpha subunit) in parkin mutant adult flies via the GeneSwitch GAL4 system (because Pdha knockdown severely influenced fly development), and compared their CoA and acetyl-CoA levels. Pdha knockdown strongly enhanced CoA accumulation, while Pdha overexpression only mildly mitigated it. Interestingly, when parkin was suppressed Pdha knockdown further reduced the acetyl-CoA level and Pdha overexpression rescued it (Supplementary Fig. 4a, b). As an important control, in the parkin mutant, the level of Pdha protein was not significantly reduced (Supplementary Fig. 4c).

These findings suggest a distinctiveness of these two genes, PINK1 and parkin, in modulating CoA/acetyl-CoA synthesis and mitochondrial metabolic homeostasis, despite their tight connection in the MQC regulation. To further study their interaction in this regard, we measured the CoA and acetyl-CoA levels in different combinations of PINK1 and parkin. Intriguingly, both parkin OE and RNAi notably restored the CoA reduction in PINK1 RNAi flies, whereas had the opposite effect on the acetyl-
**Fig. 4** fbl Does Not Genetically Interact with parkin. **a, b** No rescue effects of fbl-L OE (a) and D-pan alimentation (b) on wing posture defects in parkin mutant. 25 flies per genotype per group were tested; n = 6 biologically independent groups in assay (a); n = 8 (NF), n = 6 (D-pan) biologically independent groups in assay (b). **c, e** No rescue effects of fbl-L OE (c) and D-pan alimentation (e) on mitochonbral aggregation defects in parkin mutant muscles. Scale bars, 25 μm. **d, f** Violin plots showing the quantifications corresponding to (c) and (e). The significance was calculated by using two-proportion Z test, and the threshold was set at 3 μm²; n = 3 biologically independent samples in assays (d) and (f). **g, h** No effects of fbl-L OE (g) and D-pan alimentation (h) on ATP level reduction in parkin mutant. **i, j** No effects of fbl-L OE on CoA (i) and acetyl-CoA (j) levels in parkin mutant. n = 3 biologically independent samples in assay (g, j). For assays in **a, g, j**, 1US-GFP serves as control. **k** No rescue effect of parkin OE on wing abnormal inflation caused by fbl RNAi, and RT-PCR showing the efficiency of parkin OE, rp49 serves as loading control. **l** Effects of parkin OE on survival curve decline caused by fbl RNAi. 30 flies per group and 3 independent groups (90 flies in total) were tested per genotype. For assays in **a, b, g, j**, the significance was calculated by using two tailed unpaired t-test. For assays in **l** the significance was calculated by Log-rank (Mantel–Cox) test. Data are presented as mean values ± SEM; n.s. not statistically significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Source data are provided as a Source Data file.

CoA level (Supplementary Fig. 4d, e). In line with the elevated CoA level in the parkin mutant, similar to D-pan, 4’-PPT could not rescue the abnormal wing posture and lifespan in parkin mutant (Supplementary Fig. 4f, g). Lastly, parkin OE did not ameliorate those defects caused by fbl RNAi in our assays, such as wing inflation and shortened lifespan (Fig. 4k, l). Taken together, Parkin and Fbl act somewhat independently downstream of PINK1 in regulating CoA metabolism and maintaining the normal mitochondrial homeostasis.

Fbl rescues mitochondrial damages in PINK1 mutant through mitophagy. On damaged mitochondria, PINK1 phosphorylates Parkin and the activated Parkin subsequently generates poly-ubiquitination signal on some OMM (outer mitochondrial membrane) proteins in their first wave of actions49. Autophagy receptors can then bind with ubiquitinated targets and trigger following mitophagy processes. To investigate the role of Fbl and CoA metabolism in mitophagy induction, we examined their effects on p62, the sole autophagy receptor Ref(2)P in Drosophila30, and autophagy-related proteins (Atg proteins)31,32. In the genetic analysis, we found that in PINK1 RNAi background, knocking-down ref(2)P or Atg1 (encoding Atg1, Autophagy-related 1) could effectively diminish rescue effects from fbl OE; while OE of ref(2)P or Atg1 could significantly neutralize the severity enhanced by fbl RNAi in multiple aspects: wing posture (Fig. 5a), ATP production (Fig. 5b), and mitochondrial aggregation (Fig. 5c). Besides Atg1, genetic regulations of other Atg genes were also able to occlude the beneficial effects of fbl OE, indicating a nexus of Fbl with mitophagy (Supplementary Fig. 5a). To evaluate the promoting effect of Fbl on mitophagy, we examined the selected mitophagy marker Marf (Mitochondrial assembly regulatory factor, homolog of human Mitofusin-2)53. We found that Marf was effectively eliminated by fbl RNAi1,2 (encoding Atg1, Mitochondrial assembly regulatory factor, homolog of human PGC-1α) and mitochondrial biogenesis (Supplementary Fig. 5g, h). In addition,
consistent with genetic interactions (Supplementary Fig. 5a, b), RT-PCR analyses also revealed similar changes in mitochondrial mass, that is, mitophagy stimulated by fbl OE could be impeded by Atg1 and ref(2)P RNAi while enhanced by Atg1 OE (Supplementary Fig. 5e, f), without altering the mitochondrial biogenesis (Supplementary Fig. 5i, j).

To further study the involvement of mitophagy in Fbl’s rescue, we first visualized the occurrence of mitophagy (mitochondrial degradation in lysosome) in fly muscles by ectopically expressing the mitoQC indicator54. In PINK1 RNAi flies with fbl OE, mito-lysosomes, indicated by the signal of “spectral shifted” puncta of more acidic conditions, were found dramatically increased (Fig. 5d, e). Consequently, more ATG8a (Autophagy-related 8a)/LC3 (Microtubule-associated proteins 1 A/1B light chain 3 beta) signals were found to colocalize with the mitochondrial marker – ATP5a (ATP synthase F1 subunit alpha) when fbl was
Fig. 5 Fbl Helps Eliminate Damaged Mitochondria via Mitophagy. a Effects of ref(2)P and Atg1 on wing posture defect showing their genetic interactions with fbl in PINK1 RNAi flies. 25 flies per genotype per group were tested, n = 6 (fbl-L OE/GFP), n = 9 (fbl-L OE/ref(2)P RNAi), n = 8 (fbl-L OE/Atg1 RNAi), n = 4 (fbl-L OE/Atg1 RNAi2), n = 6 (fbl-L OE/GFP, fbl RNAi/GFP, fbl RNAi/ref(2)P 7 M, fbl RNAi/Atg1 OE) biologically independent samples. b Effects of ref(2)P and Atg1 on ATP level regulation with fbl in PINK1 RNAi flies. For assays in (a, b) UAS-GFP serves as control. c Effects of ref(2)P and Atg1 on mitochondrial aggregations in flight muscle with fbl in PINK1 RNAi flies. Scale bar, 25 μm. w- serves as control. d Mito-lysosome analysis with mitoQC in PINK1 RNAi flies showing the regulation of fbl on mitophagy. Scale bar, 25 μm. e Quantification of mito-lysosomes shown in d n = 15 from 5 biologically independent animals. w- serves as control. f Effect of chloroquine (CQ) on wing posture showing its inhibition of fbl’s rescue in PINK1 RNAi flies. 25 flies per genotype per group were tested, n = 3 biologically independent groups. g, i, k Immunostaining of mitoGFP and LC3 showing effects of human PANK2 OE, g D-Pan alimentation (i) and PANK2 RNAi (k) on mitochondrial induction in WT and PINK1 knockdown (shRNA) HEK cells upon CCCP treatment. Scale bars, 10 μm. h, j, l Quantifications of g, i, k correspondingly. n = 80/9, means 80 cells examined over 9 independent experiments (shCON/CON), n = 70/10 (shCON/PANK2 OE), n = 65/9 (shPINK1/CON), n = 80/14 (shPINK1/PANK2 OE) in assay (h); n = 60/9 (shCON/vehicle), n = 60/12 (shCON/D-pan), n = 41/7 (shPINK1/vehicle), n = 38/8 (shPINK1/D-pan), in assay (i); n = 56/8 (shCON/siCON), n = 70/8 (shCON/siPANK2), n = 71/8 (shPINK1/siCON), n = 71/8 (shPINK1/siPANK2) in assay (j). Immunoblots embedded in (h, j, and l) showing gene knockdown and overexpression efficiencies in experiments (j, i, and k) accordingly. m Immunostaining of mitoGFP and LC3 showing the mitophagy decline caused by PANK2 LOF in human control and PKAN fibroblasts upon CCCP treatments. Scale bar, 10 μm. Arrow heads, colocalization of mitoGFP and LC3. n Colocalization analysis of m. For assays in (a, f), the significance was calculated by using one way ANOVA followed by post hoc Šidák’s multiple comparison test (a) or Dunnett’s multiple comparisons test (f). For assays in (b, e, h, i, j, l), the significance was calculated by using two tailed unpaired t-test. Data are presented as mean values ± SEM; n.s. not statistically significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Source data are provided as a Source Data file.

The mechanism of Fbl/PANK2 assisting mitophagy is conserved. We next investigated whether the uncovered connection between fbl/PANK2 and PINK1 holds true in human cells. To verify the occurrence of mitophagy in human cells, multiple autophagy markers including LC3, p62 and Lamp2 (lysosomal-associated membrane glycoprotein 2), were counterstained with mitochondrial indicators in both WT and PINK1 knockout cells after 12 hours carbonyl cyanide m-chlorophenyl hydradezone (CCCP) treatment. We found that PANK2 OE did not or at most only mildly facilitate the colocalization between autophagy markers and mitochondria in WT cells whereas dramatically restored the activity of mitophagy in PINK1 knockout cells (Fig. 5g, h and Supplementary Fig. 6a–d). Immunoblotting also validated prompt degradation of autophagy receptors (p62, Optineurin/OPTN) and mitochondrial markers (Tom20 and Tim23), and LC3 lipidation (LC3-I/LC-II), indicating burst of mitophagy induced by PANK2 OE in both WT and PINK1 knockout cells (Supplementary Fig. 6e, f). Similar to the genetic regulations, D-pan administration exhibited similar rescues of mitophagy in PINK1 knockout cells by promoting colocalization between autophagy markers (such as LC3, p62 and Lamp2) with mitochondria (Fig. 5i, j and Supplementary Fig. 6g–j). It also increased the degradation of autophagy receptors (p62, OPTN) and mitochondrial markers (Tom20 and Tim23), as well as LC3 lipidation, which meant that the mitophagy impaired by PINK1 deficiency was restored (Supplementary Fig. 6k, l). As controls, neither of PANK2 OE and D-pan administration would change the basal level of mitophagy in human cells without mitochondrial stress (e.g., CCCP treatment) in either immunoblotting (Supplementary Fig. 6m) or immunofluorescence staining experiments (Supplementary Fig. 6n–s). Moreover, a mitoKeima indicator was expressed in this human cell system, and we found that D-pan treatment significantly helped cells regain the mitophagy ability in PINK1 knockdown cells (Supplementary Fig. 6i). Interestingly, RE-024, which is an alternative precursor bypassing PANK2 in CoA synthesis pathway, also significantly promoted mitophagy in PINK1 knockdown human cells, as indicated by the colocalization between LC3 and mitochondrial marker (Supplementary Fig. 6u, v).

Conversely, PANK2 knockdown in human cells after exposure to CCCP significantly inhibited mitophagy, manifesting as aloofness of autophagy markers (LC3, p62 and Lamp2) from mitochondria (Fig. 5k, l and Supplementary Fig. 7a–d) and the blockage of degradation of autophagy and mitochondrial markers (Supplementary Fig. 7e, f), while had no visible effect in non-treated cells (Supplementary Fig. 6m, 7g–i). In line with this, PKAN patient fibroblasts (loss of PANK2 activity) exhibited a paucity of mitophagy when stressed with CCCP. After 24 hours of CCCP treatment, PKAN fibroblasts could still maintain mitochondria compared to the control, and they also showed less LC3 lipidation at the basal level, indicating an impaired mitophagy ability (Supplementary Fig. 7j, k). In addition, the LC3 recruitment to damaged mitochondria was also largely diminished in PKAN fibroblasts after 4 hours treatment (Fig. 5m, n). These results suggest that the regulation of mitophagy by PANK2 and CoA metabolism is also conserved in human cells.

Fbl promotes mitophagy via p62 acetylation. To better understand the mitophagy process happened in these flies, we examined the sole autophagy receptor Ref(2)P and ubiquitin signals in fly muscle tissues. Prominent Ref(2)P puncta were present in the muscle cell cytoplasm of PINK1 RNAi flies, while general diffuse ubiquitin signals were observed (Fig. 6a, b). Recruitment of ubiquitin to Ref(2)P was strongly reinforced by overexpression of Fbl, while reduced when Fbl was inhibited (Fig. 6a, b), suggesting a boost of Ref(2)P/p62-ubiquitin binding after enhancing Fbl activity. We also found an elevation of the Ref(2)P level in PINK1 LOF flies (Supplementary Fig. 8a, b) in which it showed polyubiquitination. On this basis, fbl OE significantly strengthened it and vice versa (Fig. 6c). Intriguingly, the poly-ubiquitin signal could not be detected from the denaturing IP purified Ref(2)P protein from same samples, indicating that it is not from direct

overexpressed, and aloof from each other when fbl expression was suppressed (Supplementary Fig. 5k). To verify the biological significance, we fed flies with chloroquine (CQ), a classic autophagy inhibitor which impairs autophagosome fusion with lysosomes, resulting in the accumulation of undegraded cargoes in cells. Notably, CQ administration in PINK1 mutant flies sufficiently inhibited the wing postural rescue of fbl OE (Fig. 5i) and degradation of autophagy markers e.g. poly-ubiquitination and Ref(2)P (Supplementary Fig. 5l). Altogether, our data in Drosophila models strongly suggest that, in PINK1 LOF flies, Fbl helps maintain the mitochondria integrity via increasing the activity of mitophagy. Intriguingly, although Fbl modulates mitophagy in our study, neither overactivation nor inhibition of autophagy factors (Atg genes) and autophagy receptor (ref(2)P gene) could rescue defects in fbl knockdown flies, indicating that compromised mitophagy or autophagy may not be the main cause of PKAN pathogenesis (Supplementary Fig. 5m).
ubiquitination of Ref(2)P itself, but from the other polyubiquitinated proteins bound to it (Supplementary Fig. 8c). Accumulating evidence suggested the importance of post-translational modifications of p62, such as acetylation, on its activation and binding ability with ubiquitylated proteins under nutrient stress. While the general protein acetylation was not dramatically affected (Supplementary Fig. 8d), we still found increased acetylation on the immunoprecipitated Ref(2)P protein when fbl-L was overexpressed and declined signal when fbl expression was suppressed (Fig. 6d and Supplementary Fig. 8b).

Tip60 (Lysine acetyltransferase KAT 5) and HDAC6 (Histone deacetylase 6) have been respectively reported as the key acetyltransferase and deacetylase to balance the p62 acetylation. To analyze their functions in Fbl-promoted mitophagy, we induced them in our PINK1 LOF plus fbl OE combination. Interestingly, both Tip60 RNAi and HDAC6 OE remarkably suppressed the rescue of fbl OE in abnormal wing posture rate (Fig. 6e) and ATP production (Fig. 6f). Further, in more complete genetic analysis, we found that in PINK1 RNAi flies, Tip60 RNAi and HDAC6 OE enhanced wing abnormalities, while Tip60 OE and HDAC6 RNAi significantly mitigated them (Supplementary Fig. 8e). Interestingly, Tip60 RNAi and HDAC6 OE acted more potently in fbl OE flies (PINK1 RNAi background), whereas Tip60 OE and HDAC6 RNAi showed no further rescue, indicating that Tip60 and HDAC6 genes are downstream of fbl (Supplementary Fig. 8f). On the contrary, fbl RNAi nearly eliminated all actions of Tip60 and HDAC6 on PINK1 RNAi flies, which can be explained by the consequence of severe reduction of CoA/acetyl-CoA, the essential element for Tip60 and HDAC6 functions (Supplementary Fig. 8g).

To verify whether the regulation of Tip60 and HDAC6 on Fbl is through acetylation, we checked the acetylation of Ref(2)P in these flies. As expected, Tip60 RNAi and HDAC6 OE were found to obviously diminish the acetylation of Ref(2)P in flies (Fig. 6g), indicating similar similarity between human cell and fly models and in different subtypes of autophagy. In human cells, the K420 and K435 sites of p62 UBA domain are essential for mitophagy, as the p62 K2R (K420R, K435R, mimic of nonacylated lysine residues) mutant loses the ability to colocalize with damaged mitochondria (Supplementary Fig. 8h, i). We subsequently performed in vitro acetylation assay on purified Ref(2)P by Tip60. Although they are not conserved in Drosophila, Ref(2)P still could be acetylated by Tip60 surprisingly, indicating the existence of other potential acetylation sites that can be recognized (Supplementary Fig. 8j). Lastly, we conducted an in vitro ubiquitin-binding assay to assess the contribution of acetylation on Ref(2)P to ubiquitin binding affinity. We found that acetylation by Tip60 on Ref(2)P facilitated its binding to ubiquitin (Supplementary Fig. 8k). In sum, we suggest that in PINK1 LOF PD model, Fbl helps maintain the mitochondria integrity via acetylating/activating Ref(2)P, promoting ubiquitination and increasing mitophagy.

fbl is locally translated on OMM and governed by PINK1/Parkin pathway. A recent report alluded an increased level of pantothenate (i.e., Fbl/PANK2 substrate) in PINK1 mutant; and...
in our preliminary assays, pantothenate also demonstrated no rescue to PINK1 mutant (data not shown) as well as fbl mutant. This observation implied a latent PANK dysfunction in PINK1 LOF animals. When studying Fbl functions in these animals, we found Fbl level was suppressed in PINK1 mutant and PINK1 RNAi flies (Fig. 7a, b and Supplementary Fig 9a, b), and so was human PANK2 when PINK1 was knocked down in human cells (Supplementary Fig. 9c, d). Both Drosophila Fbl and human PANK2 are considered as the nuclear-encoded mitochondria targeted proteins, and recent studies have revealed a novel localized, mitochondrial membrane dependent, co-translation import mechanism of certain nuclear-encoded mitochondrial proteins on OMM facilitated by PINK1/Parkin pathway. To study the mechanism of PINK1 controlling fbl/PANK2

Fig. 7 PINK1/Parkin Pathway Regulates fbl mRNA Translation on OMM. a Immunoblot of Fbl protein levels in WT (w+/Y) and PINK1 mutant showing the regulation by PINK1. Tubulin serves as loading control. b Quantification of n = 3 biologically independent samples. In this and all subsequent immunoblot and RT-PCR quantifications, 3 independent biological repeats were measured per genotype. c Immunostaining of Fbl and mitoGFP signals showing the mitochondrial localization of Fbl in PINK1 RNAi fly muscles. Scale bar, 25 μm. d Colocalization analysis of e Semiquantitative RT-PCR of fbl mRNA in PINK1 mutant muscle. mRNAs prepared from total, cytosol (cyto), or mitochondrial (mito) fractions were used. Mitochondrial genome encoded gene mt:Col serves as mitochondrial loading control; rp49 serves as total and cytosol loading controls. f Quantification of f. For RT-PCRs in (e and d), n = 3 biologically independent samples. g CLIP assays showing PINK1 binding to fbl mRNA in fly. C-I30 and rp49 mRNAs served as positive and negative control, respectively. h CLIP assays showing reduced binding to fbl mRNA of PINK1G309D compared to PINK1 WT. i Quantification of h. n = 3 biologically independent samples. j Immunoblot of Fbl protein levels showing the regulation of Pum and Glo. Tubulin serves as loading control. k Quantification of j n = 3 biologically independent samples. l RT-PCR showing effects of Pum and Glo on the defective mitochondrial localization of fbl mRNA in PINK1 RNAi flies. mt:Col serves as mitochondrial loading control, while rp49 serves as total and cytosol loading controls. m Quantification of l n = 3 biologically independent samples. For assays in (b) the significance was calculated by using one way ANOVA followed by post hoc Dunnett’s multiple comparisons test. For assays in (f, i, k, m) the significance was calculated by using two tailed unpaired t-test. Data are presented as mean values ± SEM; n.s. not statistically significant; *p < 0.05; **p < 0.01; ***p < 0.001. Source data are provided as a Source Data file.
translation, we first analyzed the Fbl localization in the fly muscle tissue when PINK1 was repressed. Unexpectedly, we found that although the total amount of Fbl protein was reduced, Fbl still colocalized with mitochondria, especially aggregated mitochondria in PINK1 RNAi flies, suggesting that the aggregated mitochondria might be functionally more active (Fig. 7c, d). Notably, aggregated mitochondria showed a high membrane potential in TMRM staining indicating that aggregated mitochondria with high mitoGFP signal were formed in a compensatory protective way (Supplementary Fig. 9e). The data above suggest that Fbl could be the potential target of PINK1.

We next analyzed fbl or human PANK2 mRNA in the percoll-gradient purified mitochondrial fraction upon PINK1 expression modulations. The amount of mRNAs localized to mitochondria was significantly lessened, with a corresponding increase in the cytosolic fraction, in Drosophila PINK1 mutant or human PINK1 knockdown cells (Fig. 7e, f and Supplementary Fig. 9f, g). We then conducted the crosslinking and immunoprecipitation (CLIP) assay to detect a possible direct interaction between fbl mRNA and PINK1. Similar to the previous context, fly C-130 (NADH dehydrogenase 30 kDa subunit) mRNA, fbl and human PANK2 mRNAs were also found to robustly bind to PINK1 (Fig. 7g–i and Supplementary Fig. 9h–j). Intriguingly, mild mitochondrial stress (such as a short CCCP treatment) enhanced this interaction (Supplementary Fig. 9i), while a pathogenic form of PINK1 (PINK1 G309D) displayed a much lower binding affinity compared to WT PINK1 (Fig. 7h, i).

In addition, we purified and analyzed fbl mRNA ribonucleoprotein (mRNP) complex by generating a construct expressing fbl mRNA tagged with MS2 binding sites and using the MS2-Tagged RNA Affinity Purification (MS2-TRAP) system. Consistently, both endogenous, exogenous PINK1 and translational repressor Pumilio homolog 2 (Pum 2) were co-purified with the fbl and C-130 mRNPs in human cells (Supplementary Fig. 9k).

Translation repressors such as Pumilio (Pum; fly ortholog of human proteins Pumilio homolog 1, Pum 1 and Pumilio homolog 2, Pum 2), Glorund (Glo; fly homolog of heterogeneous nuclear ribonucleoprotein L, hRNP L) and heterogeneous nuclear ribonucleoprotein H (hRNP H) have been demonstrated having lower binding affinity compared to WT PINK1 (Fig. 7h, i). In addition, we purified and analyzed fbl mRNA ribonucleoprotein (mRNP) complex by generating a construct expressing fbl mRNA tagged with MS2 binding sites and using the MS2-Tagged RNA Affinity Purification (MS2-TRAP) system. Consistently, both endogenous, exogenous PINK1 and translational repressor Pumilio homolog 2 (Pum 2) were co-purified with the fbl and C-130 mRNPs in human cells (Supplementary Fig. 9k).

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Discussion

In this study, we established a sophisticated link between Fbl/ Parkin2 the first and speed-limiting regulatory enzyme in the CoA synthesis pathway and PINK1/Parkin, a central pathway in the MQC (Supplementary Fig. 10). fbl mutant displayed obvious mitochondrial dysfunctions in the central nervous system analogous to PINK1 mutant, which was a recurrence of the commonalities of PKAN and PD. fbl OE rescued, while fbl RNAi aggravated, multiple defects associated with Drosophila PINK1 mutant. Intracellular CoA and acetyl-CoA levels are largely influenced by Fbl/PANK2 bioavailability and they execute an important role in regulating mitophagy activity: ReI2p/p26 requires specific acetylation to facilitate its interaction with ubiquitinated proteins and initiate the downstream events in mitophagy. In the other half of the regulatory loop, reminiscent of certain nuclear-encoded mitochondrial RCC subunits, Fbl translation follows a co-translational import mechanism on OMM and is tuned by PINK1, Parkin, Pum and Glo. Accordingly, PINK1-related PD should also suffer from Fbl/PANK2 deficiency. The stimulation of mitochondrial protein translation and import is recognized as an indispensable part of MQC controlled by PINK1 pathway to fight against mild mitochondrial damages. The MQC failure would cause more severe and broader mitochondrial dysfunctions in a positive-feedback manner due to the CoA shortage. These observations and underlying mechanism provide a rational explanation for overlapping symptoms of PD and PKAN as we discussed earlier.

We conclude that fbl is downstream of PINK1 in this regulation. How could then the double knock-down of PINK1 RNAi and fbl RNAi exhibit a worsening phenotype? From genetic points of view, clear genetic epistasis relationship can only be established with null mutations for loss of function of genes. Unfortunately, it is not possible to analyze this with null mutants; complete absence of Fbl would not be possible because CoA is such a vital metabolic intermediate molecule without which life cannot be sustained. The observation that PINK1 RNAi was aggravated by fbl RNAi could be explained in two ways: (1) they may be in parallel pathways, working synergistically or together towards a specific phenotype; (2) they may be in a linear pathway, both of which, when knocked down, become limiting. In other words, aggravation of PINK1 RNAi by fbl RNAi, this fact by itself, can only indicate they are interacting, but not enough to suggest they are in a linear pathway. To establish they are in a linear pathway needs more concrete evidence, such as fbl OE rescued PINK1 mutant or RNAi (Fig. 2 and Supplementary Fig. 2), and PINK1 loss directly impacted fbl mRNA location and protein level (Fig. 7 and Supplementary Fig. 9). Considering that PINK1 loss affects fbl mRNA localization and expression, they would not be in parallel pathways. At molecular level, the explanation for fbl RNAi aggravating PINK1 RNAi is as follows: PINK1 RNAi reduced fbl mRNA positioning on the mitochondria, affecting its translation, and on top of this, fbl RNAi would make this worse. This explanation fits well with the above-mentioned scenario 2, where both are in a linear pathway and when knocked down, both are limited and in combination would worsen the phenotype. Consistent with this scenario, the Fbl protein level in PINK1 and fbl duo RNAi (or mutant) fly was dramatically lower than that of PINK1 RNAi alone (Fig. 7a and Supplementary Fig. 9a). Therefore, our data strongly supports that fbl is downstream of PINK1. Of course, Fbl, the enzyme catalyzing the key metabolic component CoA, does things more than its role in the PINK1-Fbl pathway.

Our genetic analyses indicate that Fbl/PANK2 works in parallel with Parkin, both downstream of PINK1. fbl OE could effectively rescue PINK1 LOF but not parkin LOF in our tests. But on the other hand, biochemical analyses suggest that Parkin, just like...
Pink1, is involved in Fbl translation. To better compare them, we summarized these congruent and disparate findings in a table (Supplementary Table 1). How could we understand the drastic differences in fbl rescue of the mutants of the two genes? We consider that differential roles of PINK1 and Parkin in regulating the level of cellular CoA and its derivant acetyl-CoA might be the key. Previous global analysis of metabolic changes in Drosophila PINK1 null mutant revealed a downregulation of metabolites in CoA metabolism including CoA and acetyl-CoA, while panthenol level is increased. Consistently, our data indicate that PINK1 regulates the mRNA localization and protein level of fbl, leading to the defect of CoA and acetyl-CoA synthesis in PINK1 mutant, which can be rescued by fbl OE. Unlike that in PINK1 mutant (Fig. 2h, i), on the other hand, fbl OE could not restore the acetyl-CoA level in parkin mutant (Fig. 4l, j), which implies that a possible blockage exists in the conversion of acetyl-CoA from CoA when Parkin is affected. Intriguingly, both parkin OE and knockdown increased the CoA level in PINK1 LOF, although further reduced acetyl-CoA level was found in the double knockdown flies (Supplementary Fig. 4d, e). The inability of fbl OE or D-Pan supplement to rescue Parkin LOF indicates genetically that parkin LOF is associated with a defect downstream of fbl, likely PDH, the critical enzymic protein complex catalyzing the conversion of pyruvate and CoA to acetyl-CoA. Indeed, there are reports showing that PRKN knockdown in H460 cells causes reduction of PDH complex activity and acetyl-CoA level drop. We thus suspect that PDH dysfunction is also responsible for the CoA accumulation and acetyl-CoA decline in parkin mutant observed in our experiments. Encouragingly, regulation of Pdha effectively changed CoA and acetyl-CoA levels in parkin mutant (Supplementary Fig. 4a, b). Since the protein level of Pdha did not obviously change in parkin mutant (Supplementary Fig. 4c), our data suggest that the effect of Parkin on Pdha appears to be mainly through regulation of the enzymatic activity. Thus, back to the PINK1, parkin double LOF fly, the pathological accumulation of CoA and further depletion of acetyl-CoA may be related to the strong loss of PDH activity. This can be the main reason underlying the observation that fbl OE and D-pan rescue PINK1 LOF flies, but not parkin LOF flies, since both interventions can only increase in vivo CoA levels but not mitigate the PDH complex defect resulted from parkin deficiency. In other words, fbl OE can enhance both CoA and acetyl-CoA levels in PINK1 LOF, while only CoA but not acetyl-CoA in parkin LOF, explaining the differential rescuing effects of Fbl on PINK1 and parkin LOF.

One important implication of this study is the likely accomplishable approaches for relevant disease therapies. In our study, D-panthethine (D-pan), fosmetpanthoteinate (RE-024) and 4'-phosphopantetheine (4'-PPT) were found improving multiple physiological indexes of PINK1 mutant. Similar rescues of D-pan, RE-024 and 4'-PPT strongly indicate the rescue is via the CoA synthesis pathway. D-pan has been shown to have a positive effect in MPTP-induced PD rodent models. Moreover, 4'-PPT and RE-024 were developed to treat PKAN. Although in a recent clinical trial, Fosmetpanthotenate Replacement Therapy (FORT) was shown not to be very effective. In another single-arm, open-label study, pantethine was shown to delay the progression of motor dysfunction in children with PKAN, but not rescue. Certain beneficial effects to PD are observed with therapies in our current studies. Since pathways of de novo synthesis, transport and metabolism of CoA, as well as relevant precursors are still not fully understood, differences between animal models (Drosophila and mouse) and humans may cause divergence in efficacy of treatment. It will be interesting to further confirm these effects in other systems such as PD patients’ fibroblast-derived neurons. Another promising therapeutic compound that was not included in this study, is kinetin, which has been proved to be a PINK1 activator. In our study, Fbl/ PANK2 expression is primarily determined by PINK1 activity. Kinetin treatment for mild mitochondrial damages might elevate the PINK1 activity, promote Fbl translation, augment CoA and acetyl-CoA production, boost mitophagy and help maintaining the mitochondrial health. Further evaluations can be performed in both PD and PKAN mouse models, while positive outcomes may lead to the development of improved disease therapies.

On the whole, mutations in the PKAN gene PANK2 were not found in 67 familiar or early onset PD patients, nor in 339 idiopathic late onset PD patients, suggesting PANK2 mutation is not a significant, if any, factor in PD. We suspect that PINK1 mutations in PD result less severe decrement of PANK2 activity compared to PANK2 mutations found in PKAN, since PINK1 is upstream of PANK2 and not the sole controller of its translational regulation. PANK2 mutations can cause more direct and debilitating defects; PKAN patients often show symptoms at 3–4 years old and die in their early teens. It is also worth noting that PKAN belongs to a broader category of neurodegenerative diseases, NBIA (Neurodegenerative diseases with Brain Iron Accumulation), PKAN is known as NBIA1, and another subset include PLAN or NBIA2 (PARK14), mutated for P琅2G6 (encoding PLA2G6, 85/88 kDa calcium-dependent phospholipase A2), a gene involved in lipid metabolism. NBIA2 exhibits widespread cortical alpha-synuclein-positive Lewy body pathology. A far subset of NBIA is MPAN, mutated in a poorly characterized mitochondrial protein called C19orf12. MPAN also has Lewy body-like, as well as tau-positive inclusions. These observations imply that NBIA as a whole also overlaps with PD, or they are more closely related than they appear.

Lastly, in this research, we conducted new customized statistics of mitochondrial aggregation/morphology in Drosophila muscle and DA neurons. We found the regular ONE-WAY ANOVA test or Student’s t-test was not suitable in these scenarios, since their assumption are to test if the mean values of different groups have significant difference. The underlying biological hypothesis of assumption is that the genetic regulation will equally affect each mitochondrion. However, this is not what we observed in our samples; the aggregation randomly happens in a portion of the mitochondria population, leaving a large number of smaller individuals. This observation indicates that it may be more relevant to compare the behavior of the extreme values under different experiment conditions. The two-proportion Z test is used to determine whether percentages of values above a certain threshold are significantly different between the experiment group and the control. Thus, stepwise threshold screening can be utilized to determine specific thresholds that provide significance in these tests, and in addition, help us determine whether the modifications (genetic or pharmacological) will preferentially remove smaller or larger mitochondrial aggregates. The new tests are more in line with our observation and better reflect the changes in mitochondrial aggregates in our experiments.

Methods

Drosophila stocks. Flies were normally raised at 25 °C, ~65% humidity on standard cornmeal media, unless otherwise noted. D-panthethine (Sigma) was added at a concentration of 0.8 mg/ml in the standard fly food.

Transgenic lines UAS-Fbl-L (Fbl-L OE) and UAS-Fbl-S (Fbl-St OE and Fbl-S2 OE) were generated in w1118 background by P-element mediated transformation. Fbl-L M2, M7 and yeast ND1 lines were generated by injection of pUAST-Fbl-L (K221A) and pUAST-yND1 plasmids into w1118 embryos. The expression of Fbl-L mutants (lines 2# and 7#) and ND1 were examined by RT-PCR. Some fbl RNAi lines were purchased from the Vienna Drosophila RNAi Center (VDRC, Vienna, Austria) (v101437 for fbl RNAi #1) and the Tsinghua Fly Center (THU9131.N for fbl RNAi #2). UAS-ATG2 RNAi (THU2714), UAS-ATG6 eRNA (TH1555), UAS- ATG12 RNAi (THU2715) and UAS-Ppdc RNAi (THU4283.N), were from Tsinghua Fly Center. The PINK1 mutant was a gift from Dr. Jongkeong.
The TH-Gal4 line was a gift from Dr. Serge Birman. The UAS-mitoGFP fly line was a gift from Dr. William Saxton. UAS-ref(2)P lines were gifts from Dr. L. M. Martinez.25 The UAS-PINK1 RNAi line, UAS-parkin RNAi line, UAS-parkin OE line, UAS-pum OE line, UAS-pum RNAi line, UAS-glo RNAi lines were gifts from Dr. Bingwei Li28,35. The UAS-Agt1 OE line was a gift from Dr. Tao Wang at National Institute of Biological Sciences, Beijing. The Parkin1 and Parkin2 mutants were provided by Dr. Patrik Verstreken. All the other stocks were purchased from the Stock Centers (see Supplementary Table 2).

Cell lines. Mammalian cells were cultured under normal conditions (1x DMEM medium (Hyclone), 10% fetal bovine serum (FBS, Gibco), 5% CO2, 37°C, with supplementation of penicillin/streptomycin. Human fibroblast cells from PKAN patients (genotypes: c.36 G > T/c.979 A > G; c.1561 G > A/c.1502 T > C; c.1561 G > A on PANK2 genes) were from Prof. Susan J. Hayflick (Oregon Health and Science University, USA). The HEK293T cell was a gift from Prof. Yu Li Lab (Tsinghua University, China).

Plasmids and molecular cloning. The site-directed mutagenesis was performed to generate the kinase-dead form of Fbl-L (K221A: Lys 221 — Ala)29. The DNA was cloned into pUAST vector via XhoI-EcoRI sites to generate the pUAST-Fbl-L (K221A) construct. The CDNA sequence of Drosophila Pifida (dPifida) was cloned into pUAST vector via EcoRI-XhoI sites to generate the pUAST-dPifida construct. The CDNA sequence of human PANK2 was cloned into pcDNA3.1 vector via NheI-EcoRI sites to generate pcDNA3.1-hPANK2 construct. The CDNA sequences of Drosophila ref(2)P and Tip60 was cloned into pMXB10 vector to generate pMXB10-Ref(2)P and pMXB10-Tip60. To generate the MS2-βl plasmid, βl-5’-UTR was PCR amplified and used to amplify βl-5’-UTR-CDN-3’-UTR-CDN and PCR products were cloned into pm2S vector via PmeI-XhoI. MitoRed and mitoGreen plasmids were from Prof. Yangyan Wu at Hunan Agriculture University. The MitoKeima plasmid was from Dr. Hongguang Xia of Zhejiang University.

RNA purification, RT-PCR, and CLIP assay. RNAs were extracted from different subcellular fractions by following the manufacturer’s instructions from TRIzol Reagent (Invitrogen) or RNaseasy Kit (Qiagen). At least 10–15 flies or fly thoraces were used for RNA extraction. cDNAs were synthesized using the Transcript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction. cDNAs were synthesized using the TransScript one-step gDNA Removal and cDNA Synthesis Super Mix kit (TransGen Biotech), and cDNAs were used for RNA extraction.

Mitochondria purification. Crude mitochondria fractions were isolated from 3rd instar larvae as described44. Briefly, 3rd instar larvae were gently homogenized in cold mitochondrial isolation buffer (HBS buffer: 5 mM HEPES, 70 mM Sucrose, 2 mM MgCl2, 1 mM EGTA) and protease inhibitor cocktail. The homogenate was then centrifuged at 10000 g for 10 min twice at 4 °C to remove the pellet. The supernatant was further centrifuged at 10000 g for 15 min twice, and the pellet containing mitochondria was resuspended in the isolation buffer. The mitochondrial protein concentration was measured by the BCA kit (Thermo Scientific). Complete images were taken with a Zeiss LSM710 Meta confocal microscope and analyzed by the ZEN lite Digital Imaging Software.

Immunoprecipitation and mRNP purification. For immunoprecipitation, extracts of fly tissues were prepared by homogenization in lysis buffer. After centrifugation at 10000 g for 5 min, the supernatant was subjected to immunoprecipitation using the indicated antibodies and protein A/G magnetic bead (Pierce) at 4 °C for overnight. Immunocomplexes were analyzed by SDS-PAGE and immunoblotting. For denaturing immunoprecipitation, 30 fly thoraces of each genotype were collected and homogenized with 500 μl cell lysis buffer; samples were mixed with 500 μl 2X SDS Laemmli loading buffer supplemented with 2-mercaptoethanol and denatured by boiling at 100 °C for 10 min. Then samples were diluted with pre-chilled lysis buffer at a 1:5 ratio and subjected to immunoprecipitation using rat anti-RNAi-Ref(2)P (Abcam).

For Fbl and C-1 30 mRNP purification, pcDNA3.1-PINK1.FLAG, MS2-BP, GST, and MS2-bs-C-1 30 or MS2-bs-Fbl plasmids were transiently expressed together. Sixty hours post-transfection, we UV crosslinked attached cells in the petri dish and purified mitochondria as described26. We homogenized the mitochondria fraction in lysis buffer and performed GST pull-down assays using glutathione-sepharose beads (BeaverBio) at 4 °C for 6 h with gentle shaking. Subsequently, beads were washed three times (10 min each) at 4 °C in lysis buffer, mixed with 2X SDS Sample buffer, and loaded onto SDS-PAGE gels.

shRNA and siRNA transfection. Human PARK1 shRNAs were purchased from human shRNA library in Tsinghua University (#TRCN0000199193, #TRCN0000199446), and packaged by the lentiviral system (vector, packaging, and envelope plasmids with the plasmid proportion 3:2:2) by using P2PAX, PMD2G plasmids and the HEK293T cell. Lentiviruses were harvested in 36 h and transduced into HEK293T cells with 8 μg/ml Polybrene, after 24 h, lentivirus contained
medium was exchanged to new DMEM medium with 10%FBS, 1 µg/ml puromycin was supplemented after 48 h, protein extract from the transfected cells was used to analyze human PINK1 protein level. Human PANK2 was knocked down by shRNAs (Sangon Biotech), which was transfected by Attractene Transfection Reagent (Qagen).

Analyzing mitophagy activity in mammalian cells. To analyze the mitophagy activity in WT and PINK1 RNAi cells, mitochondrial and autophagy markers were expressed in HEK cells as follows: mitoDsRed/GFP-LC3, mitoDsRed/FLAG-p62 and mitoGreen/Lamp1-RFP. Transfected cell lines were selected by treating with 200 µg/ml puromycin. Cells were subsequently treated with CCCP for 12 h, and the colocalizations of mitochondrial and autophagy markers were analyzed by confocal microscopy after immunofluorescence staining. To study the effect of CoA precursors on mitophagy, 100 ng/ml D-Pan and 300 ng/ml RE-024 were administrated above the threshold across the two groups, the null hypothesis and alternative hypothesis of the test are:

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H_0 : \alpha = 0; H_1 : \alpha > 0
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Acknowledgements
This research was supported by the National Basic Research Program of China (2018YFA0900100 to B. Zhou), the National Science Foundation of China (31971087, 91649118 to B. Zhou, 31700883 to Y. Huang, and 31700881 to Z. Wan), the National Institutes of Health (R15AG067470 to Z. Wu, and R01NS084412 to B. Lu), the Cancer Prevention and Research Institute of Texas (RP210068 to Z. Wu), the Children’s Brain Diseases Foundation (411903 to Z. Wu) and Southern Methodist University (new faculty startup fund to Z. Wu). We are grateful to the Bloomington Stock Center, the Tsinghua Fly Center, and the Vienna Drosophila RNAi Center for fly stocks. We are indebted to Dr. O. Sibon for the anti-Fbi rabbit polyclonal antibody. We thank our friends and other research labs mentioned in the manuscript for their generosity in providing us relevant fly stocks and reagents. We also thank Dr. Johnson Taylor for editing and suggestions. The Biomedical Analysis Center of Tsinghua University provided technical help and services.

Author contributions
B.Z., Z.Wu, Y.H., Z.Wan, and Y.T. designed the experiments. Y.H., Z.Wan, and Y.T. performed the experiments, analyzed data, wrote the manuscript, and contributed equally to this project. C.Y., B.Liu, and J.C. performed the Two-Proportion Z Test. J.X., B.Laboret, and S.N. helped with some of the research labs mentioned in the manuscript for their generosity in providing us relevant fly stocks and reagents. We also thank Dr. Johnson Taylor for editing and suggestions. The authors declare no competing interests.

Additional information
The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-30178-x.

Correspondence and requests for materials should be addressed to Zhihao Wu or Bing Zhou.

Peer review information Nature Communications thanks Ashley Bush and the other anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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