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14. ABSTRACT

Loss of function mutations in the genes encoding PINK1 and Parkin results in early-onset forms of Parkinson's disease (EOPD). Both enzymes are functionally linked and together direct a neuroprotective mitochondrial quality control (mitoQC) ensuring elimination of damaged organelles from cells via the autophagy-lysosome system (i.e. mitophagy), which is lost in EOPD. Given the complexity of this pathway and the general missing heritability in EOPD, it is highly likely that additional genes regulating this pathway may also be found mutated in EOPD. The overarching goals of this project are to 1) identify high confidence genetic modifiers of the PINK1/Parkin pathway by a two-tiered functional screening (overlay of genome-wide siRNA and miRNA screens) in cells, 2) to identify the underlying genetic variation and characterize the EOPD genome (whole-genome-sequencing of patients), as well as 3) to determine the pathogenicity of these novel EOPD sequence variants in functional readout studies. Using this combined functional genetics approach we are currently whole-genome sequencing the first 50 EOPD patients and overlaying results from our sequences with hits from the siRNA and miRNA screens.

15. SUBJECT TERMS
early-onset Parkinson's disease, mitochondrial quality control, mitophagy, PINK1, Parkin, functional genomic screening, genetic architecture

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1. **INTRODUCTION:** Loss of function mutations in the genes encoding PINK1 and Parkin results in early-onset forms of Parkinson's disease (EOPD). Both enzymes are functionally linked and together direct a neuroprotective mitochondrial quality control (mitoQC) ensuring elimination of damaged organelles from cells via the autophagy-lysosome system (i.e. mitophagy), which is lost in EOPD. Given the complexity of this pathway and the general missing heritability in EOPD, it is highly likely that additional genes regulating this pathway may also be found mutated in EOPD. The overarching goals of this project are to 1) identify high confidence genetic modifiers of the PINK1/Parkin pathway by a two-tiered functional screening (overlay of genome-wide siRNA and miRNA screens) in cells, 2) to identify the underlying genetic variation and characterize the EOPD genome (whole-genome-sequencing of patients), as well as 3) to determine the pathogenicity of these novel EOPD sequence variants in functional readout studies. Using this combined functional genetics approach we will determine the regulation of mitophagy as well as the genetic architecture of EOPD.

2. **KEYWORDS:** early-onset Parkinson's disease, mitochondrial quality control, mitophagy, PINK1, Parkin, functional genomic screening

3. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?**

  Major Task 1: Nomination of mitoQC candidate genes by an accelerated, two-tiered functional screen and processing through bioinformatics resource/filtering strategy – Month 1-18

  Major Task 2: Whole-Genome sequencing in patients with EOPD and nomination of disease genes/variants – Month 1-36

  Major Task 3: Validation of high-confidence mitoQC/EOPD genes and dysfunctions of sequence variants on molecular, cellular, and organismal level – Month 6-36

- **What was accomplished under these goals?**

  Towards accomplishing major goals of the project, we first completed the regulatory review and approval by Mayo Clinic IRB and the HRPO (subtask 1.1). This was finalized and approved in early 2018. We have screened our latest early-onset patients with Parkinson's disease to identify those that are negative for *PINK1* and *PARKIN* mutations, and have prioritized these subjects for whole-genome sequencing. In addition, we identified all early-onset patients for which we have collected fibroblast biopsies and grown the cell lines and extracted DNA, we will include these cell lines for sequencing which will provide an immediate opportunity to functionally assess the influence of variants identified within these subjects on the efficiency of the mitophagy pathway. We are currently whole-genome sequencing 50 patients with early-onset disease and should have the data within the first month of the second funding period.

  Working with our colleague, Dr. Xinnan wang (Stanford), we identified variants in the *Mitofilin* (*MIC60*) gene, a substrate for PINK1 phosphorylation, which may play a role in mitochondrial dysfunction and disease (Tsai et al., 2018). The MIC60 protein maintains mitochondrial functions and cellular survival, and disruption can lead to cell death. We identified a number of variants within the mitochondrial targeting series that were present either specifically in cases or controls with contrasting functional effects. We belive the deleterious mutations may be disrupting the mitochondrial targeting ability of the MIC60 protein. It would appear that mutations with *Mitofilin* have the ability to be damaging to mitochondrial function and possibly through a PINK1-dependent mechanism. In addition, a novel mutation in the *LRP10* gene was reported to cause Parkinson's disease and we are

assessing the role of mutations in early-onset forms of the disease and any possible links to mitochondrial function and protein trafficking (Ross & Bu, 2018).

We are currently identifying variants through available whole-exome and our whole-genome sequencing in early-onset patients with Parkinson's disease and overlay those results with the screens from Major Task 1. We will use a cohort of 1000 whole-genome sequences that we have collected from the Mayo Clinic Biobank cohort. In addition, we have obtained whole-genome sequence data from the Parkinson's Progression Markers Initiative (PPMI) cohort supported by the Michael J. Fox Foundation and are processing data to use a replication cohort for our sequence data. This series and other sequence data we are generating will also allow us to rapidly assess the role of variants/genes in the more frequent late-onset forms of Parkinson's disease that we identify in our studies.

- **What opportunities for training and professional development has the project provided?**
  Nothing to Report.

- **How were the results disseminated to communities of interest?**
  We published the Tsai et al. paper in Molecular Cell (Impact Factor. 14.3), and we published the editorial on *LRP10* in Lancet Neurology (Impact Factor. 27.1).

- **What do you plan to do during the next reporting period to accomplish the goals?**
  We will complete the whole-genome sequencing of 100 early-onset patients with Parkinson's disease. We will extract variants that are predicted deleterious in genes that are established within the mitophagy/autophagy pathways and assess these against our in-house control whole genome sequence data and also through additional datasets we have obtained, e.g. PPMI. We will then plan to work with Dr. Springer to assess the functional impact of the variants within his cellular model of mitophagy.

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*
- **What was the impact on the development of the principal discipline(s) of the project?**
  Nothing to Report.
- **What was the impact on other disciplines?**
  Nothing to Report.
- **What was the impact on technology transfer?**
  Nothing to Report.
- **What was the impact on society beyond science and technology?**
  Nothing to Report.

5. **CHANGES/PROBLEMS:**
- **Changes in approach and reasons for change**
  Nothing to Report

- **Actual or anticipated problems or delays and actions or plans to resolve them**
  We encountered an initial delay of >4 months due to IRB and HRPO approval. This had been solved end by the beginning of 2018. In addition, we have been attempting to establish collaboration with The American Genome Center at the Uniformed Services University in Bethesda. The cost of whole-genome sequencing would be reduced if we are able to perform the whole-genome sequencing there. We have been trying to complete a material transfer agreement and work with Dr. Clifton Dalgard, Ph.D. who is Associate Professor and Director of the Laboratory Core (Collaborative Health Initiative Research Program; CHIRP).

These discussions are still on-going and we hope we can establish a productive collaboration. We have begun sequencing with Hudson Alpha as the provider until such time as we can use The American Genome Center at the Uniformed Services University. The time line has only been marginally affected by the above and we are confident to be able to complete all sequencing as scheduled with either center.

- **Changes that had a significant impact on expenditures**
  Nothing to Report

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
  Nothing to Report

6. **PRODUCTS:**
   Nothing to Report at this point.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**
   - **What individuals have worked on the project?**

Name:	*Owen A. Ross, PhD*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2.0*
Contribution to Project:	*Together with the co-PI Dr. Springer, Dr. Ross has supervised the project, collected all regulatory material and ensured all necessary steps towards completion of the milestones*
Funding Support:	
Name:	*Ana Kolicheski, PhD*
Project Role:	*Research Fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2.0*
Contribution to Project:	*Dr. Kolicheski has coordinated the analysis of the next-generation sequence data in consultation with biostatisticians and bioinformaticians.*
Funding Support:	
Name:	*Ronald Walton, BSc*
Project Role:	*Laboratory Technician*

Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*4.5*
Contribution to Project:	*Mr. Walton processed and organized all the samples from patients with early-onset Parkinson's disease. He completed the gene screening of PINK1 and PARKIN to identify and exclude carriers of known mutations prior to selection for whole-genome sequencing. He prepares and is responsible for shipping samples for sequencing, retrieval of data and facilitating bioinformatics processing.*
Funding Support:	

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

  Owen A. Ross, PhD

  Changes in Active Support:

  New

  Title: Functional genomics of PINK1/PARKIN-related disease using –omics data from the PPMI series
  Committed Time: 0.60
  Supporting Agency: Michael J Fox Foundation for Parkinson's Research
  Contracting/Grants Officer: Anna Naito, Research Programs Officer
  Performance Period: **7/10/2018** - 7/9/2019
  Level of Funding: $125,000 (current annual direct costs)
  Goals & Specific Aims:
  Role: PD/PI

  Title: Mayo Clinic APDA Center for Advanced Research
  Committed Time: 0.12
  Supporting Agency: American Parkinson Disease Association
  Contracting/Grants Officer: Heather Gray, Research Manager
  Performance Period: **09/01/2018**-08/31/2019
  Level of Funding: $100,000 (current annual direct costs)
  Goals & Specific Aims:
  Role: Co-Investigator

  Ended

- **What other organizations were involved as partners?**
  "Nothing to Report."

8. **SPECIAL REPORTING REQUIREMENTS**
   - **COLLABORATIVE AWARDS:**
     For an update on Major Task 1 see report from the co-PI Dr. Springer.
   - **QUAD CHARTS:**
     See appendix
9. **APPENDICES:** *Quad chart, Tsai et al., 2018, Ross & Bu, 2018.*

Early-Onset Parkinson's Disease Is a Mitochondrial Disease: A Nigral Mitochondrial Cytopathy

PR160606P1

W81XWH-17-1-0249

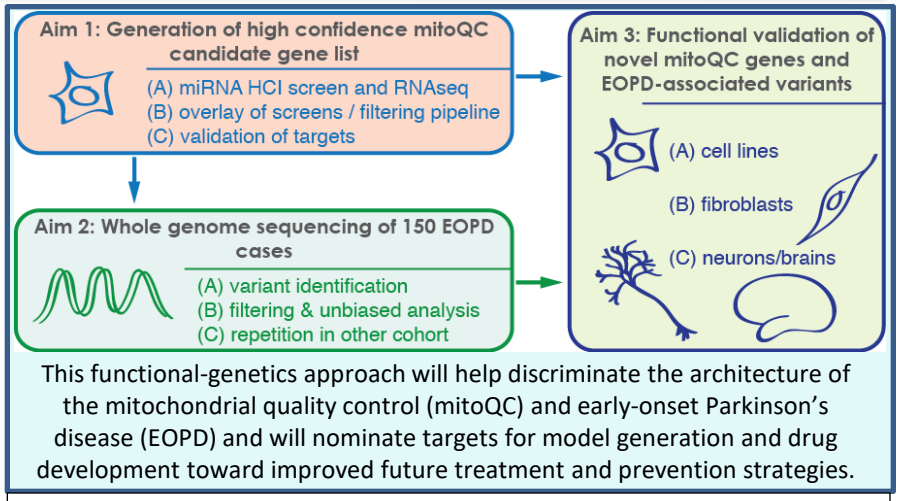
PI: Owen A. Ross, PhD **Org:** Mayo Clinic Jacksonville **Award Amount:** $1,176,917

Study/Product Aim(s)

- Specific Aim 1: To identify high-confidence genetic modifiers of PINK1/PARK2-directed mitochondrial quality control (mitoQC)
- Specific Aim 2: To identify the underlying genetic variation and characterize the early-onset Parkinson's disease (EOPD) genome
- Specific Aim 3: To determine pathogenicity of novel EOPD sequence variants in functional readout studies

Approach

We hypothesize that EOPD is a mitochondrial disease and that its genetic causes cluster around loss of mitoQC functions resulting in failure to safely dispose of damaged organelles. Our overarching goal is to delineate this pathway and the disease relevance of individual key players and their variants towards rationalized biomarker and drug development. This will be achieved through combining whole-genome-sequencing data from EOPD patients with functional genetic screening of genes/variants.



Accomplishment: Complete regulatory review and approval by Mayo Clinic IRB and by HRPO.

Timeline and Cost

Activities CY	17	18	19	20
Aim 1: Functional screening				
Aim 2: WGS & analysis				
Aim 3: Validation & pathogenicity				
Estimated Budget ($K)	**$196**	**$392**	**$392**	**$196**

Updated: (09/14/2018)

Goals/Milestones

CY17 Goal – Regulatory review and roll-out of study

✓Complete regulatory review and approval by HRPO

✓Whole-genome sequencing of 50 EOPD patients

CY18 Goals – First-tier filtering of genomic variants

☐ Whole-genome sequencing of 100 EOPD patients

☐ Critical variant identification of mitoQC candidates

CY19 Goal – Second-tier filtering of genomic variants

☐Whole-genome sequencing of 150 EOPD patients

☐Critical variant identification with informed and unbiased strategies

CY20 Goal – Third-tier filtering and replication of candidate variants

☐Pathogenic variant identification in EOPD/mitoQC genes

☐Variant replication in additional cases of EOPD, LOPD & controls

☐**Comments/Challenges/Issues/Concerns**

- If timelines change, comment here.
- If off by more than one quarter in spending, comment here.

Budget Expenditure to Date

Projected Expenditure:

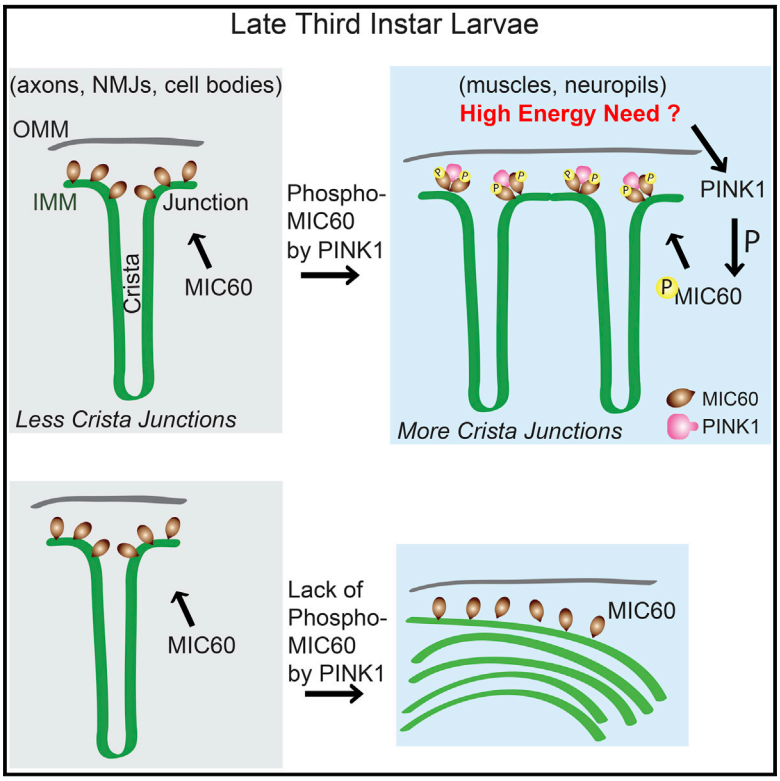
Actual Expenditure: $117,819.98

Article

Molecular Cell

PINK1 Phosphorylates MIC60/Mitofilin to Control Structural Plasticity of Mitochondrial Crista Junctions

Graphical Abstract



Highlights

- Mitochondria remodel crista junctions in selective subcellular areas in *Drosophila*
- This structural remodeling requires PINK1 to phosphorylate MIC60
- PINK1-mediated phosphorylation of MIC60 stabilizes MIC60 oligomerization
- Rare coding variants of *MIC60* found in Parkinson's patients are damaging in flies

Authors

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In Brief

Tsai et al. discover that mitochondria increase crista junctions and numbers in selective subcellular areas in *Drosophila*. This structural remodeling requires Parkinson's-linked PINK1 to phosphorylate the inner mitochondrial membrane protein MIC60, which stabilizes MIC60 oligomerization. MIC60 functions downstream of PINK1 to maintain mitochondrial functions and cellular survival.

Tsai et al., 2018, Molecular Cell *69*, 1–13
March 1, 2018
https://doi.org/10.1016/j.molcel.2018.01.026

CellPress

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Molecular Cell
Article

PINK1 Phosphorylates MIC60/Mitofilin to Control Structural Plasticity of Mitochondrial Crista Junctions

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SUMMARY

Mitochondrial crista structure partitions vital cellular reactions and is precisely regulated by diverse cellular signals. Here, we show that, in *Drosophila*, mitochondrial cristae undergo dynamic remodeling among distinct subcellular regions and the Parkinson's disease (PD)-linked Ser/Thr kinase PINK1 participates in their regulation. Mitochondria increase crista junctions and numbers in selective subcellular areas, and this remodeling requires PINK1 to phosphorylate the inner mitochondrial membrane protein MIC60/mitofilin, which stabilizes MIC60 oligomerization. Expression of MIC60 restores crista structure and ATP levels of *PINK1*-null flies and remarkably rescues their behavioral defects and dopaminergic neurodegeneration. In an extension to human relevance, we discover that the PINK1-MIC60 pathway is conserved in human neurons, and expression of several *MIC60* coding variants in the mitochondrial targeting sequence found in PD patients in *Drosophila* impairs crista junction formation and causes locomotion deficits. These findings highlight the importance of maintenance and plasticity of crista junctions to cellular homeostasis *in vivo*.

INTRODUCTION

Efficient mitochondrial oxidative phosphorylation and ATP synthesis rely heavily on the exquisite membrane organization of the mitochondrial cristae. The inner mitochondrial membrane (IMM) protrudes into the matrix to form cristae that harbor the electron transport chain (ETC) machinery and ATP synthase. Each individual crista contains a tubular invagination, with an opening to the intermembrane space called crista junction and a bottom called crista tip (Figure 1A). Crista membranes bend extensively at crista junctions and tips to sustain the remarkably narrow and elongated crista space, and this unique shape is required for maintenance of solute gradients and localization of the ETC complexes (Mannella et al., 2013). Studies in yeast have revealed several crucial factors involved in maintenance of crista structure, including MIC60 (IMMT/Fcj1/mitofilin), mitochondrial fission-fusion machinery, F_oF_1-ATP synthase, and mdm33 (Frezza et al., 2006; Meeusen et al., 2006; Messerschmitt et al., 2003; Rabl et al., 2009; Strauss et al., 2008). MIC60 is an IMM integral protein and a major component of the mitochondrial contact site and cristae organizing system (MICOS) complex (Pfanner et al., 2014). Mitochondrial crista structure is not always static; instead, it undergoes dynamic remodeling tightly correlated with the mitochondrial aerobic respiration rates (Hackenbrock, 1966; John et al., 2005; Mannella et al., 2013). In high-energy-demanding cells, mitochondria perform higher respiratory activities. However, it remains elusive as to how cellular signals instruct mitochondria to remodel the crista architecture, particularly in an *in vivo* setting.

Mutations in the Ser/Thr kinase PINK1 cause autosomal recessive early-onset Parkinson's disease (PD) (Valente et al., 2004). The hallmark of PD is age-dependent degeneration of dopaminergic (DA) neurons in the substantia nigra. PINK1 is imported into healthy mitochondria with the polarized mitochondrial membrane potential ($\Delta\Psi m$) (Greene et al., 2012; Morais et al., 2014; Thomas et al., 2014). PINK1 is blocked from import into depolarized mitochondria and stabilized on the outer mitochondrial membrane (OMM), where it phosphorylates its substrates to trigger mitophagy (Chen and Dorn, 2013; Kondapalli et al., 2012; Narendra et al., 2008; Vives-Bauza and Przedborski, 2011; Wang et al., 2011; Whitworth and Pallanck, 2009).

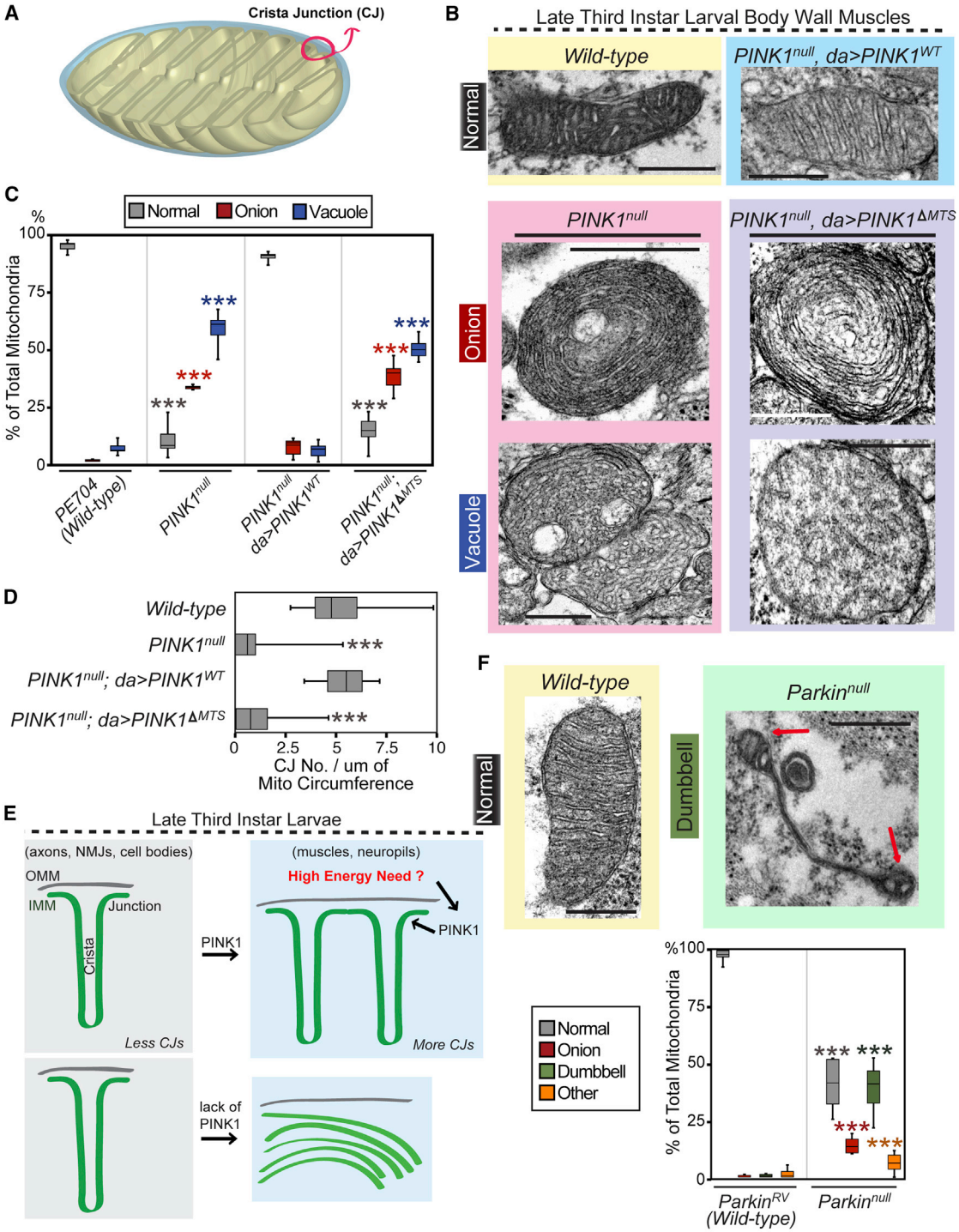

Figure 1. PINK1 Maintains Crista Junctions

(A) Cartoon depicting a mitochondrial crista junction (red circle).

(B and F) TEM images of body wall muscles of late third instar larvae (120 hr AEL) of *PINK1* null (B) or *Parkin* null (F).

(C and F) Quantification of the percentage of total mitochondria per image for *PINK1* null (C) or *Parkin* null (F). n = 307–916 mitochondria from 32–98 images obtained from 6–8 flies.

(D) Quantification of the number of crista junctions normalized to the length of the mitochondrial circumference. n = 30 mitochondria from 30 images from 6–8 flies.

(E) Schematic representation of PINK1-mediated plasticity of crista structure in third instar larvae.

(legend continued on next page)

However, whether PINK1 has kinase activity inside healthy mitochondria remains controversial. Although PINK1 has been shown to be crucial for the mitochondrial complex I activity, direct PINK1 kinase substrates in the ETC have not yet been identified (Morais et al., 2014; Pogson et al., 2014). In this work, we discover a novel substrate of PINK1 inside healthy mitochondria—the IMM protein MIC60, and reveal that the PINK1-MIC60 pathway maintains remodeling of crista junctions, the complex I activity, and DA neuronal survival *in vivo*.

RESULTS

Inside Mitochondria, PINK1 Is Required for Maintenance of Crista Junctions

We determined whether PINK1 is present inside the mitochondria of *Drosophila*. By immuno-gold staining under transmission electron microscopy (TEM), we found that transgenic PINK1-Flag was evenly distributed inside the mitochondria, likely in the matrix or intermembrane space, as well as outside the mitochondria in the cytosol (Figure S1A). Human PINK1 transgene was used here and throughout the paper, owing to the functional conservation between human and fly PINK1 (Clark et al., 2006). Using proteinase K assays (Morais et al., 2014), we found that a fraction of endogenous *Drosophila* PINK1 (dPINK1) was present inside the mitochondria at the steady state (Figure S1B). We next examined mitochondrial crista structure under TEM and identified crista phenotypes in body wall muscles of *PINK1*-null late third instar larvae 120 hr after egg laying (AEL). Approximately 34.45% of total mitochondria appeared like an "onion" with concentric multi-layered and heavily packed crista membranes, and 58.13% were filled with small "vacuole"-like crista membranes. In both cases, crista junctions were significantly reduced. In *PINK1*-null mitochondria, there were only 1.03 ± 0.29 crista junctions/μm of mitochondrial circumference, whereas in wild-type mitochondria, there were 5.08 ± 0.32 crista junctions/μm (Figures 1B–1D). These results indicate that PINK1 is required for maintenance of crista junctions in late third instar larval body wall muscles. The mitochondrial shape became round in *PINK1*-null larvae, although the mitochondrial size was not significantly altered (Figure S1C). No pronounced muscle degeneration was observed in *PINK1*-null larvae (Figure S1D). We found the same onion- and vacuole-like mitochondria in larval body wall muscles using another independent *PINK1*-null allele (Figure S1E). In the nervous system, the phenotype of the onion-like cristae and loss of crista junctions existed only in the neuropils (enriched with synapses, dendrites, and axons) at the ventral nerve cords (VNCs), but not in the cell bodies at the VNC, in the segmental nerves (axons), or at the neuromuscular junctions (NMJs) in *PINK1*-null larvae (Figure S2). We then considered the possibility that mitochondrial cristae undergo PINK1-dependent remodeling when mitochondria move from the cell bodies into the neuropils. To explore this possibility, we measured the mitochondrial crista density (the number of cristae/mitochondrial area) and found that mitochondria in neuropils contained significantly more dense cristae with increased crista junctions than those in neuronal cell bodies, axons, or NMJs (Figure S3A), indicating that mitochondrial remodeling of crista structure occurs when mitochondria move into the neuropils from the cell body and mitochondrial respiratory activity increases (Hackenbrock, 1966; John et al., 2005). In addition, neuropils had more mitochondria (the volume mitochondria occupy/total volume) than neuronal cell bodies, axons, or NMJs (Figure S3B), suggesting that synapse and dendrite-enriched neuropils consume more energy (Lajtha et al., 2007). Therefore, when mitochondria move to subcellular compartments that may have elevated energy demands, they condense their cristae and increase crista junctions in third instar larvae. This structural plasticity requires PINK1, because in those regions of *PINK1*-null larvae mitochondrial cristae fail to remodel as in wild-type, and instead they display the onion-like membranes with loss of crista junctions (Figures 1, S1, and S2). Taken together, PINK1 is essential for mitochondria to increase crista junctions in selective subcellular areas in third instar larvae (Figure 1E).

We next determined the extent to which the physical presence of PINK1 inside the healthy mitochondria is required for maintenance of crista junctions. To do this, we ubiquitously expressed full-length PINK1, or PINK1$^{\Delta MTS}$ without the mitochondrial targeting sequence (MTS) that leads PINK1 import into mitochondria (Weihofen et al., 2009), in *PINK1*-null larvae. Both PINK1 transgenes were inserted in the same genomic site using the PhiC31 integrase-mediated transgenesis systems to ensure the same genomic regulations (Markstein et al., 2008), and their protein expression levels were comparable (Figure S4A). The aberrant crista structure and loss of crista junctions in *PINK1*-null flies were fully rescued by expressing full-length PINK1, but not by PINK1$^{\Delta MTS}$, in late third instar larval body wall muscles (Figures 1B–1D). Therefore, the import of PINK1 into healthy mitochondria is required for maintenance of crista junctions in larval muscles.

The onion- and vacuole-like mitochondria found in *PINK1*-null larvae differed from the previously reported "vacuolated (empty)" mitochondria with crista fragmentation in thoracic indirect flight muscles of *PINK1*-null adults (Clark et al., 2006; Park et al., 2006). We also observed the predominant vacuolated mitochondria in *PINK1*-null adults but did detect about 25% of the onion-like mitochondria at an earlier adult stage (day 3), which all converted to vacuolated mitochondria at day 5 (Figure S4B). This result suggests that the onion-like mitochondria may represent an earlier stage of mitochondrial pathology in adults. Expression of PINK1$^{\Delta MTS}$ failed to rescue the mitochondrial phenotypes and muscle degeneration in *PINK1*-null adult flies (Figures S4B and S4C), demonstrating the importance of PINK1 inside healthy mitochondria for adults as well.

It has been reported that *Parkin*-null adult flies exhibit similar vacuolated mitochondria as *PINK1*-null adults (Greene et al., 2003; Pesah et al., 2004). To answer the question of whether

The scale bars represent 500 nm. *Parkin*^{null} (*Park*^{25}); *Parkin*^{RV} (*Park*^{rva}); *PINK1*^{nul} (*PINK1*^{5}/*Y*; *da-GAL4*); *PINK1*^{null},*da* > *PINK1*$^{WT/\Delta MTS}$ (*PINK1*^{5}/*Y*;*UAS-hPINK1*$^{WT/\Delta MTS}$*-Flag*;*da-GAL4*); wild-type (*PE704*/*Y*, precise excision control males for *PINK1*^{5}/*Y*); WT, wild-type. Genotypes are written in the same way here and for all figures except otherwise stated. Comparisons with "wild-type" are shown. *p < 0.05, **p < 0.01, ***p < 0.001, and the box-whisker plots are used for all figures unless otherwise stated. See also Figures S1, S2, S3, and S4.

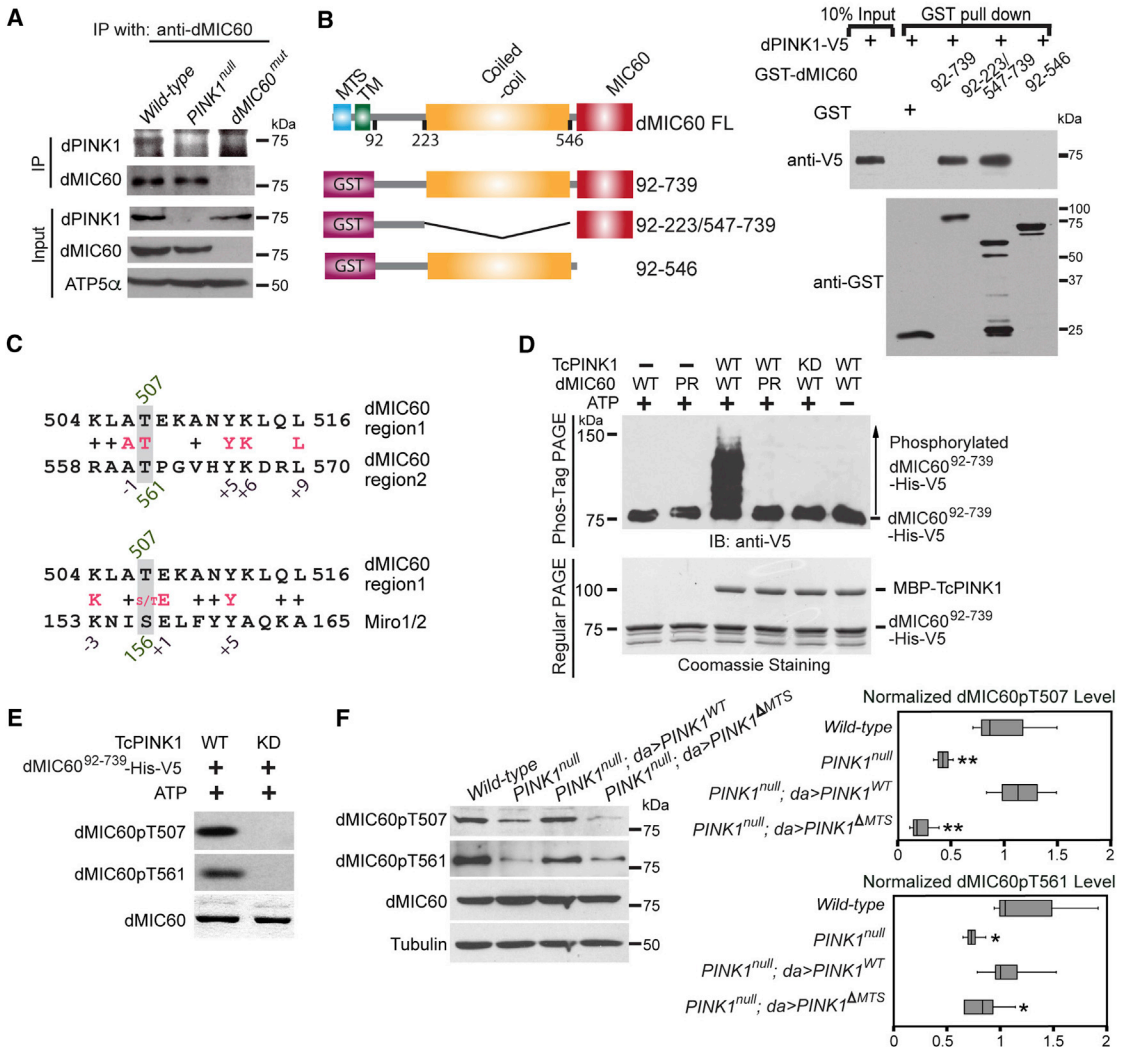

Figure 2. PINK1 Interacts with and Phosphorylates dMIC60

(A) Immunoprecipitations with anti-dMIC60 were performed using the mitochondrial fractions of pupae 72 hr after pupa formation (AFP).

(B) Schematic representation of the truncated GST-dMIC60 used in the *in vitro* co-precipitation. FL, full length. Samples were immunoblotted as indicated.

(C) Sequence alignments between the flanking regions of the two threonine sites in dMIC60 or between the phospho-peptides of dMIC60 and human Miro1/2.

(D) The upper blot is the phos-tag gel immunoblotted with anti-V5. The lower blot is the Coomassie-stained gel revealing the proteins in the reactions.

(E) The *in vitro* phosphorylation reactions were immunoblotted as indicated.

(F) Lysates of adults 5 days after eclosion were immunoblotted as indicated, and the band intensities of phospho-dMIC60 are normalized to those of total dMIC60. n = 3–6 independent experiments. Comparisons with wild-type are shown.

See also Figure S5.

Parkin-null larvae also show the onion- and vacuole-like mitochondria in their muscles as observed in *PINK1*-null larvae, we performed TEM on *Parkin*-null third instar larval body wall muscles. Surprisingly, about half of total mitochondria were normal (40.07%), and the majority of abnormal mitochondria (38.44%) displayed a "dumbbell" shape, in which two ends of one mitochondrion stretch extensively in the opposite directions while the OMM and crista junctions are intact (Figure 1F), implying a failure in fission. Thus, *PINK1* and *Parkin* mutant larvae show distinct phenotypes in crista structure. Collectively, our results reveal a novel function for PINK1 inside healthy mitochondria to regulate structural plasticity of mitochondrial crista junctions.

PINK1 Phosphorylates MIC60

We sought the mechanism by which PINK1 maintains crista junctions. A previous study has reported human MIC60 in a mass spectrometry screen searching for PINK1's binding partners (Weihofen et al., 2009). Interestingly, MIC60 has been demonstrated to play a crucial role in crista junction formation (John et al., 2005; Rabl et al., 2009). In flies, we found that endogenous dPINK1 physically interacted with endogenous *Drosophila* MIC60 (dMIC60) *in vivo* (Figure 2A), which was detected by a polyclonal antibody against dMIC60 protein (anti-dMIC60) generated by us. A band of the predicted size of dMIC60 protein was recognized by anti-dMIC60 in wild-type,

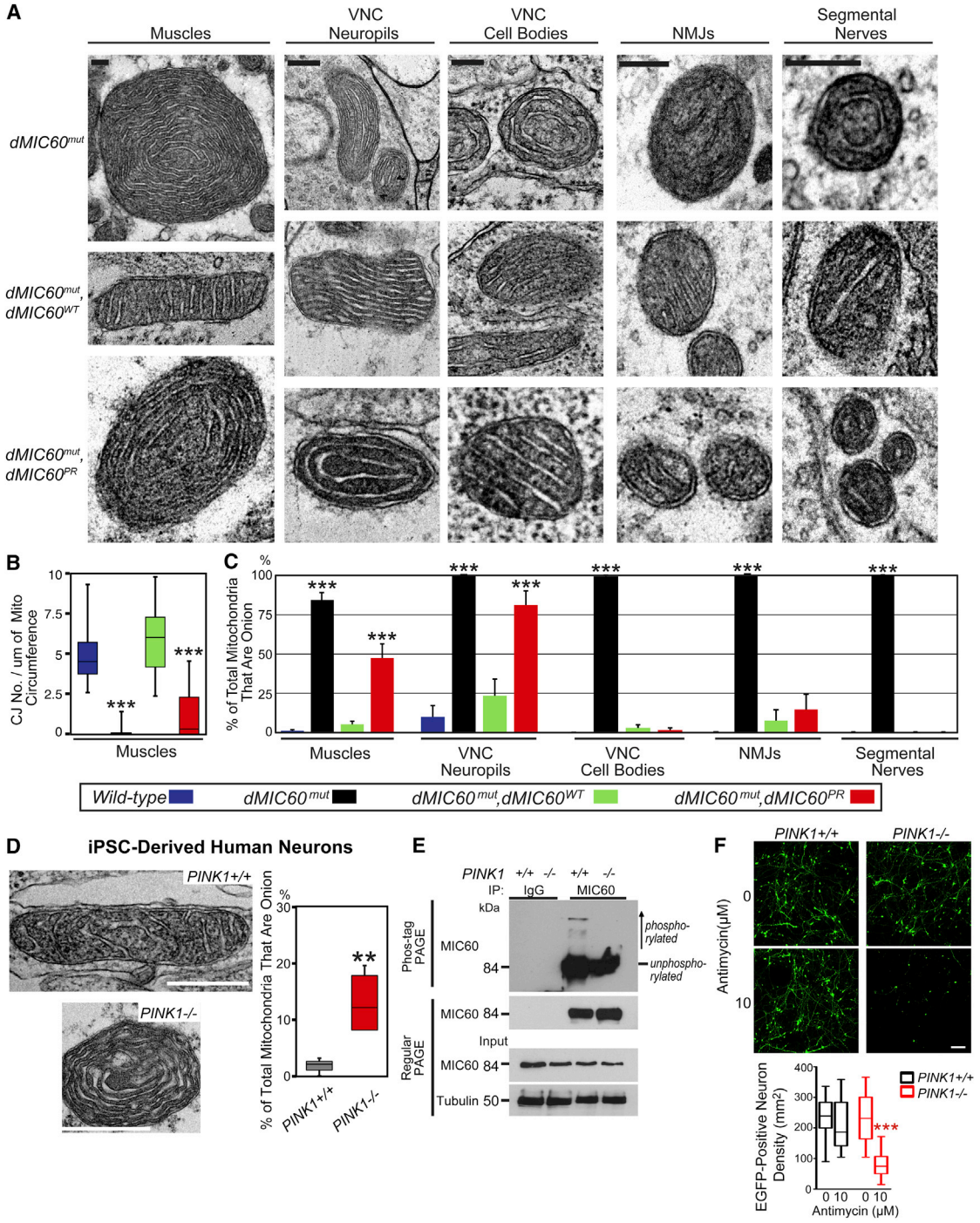

Figure 3. PINK1-Mediated Phosphorylation of dMIC60 Maintains Crista Junctions

(A) TEM images of body wall muscles, VNC, NMJs, or segmental nerves of late third instar larvae (120 hr AEL).

(B) Quantification of the number of crista junctions normalized to the length of the mitochondrial circumference. n = 30 mitochondria from 30 images obtained from 4–6 flies.

(C) Quantification of the percentage of total mitochondria per image. n = 17–236 mitochondria from 8–15 images from 4–6 larvae. Bar graphs and mean ± SEM are shown.

For (A)–(C), *dMIC60*^{mut} (*Tubulin-GAL4_dMIC60*$^{LL02849/LL02849}$); *dMIC60*^{mut},*dMIC60*$^{WT/PR}$ (*UAS-dMIC60*$^{WT/PR}$-Myc;*Tubulin-GAL4_dMIC60*$^{LL02849/LL02849}$); wild-type (*w*^{1118}). Comparisons with wild-type are shown.

(D) TEM images of iPSC-derived neurons. n = 172–198 mitochondria from 30 images from 3 independent experiments.

(legend continued on next page)

but not in a *dMIC60* mutant (*dMIC60^{mut}*) (described later), confirming the specificity of this antibody (Figure 2A). We determined that dPINK1 and dMIC60 also interacted *in vitro* and mapped the region of dMIC60 required for binding to dPINK1 (Figure 2B). We bacterially expressed N-terminal glutathione S-transferase (GST)-tagged dMIC60 with different truncations: GST-dMIC60$^{92–739}$ (lacking the N-terminal MTS and transmembrane [TM] domains); GST-dMIC60$^{92–223_547–739}$ (lacking the N-terminal MTS and TM and the coiled-coil domains); or GST-dMIC60$^{92–546}$ (lacking the N-terminal MTS and TM and the C-terminal domains; Körner et al., 2012) and incubated it with glutathione Sepharose beads before incubation with bacterially purified C-terminal V5-tagged dPINK1. We found that dPINK1 co-precipitated with GST-dMIC60$^{92–739}$ and GST-dMIC60$^{92–223_547–739}$, but not with GST-dMIC60$^{92–546}$ (Figure 2B), suggesting that the C-terminal amino acids (aas) 547-739 of dMIC60 are required for directly binding to dPINK1.

Because PINK1 is a Ser/Thr kinase, we next determined whether dMIC60 is a substrate of PINK1. To do this, we performed an *in vitro* PINK1 kinase assay on bacterially expressed dMIC60. dMIC60$^{92–739}$ was incubated with either purified *Tribolium castaneum* PINK1 (TcPINK1)—the known form of PINK1 that remains active *in vitro* (Woodroof et al., 2011)—or inactive kinase-dead TcPINK1 (TcPINK1KD) prior to mass spectrometric and phos-tag acrylamide analysis. Using mass spectrometry, we identified two dMIC60 sites, threonine 507 and 561, which were phosphorylated; these two sites were not phosphorylated in the other negative controls, although the unphosphorylated peptides were detected with the same efficiency among all reactions (Figures 2C, 2D, and S5). In the mass spectrometric analysis, we encompassed approximately 90% of the total residues of dMIC60$^{92–739}$. Using phos-tag acrylamide, where phosphorylated proteins migrate slower because of binding to the phos-tag ligands, we detected phosphorylated dMIC60 only in the reaction with ATP and TcPINK1 both present, but not when ATP or TcPINK1 was absent (Figure 2D). When both the two phosphorylation sites were mutated to phosphorylation-resistant (PR) alanine, dMIC60 was no longer phosphorylated by TcPINK1 (Figure 2D), which indicates that these two sites are the main phosphorylation residues. We generated two antibodies against phosphorylated dMIC60 at threonine 507 and 561, respectively, and found that the band intensities recognized by anti-phospho-dMIC60 were completely abolished by kinase-dead TcPINK1KD *in vitro* (Figure 2E), confirming the specificity of these antibodies. Phosphorylation at both sites was significantly reduced in *PINK1*-null flies and required the physical presence of PINK1 inside the mitochondria (Figure 2F). Taken together, PINK1 phosphorylates dMIC60 both *in vivo* and *in vitro*.

PINK1-Mediated Phosphorylation of dMIC60 Maintains Crista Junctions in *Drosophila*

Our finding that the IMM protein dMIC60 is a substrate of PINK1 (Figure 2) suggests the intriguing possibility that PINK1 might maintain crista junctions by phosphorylating dMIC60. If this hypothesis were true, blocking phosphorylation of dMIC60 by mutating the phosphorylation sites (Figure 2) might cause similar crista phenotypes as removing PINK1. To test this hypothesis directly, we ubiquitously expressed wild-type dMIC60-upstream activating sequence (UAS)-dMIC60^{WT}- or UAS-dMIC60^{PR} (Figure 2) in a *dMIC60* mutant background (*dMIC60^{mut}*) (Tsai et al., 2017) without the expression of endogenous *dMIC60* (Figures 2A, S6A, and S6B). This allele of *dMIC60* is pupal lethal (Tsai et al., 2017) and caused loss of crista junctions ubiquitously in late third instar larvae (Figures 3A–3C). Both the wild-type and mutant dMIC60 transgenes were inserted in the same genomic location (Markstein et al., 2008), and their expression levels in *dMIC60^{mut}* were comparable (Figure S6B). Ubiquitous expression of either transgene in *dMIC60^{mut}* flies did not exceed the endogenous dMIC60 level (Figure S6B) and thus circumvented the potential adverse effect by overexpression. dMIC60^{WT} in *dMIC60^{mut}* larvae completely restored their crista structure (Figures 3A–3C). In contrast, dMIC60^{PR} in *dMIC60^{mut}* failed to restore crista junctions in muscles and neuropils but could rescue crista phenotypes in neuronal cell bodies, axons, and NMJs in third instar larvae (Figures 3A–3C). Thus, dMIC60^{PR} in *dMIC60^{mut}* mirrors the crista phenotypes of *PINK1*-null flies: mitochondria lose their crista junctions in muscles and neuropils (Figures 1, 3, and S2). These results provide evidence that PINK1-mediated phosphorylation of dMIC60 is required for mitochondria to increase crista junctions in selective subcellular areas in *Drosophila*.

The PINK1-MIC60 Pathway Is Conserved in Humans

To determine whether the phosphorylation sites are conserved in humans, we knocked down endogenous human MIC60 by RNAi in HEK293T cells and expressed either RNAi-resistant wild-type or phospho-resistant human MIC60 with the two conserved sites (serine 518 and threonine 587) mutated to alanine (Figure S6C). We found that MIC60 RNAi knockdown in HEK293T cells resulted in the onion-like mitochondria with loss of crista junctions under TEM, which was fully rescued by the expression of MIC60^{WT}, but not by MIC60^{PR} (Figure S6D). We confirmed that expression of MIC60^{PR} led to loss of phosphorylation of MIC60 in those cells as detected by phos-tag gels (Figure S6E), suggesting that the two conserved sites are also the main phosphorylation residues in human MIC60. Notably, cells with MIC60^{PR} were significantly more vulnerable to oxidative stress induced by H_2O_2 treatment than cells with MIC60^{WT}, detected by the propidium iodide (PI) staining (Figure S6F). We also utilized induced pluripotent stem cell (iPSC)-derived human neurons with *PINK1* knockout and their isogenic wild-type controls. Under TEM, we detected about 15% of total neuronal mitochondria exhibiting the onion-like structure in *PINK1* loss-of-function neurons compared to only 2% in wild-type (Figure 3D). We confirmed that phosphorylation of endogenous

(E) Immunoprecipitates from iPSC-derived neurons as indicated were run in a phos-tag gel and immunoblotted with anti-MIC60. The lower blots are the regular PAGE showing immunoprecipitation (IP) and input.

(F) iPSC-derived neurons were transfected with EGFP and treated with antimycin A for 6 hr. The density of EGFP-positive neurons, which indicate live neurons, was calculated and compared to that without Antimycin A treatment. n = 30 imaging fields from 3 independent transfections.

The scale bars represent (A) 100 nm, (D) 500 nm, and (F) 100 μm. See also Figure S6.

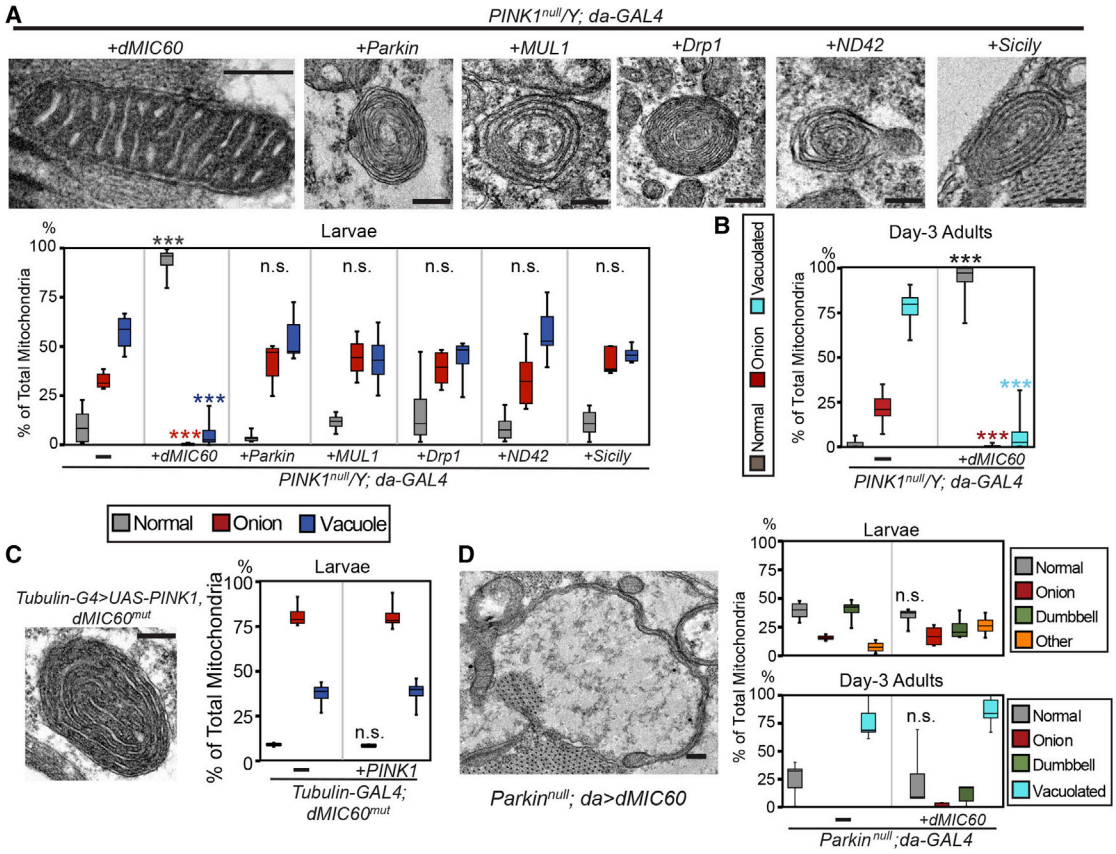

Figure 4. dMIC60 Restores Crista Structure of *PINK1*-Null

(A, C, and D) TEM images of body wall muscles of late third instar larvae (120 hr AEL) with the background of *PINK1* null (A), $dMIC60^{mut}$ (C), or *Parkin* null (D). (A–D) Quantification of the percentage of total mitochondria per TEM image from body wall muscles of late third instar larvae or thoracic indirect flight muscles of 3-day-old adults with the background of *PINK1* null (A, larvae; B, adults), $dMIC60^{mut}$ (C, larvae), or *Parkin* null (D, both). For larvae, n = 219–371 mitochondria from 27–32 images obtained from 4–6 flies. For adults, n = 106–331 mitochondria from 30–31 images obtained from 5 or 6 flies. Comparisons with the group to the left are shown. The scale bars represent 500 nm.

See also Figure S6.

human MIC60 was lost in *PINK1* knockout neurons using phos-tag gels (Figure 3E), verifying that PINK1 also mediates MIC60 phosphorylation in human neurons. Importantly, these *PINK1* loss-of-function neurons were more sensitive to oxidative stress triggered by antimycin A treatment (Figure 3F), similar to a key feature of PD patients-derived neurons (Hsieh et al., 2016). These results validate the PINK1-MIC60 pathway in human cells, including neurons, and its importance to crista junction formation and cellular survival.

dMIC60 Rescues the Defect in Crista Structure of *PINK1*-Null Flies

Because dMIC60 is a substrate of PINK1 for maintaining crista junctions (Figures 2 and 3), this places *dMIC60* genetically downstream of *PINK1*. To confirm their epistatic relationship, we ubiquitously expressed UAS-dMIC60 in a *PINK1*-null background or UAS-PINK1 in $dMIC60^{mut}$. Remarkably, upregulating dMIC60 completely rescued all the abnormal crista phenotypes in both *PINK1*-null larval and adult muscles (Figures 4A and 4B); on the contrary, PINK1 expression did not rescue the crista phenotypes in $dMIC60^{mut}$ larval muscles (Figure 4C). Importantly, these results suggest that overexpression of dMIC60 that is not phosphorylated by PINK1 compensates for the lack of PINK1. Upregulation of dMIC60 in *Parkin*-null flies did not rescue their crista and behavioral phenotypes (Figures 4D and S6G). Thus, *dMIC60* functions downstream of *PINK1* to maintain crista structure in muscles.

A few other factors have also been reported as being downstream of PINK1 at the adult stage, such as Parkin (Clark et al., 2006; Park et al., 2006), the complex I (Morais et al., 2014; Pogson et al., 2014), the mitochondrial fission-fusion machinery (Poole et al., 2010; Yang et al., 2008; Ziviani et al., 2010), and MUL1 (Yun et al., 2014). To determine whether these known PINK1-dependent pathways interplay with the PINK1-MIC60 pathway for crista structure maintenance in larval muscles, we expressed UAS-Parkin (a ubiquitin E3 ligase), UAS-ND42 (a complex I subunit), UAS-Sicily (co-chaperone of ND42), UAS-Drp1 (controls mitochondrial fission), or UAS-MUL1 (a ubiquitin E3 ligase) in *PINK1*-null larvae. All five transgenes have been shown to rescue the mitochondrial morphological phenotypes

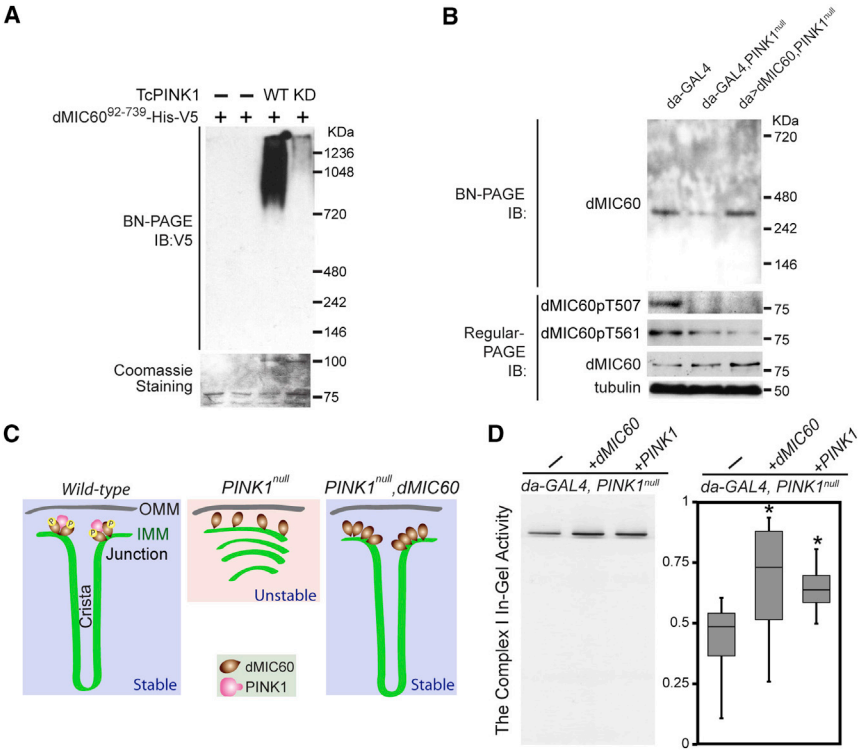

Figure 5. Both Phosphorylation and Overexpression of dMIC60 Stabilize dMIC60 Oligomerization

(A) BN-PAGE was immunoblotted with anti-V5. Note that the shifted bands may not represent the physiological forms of oligomers due to the large amount of proteins present *in vitro*.

(B) dMIC60 or phosphorylated dMIC60 was immunodetected in whole-body lysates of 5-day-old adults as indicated, using either BN-PAGE or regular-PAGE. dMIC60 migrates as a tetramer or oligomerizes with other proteins based on the size in BN-PAGE. The same results were observed for 3 times.

(C) Schematic representation of the potential impact of PINK1-mediated phosphorylation or overexpression of dMIC60 on dMIC60 oligomerization and crista curvatures.

(D) The in-gel activity of complex I was measured. The representative native gel and quantification of the band intensities compared to "*PINK1*^{null}, *da-GAL4*" are shown. n = 100 adults 5 days after eclosion for each experiment and total 4 independent experiments.

in *PINK1*-null adult muscles to varying degrees. In striking contrast to *dMIC60*, none of them rescued the onion- or vacuole-like mitochondria in *PINK1*-null larval muscles (Figure 4A). These results indicate *dMIC60* as the strongest downstream factor of *PINK1* in larvae to maintain crista structure.

Both Phosphorylation and Upregulation of dMIC60 Promote dMIC60 Oligomerization

Here, we have shown that both PINK1-mediated phosphorylation of dMIC60 (Figures 1, 2, and 3) and overexpression of dMIC60 that is not phosphorylated by PINK1 (Figures 4A and 4B) maintain crista junctions. These results suggest that upregulated and phosphorylated dMIC60 cause the same functional impact on crista junctions. Because homo-oligomerization of MIC60 has been shown to be crucial for the formation of crista junctions (John et al., 2005; Mun et al., 2010), we then reasoned that upregulated and phosphorylated dMIC60 both promote dMIC60 oligomerization. We immunoblotted dMIC60 using blue-native (BN) SDS-PAGE to detect dMIC60 oligomerization. Phosphorylation of dMIC60 by wild-type TcPINK1 *in vitro* caused retardation of dMIC60 migration above 720 kDa, indicative of dMIC60 oligomers, and this oligomerization was abolished by kinase-dead TcPINK1KD (Figure 5A). This reveals that PINK1-mediated phosphorylation of dMIC60 promotes dMIC60 oligomerization *in vitro*. To detect the dMIC60 complex *in vivo*, we immunoblotted dMIC60 from fly lysates. We found that, in wild-type background, dMIC60 migrated as an oligomer (Mun et al., 2010; Figure 5B). dMIC60 oligomerization was significantly inhibited in *PINK1*-null (Figure 5B), indicating that PINK1-mediated phosphorylation of dMIC60 promotes dMIC60 oligomerization *in vivo*. Overexpressed dMIC60 in *PINK1*-null fully restored its oligomerization (Figure 5B), mimicking the effect of phosphorylated dMIC60 on the dMIC60 complex. Importantly, overexpression of dMIC60 in *PINK1*-null did not cause more phosphorylation at either threonine 507 or 561 than that in *PINK1*-null alone without dMIC60 overexpression (Figure 5B), excluding the possibility that the restoration of oligomerization is caused by increased phosphorylation by a kinase other than PINK1. Therefore, both upregulation and phosphorylation of dMIC60 stabilize dMIC60 oligomerization, yielding the same favorable functional effect for crista junctions (Figure 5C).

dMIC60 Rescues Diverse Organelle and Organismal Phenotypes of *PINK1*-Null Flies

Aberrant crista structure may compromise mitochondrial health and accumulate oxidative stress, leading to detrimental cellular and organismal consequences. Indeed, *PINK1*-null flies display numerous defects in the complex I activity, ATP level, $\Delta\Psi$m, locomotor, and DA neuronal survival (Clark et al., 2006; Morais et al., 2014; Park et al., 2006; Pogson et al., 2014; Tsai et al., 2014). We determined whether an impaired mitochondrial crista structure underlies these dysfunctions. We ubiquitously expressed UAS-dMIC60 in *PINK1*-null flies to restore their crista structure (Figures 4A and 4B) and determined whether this could alleviate these phenotypes. We found that dMIC60 completely rescued *PINK1*-null's defects in the complex I activity (Figure 5D), ATP level (Figure 6A), $\Delta\Psi$m detected by tetramethylrhodamine (TMRM) in larval muscles (Figure 6B), and larval crawling ability (Figure 6C). On the contrary, expression of Parkin, ND42, or Drp1, which fails to rescue the crista phenotypes in *PINK1*-null larvae (Figure 4A), did not fully rescue their crawling deficit (Figure 6C). Additionally, upregulating dMIC60 in *PINK1*-null flies

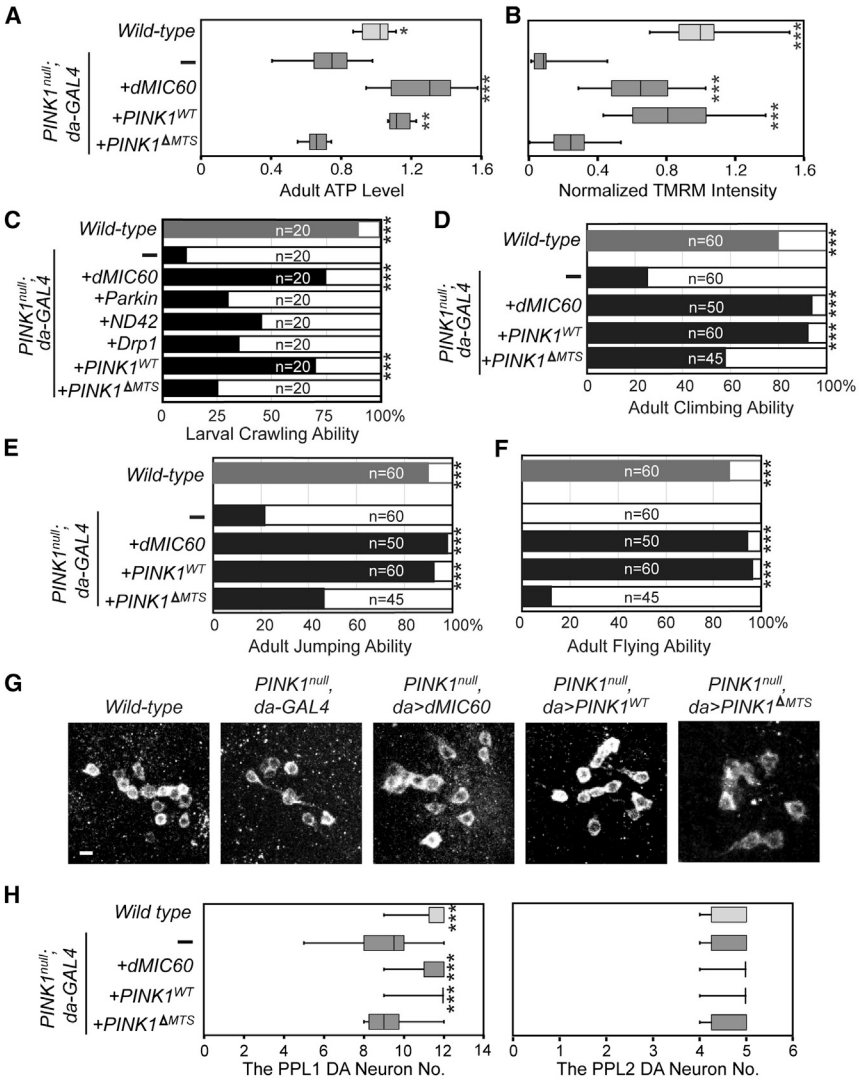

Figure 6. dMIC60 Rescues Various Phenotypes of *PINK1*-Null

(A) Quantification of the total ATP level in adult flies 5 days after eclosion. n = 5 adults for each experiment and total 6 independent experiments.

(B) Quantification of the mitochondrial/cytoplasmic TMRM fluorescent intensity in body wall muscles of third instar larvae. n = 6 larvae.

(C–F) The crawling ability of third instar larvae (C) and the climbing (D), jumping (E), and flying (F) abilities of adult flies 5 days after eclosion were quantified. n = 20–60 flies. The percentage of flies that are scored as a "1" (able to do it) is shown in black/gray bars, and the percentage of flies that are scored as a "0" (unable to do it) is shown in white bars. The chi-square test is used as the data are categorical.

For (A)–(F), wild-type, *PE704/Y*.

(G) The PPL1 clusters of DA neurons visualized by anti-TH in adult brains 15 days after eclosion. *PINK1*^{null}, *PINK1*^{B9}*/Y*; wild-type, *PINK1*^{RV}*/Y* (precise excision control males for *PINK1*^{B9}*/Y*). *PINK1*^{B9}*/Y* exhibits the same crista impairments as *PINK1*^{5}*/Y* used in the other figures (Figure S1E).

(H) Quantification of the DA neuron number in one PPL1 or one PPL2 cluster per brain of adult flies 15 days after eclosion. n = 10–14 brains. Genotypes are the same as in (G). For all panels, comparisons with *PINK1*^{null}, *da-GAL4* are shown. The scale bar represents 5 μm.

completely rescued their climbing, jumping, and flying defects 5 days after eclosion and DA neurodegeneration in the protocerebral posterior lateral 1 (PPL1) cluster 15 days after eclosion (Figures 6D–6H). Importantly, expression of the non-mitochondrial-targeting mutant PINK1$^{\Delta MTS}$ (Weihofen et al., 2009), which does not restore the crista structure in *PINK1*-null flies (Figures 1 and S4), did not rescue their impairments in the $\Delta\Psi$m, ATP level, behavior, and DA neuronal number (Figure 6). In summary, *dMIC60* functions downstream of *PINK1* to maintain mitochondrial functions and locomotion and to prevent DA neurodegeneration. In a broader sense, this discovery adds a new player, MIC60, to a cellular pathway with a key role in PD.

Rare Coding Variants in the MTS of *MIC60* Found in PD Patients Are Damaging in Flies

Recessive loss-of-function *PINK1* mutations are a well-established cause of familial forms of early-onset PD (Valente et al., 2004). Our discovery of a new PINK1-MIC60 pathway crucial for mitochondrial functions and neuronal integrity suggests the possibility for *MIC60* coding mutations in PD. We first sequenced the entire coding region of *MIC60* gene (*IMMT*: 2p11.2, OMIM*600378; Table S1) in 100 familial PD probands, 250 apparently sporadic early-onset PD patients, and 350 age/gender/ethnicity-matched controls of East Asian origin, recruited from National Taiwan University Hospital. We identified one heterozygous missense mutation in *MIC60*, c.G50T (p.C17F), in 1 familial dominant patient (Figures S7A and S7B) but no variants in controls. We genotyped this variant in additional 602 independent sporadic late-onset patients and 581 age/gender/ethnicity-matched control subjects of East Asian origin. We detected the heterozygous p.C17F mutation in 1 sporadic patient (Figures S7C and S7D), but not in controls. The p.C17F variant is located in the MTS region of MIC60. Interestingly, recent studies have suggested that rare MTS variants in the PD-linked gene *CHCHD2* play a role in the risk of PD (Ogaki et al., 2015). We sequenced the MTS region in additional 859 Caucasian PD patients and 871 control individuals recruited at Mayo Clinic, USA. We identified two heterozygous MTS missense mutations (p.A4V and p.R25H) in 2 sporadic PD patients and one heterozygous MTS missense mutation (p.R31C) in 2 control individuals (Figure 7A; Table S2). We next analyzed the exome sequencing data released by Parkinson's Progression Markers Initiative (PPMI) (http://www.ppmi-info.org). This study includes 422 recently diagnosed PD patients and 163 healthy controls of Caucasian origin, recruited from

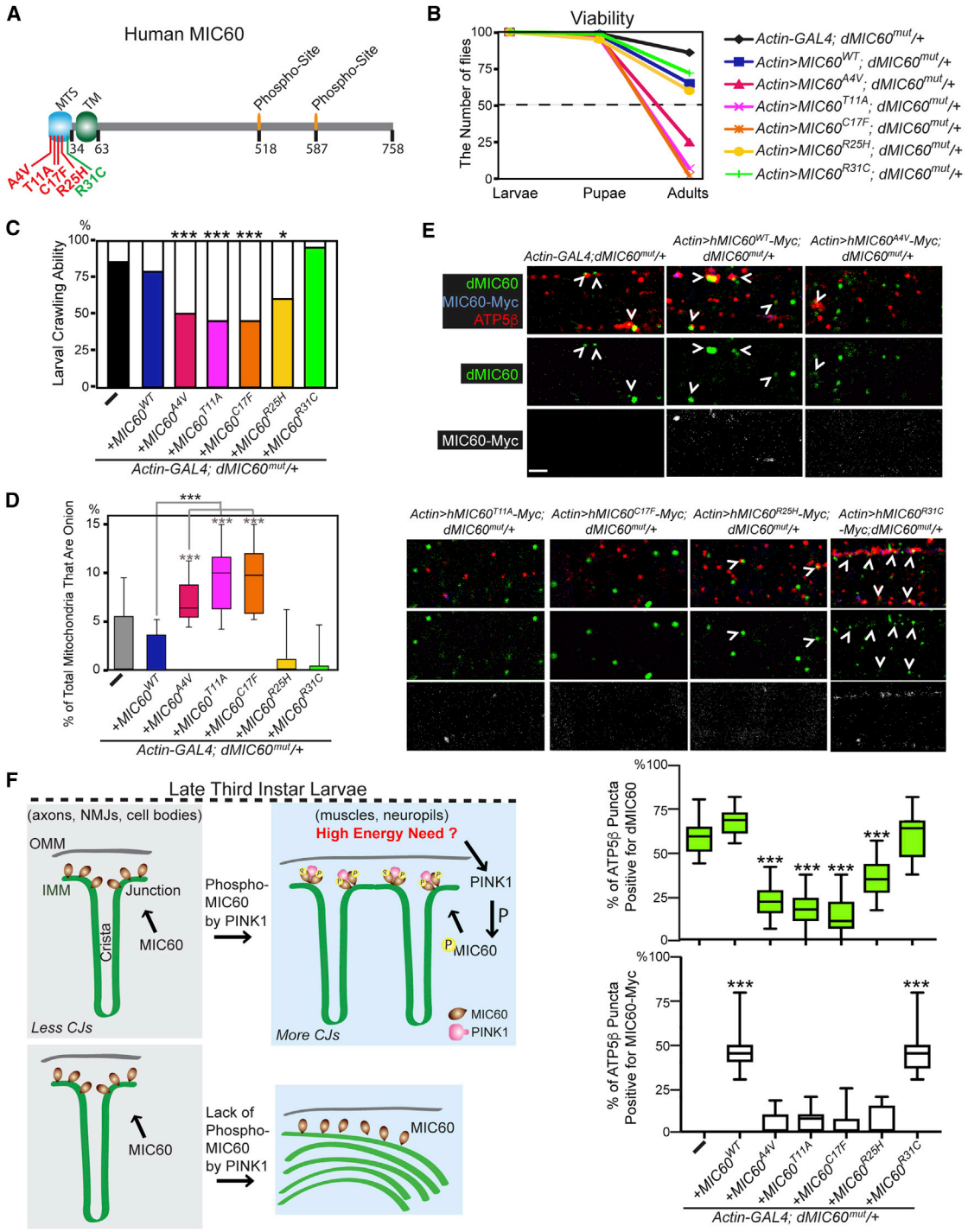

Figure 7. *MIC60* Variants in Humans and *Drosophila*

(A) Depiction of the *MIC60* MTS variants identified in this study.

(B) Viability analysis. One hundred late third instar larvae of each genotype were collected, and the numbers of their pupae and adults were subsequently counted.

(C) The crawling ability of third instar larvae with genotypes as indicated. n = 20 larvae. The percentage of flies that are scored as a "1" is shown in black/color bars, and the percentage of flies that are scored as a "0" is shown in white bars. The chi-square test is used.

(D) Quantifications of mitochondria in TEM images from body wall muscles of late third instar larvae (120 hr AEL; representative images in Figure S7F). n = 20 images from 6 flies.

(E) Single sections of confocal images showing immunostaining against endogenous dMIC60 (green) or exogenously expressed human MIC60-Myc (blue/white) in body wall muscles of third instar larvae. Red is the mitochondrial marker ATP5β. Note that anti-dMIC60 does not recognize expressed human MIC60. White

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the United States and Europe. We again identified one heterozygous MTS mutation (p.T11A) in 1 sporadic PD patient (Figure 7A), which was not present in any SNP database (Table S2). As all variants identified so far are rare (minor allele frequency < 0.5%; Table S2), we compared the cumulative allele frequency of MTS coding variants in PD patients with that in the respective ethnic groups in the genome Aggregation Database (gnomAD) (http://gnomad.broadinstitute.org), the largest internationally combined database aggregating population variant frequencies, and combined the results using meta-analysis. We found that the occurrence of MTS coding variants was significantly higher in PD patients than that in gnomAD (meta odds ratio [OR] = 3.610; meta p = 0.023; Table S2).

We conducted an unbiased screen in flies to determine the functional pathogenicity of those *MIC60* MTS mutations. Because all the identified variants are heterozygous, we ubiquitously expressed the human MIC60 transgenes in a heterozygous *dMIC60^{mut}* background (*dMIC60^{mut}*/+). All the wild-type and mutant human *MIC60* transgenes were inserted in the same genomic location (Markstein et al., 2008) and expressed at similar levels (Figure S7E). Strikingly, expression of MIC60A4V, T11A, or C17F found in PD patients in "*dMIC60^{mut}*/+" flies, but not of wild-type MIC60 or R31C found in healthy controls, led to severe adult lethality and significantly impaired the larval crawling ability, although flies with the *dMIC60^{mut}*/+ background genotype were normal (Figures 7B and 7C). Expression of the R25H variant, found in one PD patient, compromised the larval crawling ability, but not viability (Figures 7B and 7C). Importantly, expression of MIC60A4V, T11A, or C17F in *dMIC60^{mut}*/+ impaired mitochondrial crista junction formation causing the onion-like mitochondria in late third instar larval body wall muscles (Figures 7D and S7F). Because these mutations reside in the MTS, we reasoned that they may disrupt the mitochondrial targeting ability of MIC60. To test this hypothesis, we immunostained endogenous fly dMIC60, exogenously expressed human MIC60 by the Myc tag, and the subunit of the mitochondrial ATP synthase ATP5β as a mitochondrial marker in muscles (Morais et al., 2014). We verified the specificity of the immunostaining signals of both anti-dMIC60 and anti-Myc: the anti-dMIC60 signals disappeared in *dMIC60^{mut}* flies (Figure S7G) and the anti-Myc signals were undetectable in non-transgenic flies (Figure 7E). We observed that wild-type human MIC60 or MIC60R31C found in healthy controls largely localized to mitochondria, whereas MIC60A4V, T11A, C17F, or R25H found in patients exhibited a non-mitochondrial diffuse pattern (Figure 7E), suggesting that patients-linked variants disrupt the mitochondrial localization of MIC60. Interestingly, endogenous dMIC60 significantly localized to mitochondria in *dMIC60^{mut}*/+ flies or when exogenous wild-type human MIC60 or MIC60R31C was present, but this mitochondrial localization was greatly reduced when human MIC60A4V, T11A, C17F, or R25H was expressed (Figure 7E). These results demonstrate that *MIC60* MTS variants found in PD patients damage the mitochondrial-targeting ability of MIC60 in a dominant-negative way in *Drosophila*. Our novel strategies combining human genetics and functional screen focused on a defined coding region thus identify *MIC60* variants that are highly damaging *in vivo*.

DISCUSSION

In the present study, we have determined that the PD-causing kinase PINK1 phosphorylates MIC60 to maintain crista junctions in *Drosophila* and in human neurons. This mechanism represents a novel form of PINK1-mediated phosphorylation, as PINK1 is well-known to mediate mitophagy by phosphorylating its substrates on the surface of unhealthy mitochondria, dependent on the ΔΨm. In this new mechanism, PINK1-mediated phosphorylation of MIC60 is dependent on the physical presence of PINK1 inside healthy mitochondria and is likely activated by elevation of cellular energy demands (Figure 7F).

We have shown that overexpressed dMIC60 is able to compensate for the loss of phosphorylation by PINK1 in *Drosophila* (Figures 4, 5, and 6). This bears a resemblance to the regulation of Parkin or ND42, which has been reported to be phosphorylated by PINK1 directly or indirectly (Kazlauskaite et al., 2014; Kondapalli et al., 2012; Morais et al., 2014). Overexpression of wild-type Parkin or ND42 rescues some of the *PINK1*-null's phenotypes (Clark et al., 2006; Park et al., 2006; Pogson et al., 2014), although how this is achieved by unphosphorylated Parkin or ND42 remains unclear. Here, we have revealed that overexpressed dMIC60 mimics phosphorylated dMIC60 to stabilize MIC60 oligomerization (Figure 5), which is essential for crista junction formation (John et al., 2005; Mun et al., 2010). When phosphorylation of MIC60 is absent in *PINK1*-null, overexpressed MIC60 can stabilize its own oligomerization (Figure 5). Future structural work could help define the impact of PINK1-mediated phosphorylation on the stoichiometry of the MIC60 complex.

The onion-like mitochondria with loss of crista junctions are a well-established feature of impairments in the MICOS complex (von der Malsburg et al., 2011). This unique aberration in crista membrane topology disrupts complex assembly and solute gradients, both required for mitochondria oxidative phosphorylation (Mannella et al., 2013), and consequently leads to severe defects at the cellular and organismal levels (John et al., 2005; Mun et al., 2010; von der Malsburg et al., 2011). Our findings extend this mitochondrial phenomenon to the PD-causing gene PINK1. Future work is warranted to determine whether this mitochondrial phenotype exists in PD patients and whether it contributes to PD pathogenesis. Plasticity of crista structure must be seamlessly tailored to shifts in energy needs in highly energetic neurons to allow for vigorous alterations in their activities and circuitry. Our work thus implicates the vital importance of mitochondrial crista structure and its ability to remodel for dynamically balancing the metabolic homeostasis of a cell.

arrow heads show colocalization between endogenous dMIC60 and ATP5β. The scale bar represents 5 μm. Quantification of the percentage of ATP5β puncta that are also positive for dMIC60 or MIC60-Myc is shown. n = 12 images from 4–6 larvae.

(C–E) Comparisons with "*Actin-GAL4;dMIC60^{mut}*/+" are shown except indicated otherwise.

(F) Schematic representation of PINK1-dependent plasticity of crista junctions in third instar larvae.

See also Figure S7 and Tables S1 and S2.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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  - Immunocytochemistry and Confocal Microscopy
  - Mitochondrial Assays
  - Protein Biochemical Assays
  - TcPINK1 Kinase Assay
  - Mass Spectrometry
  - Transmission Electron Microscopy
  - Behavior Assay
  - Human Cell Experiments
- QUANTIFICATION AND STATISTICAL ANALYSES

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and two tables and can be found with this article online at https://doi.org/10.1016/j.molcel.2018.01.026.

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AUTHOR CONTRIBUTIONS

P.-I.T., C.-H.H., A.M.P., and M.J.K. designed and performed the fly and cell experiments and made the figures. C.-H.L., R.-M.W., Z.K.W., and O.A.R. sequenced the patients. V.N., M.D.G., O.A.R., and J.C. analyzed the human genetics data. C.S. and D.W. performed mass spectrometry. X.W. conceived and supervised the project, designed the experiments, and wrote the paper with the assistance from all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES

Chang, C.C., Chow, C.C., Tellier, L.C., Vattikuti, S., Purcell, S.M., and Lee, J.J. (2015). Second-generation PLINK: rising to the challenge of larger and richer datasets. Gigascience *4*, 7.

Chen, Y., and Dorn, G.W., 2nd (2013). PINK1-phosphorylated mitofusin 2 is a Parkin receptor for culling damaged mitochondria. Science *340*, 471–475.

Clark, I.E., Dodson, M.W., Jiang, C., Cao, J.H., Huh, J.R., Seol, J.H., Yoo, S.J., Hay, B.A., and Guo, M. (2006). Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. Nature *441*, 1162–1166.

Frezza, C., Cipolat, S., Martins de Brito, O., Micaroni, M., Beznoussenko, G.V., Rudka, T., Bartoli, D., Polishuck, R.S., Danial, N.N., De Strooper, B., and Scorrano, L. (2006). OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. Cell *126*, 177–189.

Greene, J.C., Whitworth, A.J., Kuo, I., Andrews, L.A., Feany, M.B., and Pallanck, L.J. (2003). Mitochondrial pathology and apoptotic muscle degeneration in Drosophila parkin mutants. Proc. Natl. Acad. Sci. USA *100*, 4078–4083.

Greene, A.W., Grenier, K., Aguileta, M.A., Muise, S., Farazifard, R., Haque, M.E., McBride, H.M., Park, D.S., and Fon, E.A. (2012). Mitochondrial processing peptidase regulates PINK1 processing, import and Parkin recruitment. EMBO Rep. *13*, 378–385.

Groth, A.C., Fish, M., Nusse, R., and Calos, M.P. (2004). Construction of transgenic Drosophila by using the site-specific integrase from phage phiC31. Genetics *166*, 1775–1782.

Hackenbrock, C.R. (1966). Ultrastructural bases for metabolically linked mechanical activity in mitochondria. I. Reversible ultrastructural changes with change in metabolic steady state in isolated liver mitochondria. J. Cell Biol. *30*, 269–297.

Hsieh, C.H., Shaltouki, A., Gonzalez, A.E., Bettencourt da Cruz, A., Burbulla, L.F., St Lawrence, E., Schüle, B., Krainc, D., Palmer, T.D., and Wang, X. (2016). Functional impairment in miro degradation and mitophagy is a shared feature in familial and sporadic Parkinson's disease. Cell Stem Cell *19*, 709–724.

Hughes, A.J., Daniel, S.E., Kilford, L., and Lees, A.J. (1992). Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. J. Neurol. Neurosurg. Psychiatry *55*, 181–184.

John, G.B., Shang, Y., Li, L., Renken, C., Mannella, C.A., Selker, J.M., Rangell, L., Bennett, M.J., and Zha, J. (2005). The mitochondrial inner membrane protein mitofilin controls cristae morphology. Mol. Biol. Cell *16*, 1543–1554.

Kazlauskaite, A., Kondapalli, C., Gourlay, R., Campbell, D.G., Ritorto, M.S., Hofmann, K., Alessi, D.R., Knebel, A., Trost, M., and Muqit, M.M. (2014). Parkin is activated by PINK1-dependent phosphorylation of ubiquitin at Ser65. Biochem. J. *460*, 127–139.

Kondapalli, C., Kazlauskaite, A., Zhang, N., Woodroof, H.I., Campbell, D.G., Gourlay, R., Burchell, L., Walden, H., Macartney, T.J., Deak, M., et al. (2012). PINK1 is activated by mitochondrial membrane potential depolarization and stimulates Parkin E3 ligase activity by phosphorylating Serine 65. Open Biol. *2*, 120080.

Körner, C., Barrera, M., Dukanovic, J., Eydt, K., Harner, M., Rabl, R., Vogel, F., Rapaport, D., Neupert, W., and Reichert, A.S. (2012). The C-terminal domain of Fcj1 is required for formation of crista junctions and interacts with the TOB/SAM complex in mitochondria. Mol. Biol. Cell *23*, 2143–2155.

Lajtha, A., Gibson, G.E., and Dienel, G.A. (2007). Handbook of Neurochemistry and Molecular Neurobiology: Brain Energetics. Integration of Molecular and Cellular Processes (Springer).

Lin, C.H., Tzen, K.Y., Yu, C.Y., Tai, C.H., Farrer, M.J., and Wu, R.M. (2008). LRRK2 mutation in familial Parkinson's disease in a Taiwanese population: clinical, PET, and functional studies. J. Biomed. Sci. *15*, 661–667.

Mannella, C.A., Lederer, W.J., and Jafri, M.S. (2013). The connection between inner membrane topology and mitochondrial function. J. Mol. Cell. Cardiol. *62*, 51–57.

Markstein, M., Pitsouli, C., Villalta, C., Celniker, S.E., and Perrimon, N. (2008). Exploiting position effects and the gypsy retrovirus insulator to engineer precisely expressed transgenes. Nat. Genet. *40*, 476–483.

Meeusen, S., DeVay, R., Block, J., Cassidy-Stone, A., Wayson, S., McCaffery, J.M., and Nunnari, J. (2006). Mitochondrial inner-membrane fusion and crista maintenance requires the dynamin-related GTPase Mgm1. Cell *127*, 383–395.

Messerschmitt, M., Jakobs, S., Vogel, F., Fritz, S., Dimmer, K.S., Neupert, W., and Westermann, B. (2003). The inner membrane protein Mdm33 controls mitochondrial morphology in yeast. J. Cell Biol. *160*, 553–564.

Morais, V.A., Haddad, D., Craessaerts, K., De Bock, P.J., Swerts, J., Vilain, S., Aerts, L., Overbergh, L., Grünewald, A., Seibler, P., et al. (2014). PINK1 loss-of-function mutations affect mitochondrial complex I activity via NdufA10 ubiquinone uncoupling. Science *344*, 203–207.

Mun, J.Y., Lee, T.H., Kim, J.H., Yoo, B.H., Bahk, Y.Y., Koo, H.S., and Han, S.S. (2010). Caenorhabditis elegans mitofilin homologs control the morphology of mitochondrial cristae and influence reproduction and physiology. J. Cell. Physiol. *224*, 748–756.

Narendra, D., Tanaka, A., Suen, D.F., and Youle, R.J. (2008). Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. J. Cell Biol. *183*, 795–803.

Ogaki, K., Koga, S., Heckman, M.G., Fiesel, F.C., Ando, M., Labbé, C., Lorenzo-Betancor, O., Moussaud-Lamodière, E.L., Soto-Ortolaza, A.I., Walton, R.L., et al. (2015). Mitochondrial targeting sequence variants of the CHCHD2 gene are a risk for Lewy body disorders. Neurology *85*, 2016–2025.

Park, J., Lee, S.B., Lee, S., Kim, Y., Song, S., Kim, S., Bae, E., Kim, J., Shong, M., Kim, J.M., and Chung, J. (2006). Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin. Nature *441*, 1157–1161.

Park, Y.U., Jeong, J., Lee, H., Mun, J.Y., Kim, J.H., Lee, J.S., Nguyen, M.D., Han, S.S., Suh, P.G., and Park, S.K. (2010). Disrupted-in-schizophrenia 1 (DISC1) plays essential roles in mitochondria in collaboration with Mitofilin. Proc. Natl. Acad. Sci. USA *107*, 17785–17790.

Parkinson Progression Marker Initiative (2011). The Parkinson Progression Marker Initiative (PPMI). Prog. Neurobiol. *95*, 629–635.

Pesah, Y., Pham, T., Burgess, H., Middlebrooks, B., Verstreken, P., Zhou, Y., Harding, M., Bellen, H., and Mardon, G. (2004). Drosophila parkin mutants have decreased mass and cell size and increased sensitivity to oxygen radical stress. Development *131*, 2183–2194.

Pfanner, N., van der Laan, M., Amati, P., Capaldi, R.A., Caudy, A.A., Chacinska, A., Darshi, M., Deckers, M., Hoppins, S., Icho, T., et al. (2014). Uniform nomenclature for the mitochondrial contact site and cristae organizing system. J. Cell Biol. *204*, 1083–1086.

Pogson, J.H., Ivatt, R.M., Sanchez-Martinez, A., Tufi, R., Wilson, E., Mortiboys, H., and Whitworth, A.J. (2014). The complex I subunit NDUFA10 selectively rescues Drosophila pink1 mutants through a mechanism independent of mitophagy. PLoS Genet. *10*, e1004815.

Poole, A.C., Thomas, R.E., Andrews, L.A., McBride, H.M., Whitworth, A.J., and Pallanck, L.J. (2008). The PINK1/Parkin pathway regulates mitochondrial morphology. Proc. Natl. Acad. Sci. USA *105*, 1638–1643.

Poole, A.C., Thomas, R.E., Yu, S., Vincow, E.S., and Pallanck, L. (2010). The mitochondrial fusion-promoting factor mitofusin is a substrate of the PINK1/parkin pathway. PLoS ONE *5*, e10054.

Rabl, R., Soubannier, V., Scholz, R., Vogel, F., Mendl, N., Vasiljev-Neumeyer, A., Körner, C., Jagasia, R., Keil, T., Baumeister, W., et al. (2009). Formation of cristae and crista junctions in mitochondria depends on antagonism between Fcj1 and Su e/g. J. Cell Biol. *185*, 1047–1063.

Schuldiner, O., Berdnik, D., Levy, J.M., Wu, J.S., Luginbuhl, D., Gontang, A.C., and Luo, L. (2008). piggyBac-based mosaic screen identifies a postmitotic function for cohesin in regulating developmental axon pruning. Dev. Cell *14*, 227–238.

Strauss, M., Hofhaus, G., Schröder, R.R., and Kühlbrandt, W. (2008). Dimer ribbons of ATP synthase shape the inner mitochondrial membrane. EMBO J. *27*, 1154–1160.

Thomas, R.E., Andrews, L.A., Burman, J.L., Lin, W.Y., and Pallanck, L.J. (2014). PINK1-Parkin pathway activity is regulated by degradation of PINK1 in the mitochondrial matrix. PLoS Genet. *10*, e1004279.

Tsai, P.I., Course, M.M., Lovas, J.R., Hsieh, C.H., Babic, M., Zinsmaier, K.E., and Wang, X. (2014). PINK1-mediated phosphorylation of Miro inhibits synaptic growth and protects dopaminergic neurons in Drosophila. Sci. Rep. *4*, 6962.

Tsai, P.I., Papakyrikos, A.M., Hsieh, C.H., and Wang, X. (2017). Drosophila MIC60/mitofilin conducts dual roles in mitochondrial motility and crista structure. Mol. Biol. Cell *28*, 3471–3479.

Valente, E.M., Abou-Sleiman, P.M., Caputo, V., Muqit, M.M., Harvey, K., Gispert, S., Ali, Z., Del Turco, D., Bentivoglio, A.R., Healy, D.G., et al. (2004). Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science *304*, 1158–1160.

Vives-Bauza, C., and Przedborski, S. (2011). Mitophagy: the latest problem for Parkinson's disease. Trends Mol. Med. *17*, 158–165.

von der Malsburg, K., Müller, J.M., Bohnert, M., Oeljeklaus, S., Kwiatkowska, P., Becker, T., Loniewska-Lwowska, A., Wiese, S., Rao, S., Milenkovic, D., et al. (2011). Dual role of mitofilin in mitochondrial membrane organization and protein biogenesis. Dev. Cell *21*, 694–707.

Wang, X., Winter, D., Ashrafi, G., Schlehe, J., Wong, Y.L., Selkoe, D., Rice, S., Steen, J., LaVoie, M.J., and Schwarz, T.L. (2011). PINK1 and Parkin target Miro for phosphorylation and degradation to arrest mitochondrial motility. Cell *147*, 893–906.

Weihofen, A., Thomas, K.J., Ostaszewski, B.L., Cookson, M.R., and Selkoe, D.J. (2009). Pink1 forms a multiprotein complex with Miro and Milton, linking Pink1 function to mitochondrial trafficking. Biochemistry *48*, 2045–2052.

Whitworth, A.J., and Pallanck, L.J. (2009). The PINK1/Parkin pathway: a mitochondrial quality control system? J. Bioenerg. Biomembr. *41*, 499–503.

Winter, D., Seidler, J., Ziv, Y., Shiloh, Y., and Lehmann, W.D. (2009). Citrate boosts the performance of phosphopeptide analysis by UPLC-ESI-MS/MS. J. Proteome Res. *8*, 418–424.

Woodroof, H.I., Pogson, J.H., Begley, M., Cantley, L.C., Deak, M., Campbell, D.G., van Aalten, D.M., Whitworth, A.J., Alessi, D.R., and Muqit, M.M. (2011). Discovery of catalytically active orthologues of the Parkinson's disease kinase PINK1: analysis of substrate specificity and impact of mutations. Open Biol. *1*, 110012.

Yang, Y., Gehrke, S., Imai, Y., Huang, Z., Ouyang, Y., Wang, J.W., Yang, L., Beal, M.F., Vogel, H., and Lu, B. (2006). Mitochondrial pathology and muscle and dopaminergic neuron degeneration caused by inactivation of Drosophila Pink1 is rescued by Parkin. Proc. Natl. Acad. Sci. USA *103*, 10793–10798.

Yang, Y., Ouyang, Y., Yang, L., Beal, M.F., McQuibban, A., Vogel, H., and Lu, B. (2008). Pink1 regulates mitochondrial dynamics through interaction with the fission/fusion machinery. Proc. Natl. Acad. Sci. USA *105*, 7070–7075.

Yun, J., Puri, R., Yang, H., Lizzio, M.A., Wu, C., Sheng, Z.H., and Guo, M. (2014). MUL1 acts in parallel to the PINK1/parkin pathway in regulating mitofusin and compensates for loss of PINK1/parkin. eLife *3*, e01958.

Ziviani, E., Tao, R.N., and Whitworth, A.J. (2010). Drosophila parkin requires PINK1 for mitochondrial translocation and ubiquitinates mitofusin. Proc. Natl. Acad. Sci. USA *107*, 5018–5023.

A novel link between trafficking and Lewy body disorders

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Elucidating the natural history and underlying genetic causes of Parkinson's disease and related Lewy body disorders is crucial in guiding our development of therapeutic intervention strategies. Approximately 15% of patients with Parkinson's disease report a family history of the disease, and studies have identified or nominated a small number of disease-associated genes from multi-incident families. However, these genes account for only 5–10% of familial cases of Parkinson's disease. As such, many more rare genetic forms probably exist. Population-based genome-wide association studies have also nominated 41 loci that can alter individual susceptibility to Parkinson's disease, and this number continues to grow.[1]

In *The Lancet Neurology*, a study by Marialuisa Quadri and colleagues[2] nominates a mutation in the *LRP10* gene as causative in a large multi-incident pedigree from Italy, by use of single nucleotide polymorphism-based linkage analysis and whole exome sequencing. The *LRP10* mutation 1807G→A (Gly603Arg) was selected as the pathogenic candidate over mutations in other genes (*OR11H12* and *POTEG*) on the basis of results from in silico predictive tools. It should be noted that, when such rare genetic causes are nominated by whole exome rather than whole genome sequencing, there remains a possibility of missing small copy number variants or repeats on the disease-segregating haplotype. Subsequent gene screening and mutation burden analysis suggested the presence of other rare variants in probands from families with Lewy body disorders, supporting a role for *LRP10* mutations in disease. However, as with all genetic results, the test for a true association will be assessed by the replication of these findings, with the possibility that rare variants might not be found again. Therefore, the replication of these results will probably rely on rare variant gene burden-based testing, the results of which can depend on the variant filtering steps employed. Indeed, the interpretation of the presence of rare, or perhaps even unique, variants in patients will be one of the major challenges to face as we try to implement genetics into clinical practice.

Functionally, the study by Quadri and colleagues shows that LRP10 colocalises with markers for the trans-Golgi network and endosomes in induced pluripotent stem cell-derived human neurons, although in a transfected overexpression model. More mutant-specific work is needed to show functional consequences, and even then this sort of results do not prove pathogenicity, but rather show a functional consequence of the mutation that might or might not be related to the disease phenotype. The *LRP10* variants associated with the disease risk are predicted to be loss-of-function, because several of them appear to reduce LRP10 protein stability when overexpressed in human neurons. However, again, these findings require further validation with use of patient-derived cells or tissues, and in model systems with genetic knocking in of specific *LRP10* mutants. The effect of *LRP10* deletion or reduction on brain function and the Lewy body-related pathology is not clear, because *Lrp10* gene knockout mice have not been studied. Depending on the phenotypes or potential lethality, a conditional knockout of *Lrp10* in mice that is specific to brain cell types might also be needed, in the absence or presence of Lewy body-related pathologies. The LRP10 protein is a member of the low-density lipoprotein receptor family; receptors in this family share structural and functional similarities, including their ability to bind common ligands, such as apolipoprotein E and amyloid β,[3,4] providing a link to Lewy body dementia, Alzheimer's disease, and neurodegeneration. *LRP10* is expressed in multiple brain cell types, including endothelial cells, astrocytes, microglia, and neurons.[5] The specific functions of *LRP10* in each of these cell types require in-depth investigation using human cell or animal models.

Although little is known about LRP10 biology, an intriguing aspect of this receptor is its localisation in the trans-Golgi network, endosomes, and, to a lesser degree, at the plasma membrane. The ability of LRP10 to traffic between the trans-Golgi network and endosomes appears to be mediated by two motifs within its cytoplasmic domain, interacting with the clathrin adaptors GGAs and adaptor proteins (AP-1 and AP-2), which suggests that LRP10 can have a potential role in mediating intracellular protein trafficking.[6,7] Indeed, LRP10 has been shown to regulate the trafficking and processing of amyloid precursor protein, affecting the production

of Alzheimer's disease-related amyloid β.[8] This function of regulating trafficking resembles those of several other Lewy body-related proteins, including α-synuclein, LRRK2, GBA, and VPS35.[3,4,9] LRP10 localisation at the plasma membrane was substantially reduced in cells with VPS35-depleted retromers.[10] The potential relationship between LRP10 and VPS35 is also highlighted in the study by Quadri and colleagues, which shows that LRP10 colocalises with VPS35, as well as with markers for the trans-Golgi network and endosomes. However, whether LRP10 and other Parkinson's disease proteins have related or distinct functions in vesicle trafficking and protein transport needs further investigation.

To confirm and apply genetics findings, such as these *LRP10* results, in the clinical setting, a functional genomics approach will be crucial. Geneticists can no longer rely solely on genetic data to assess the pathogenicity of rare or unique variants and will probably require functional readout from validated and robust disease-related assays. The importance of finding these rare genetic causes, though, remains: with each discovery we get another piece of the puzzle and move closer to finally generating therapeutics that can halt progression and prevent Lewy body disorders.

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1 Chang D, Nalls MA, Hallgrimsdottir IB, et al. A meta-analysis of genome-wide association studies identifies 17 new Parkinson's disease risk loci. *Nat Genet* 2017; **49:** 1511–16.
2 Quadri M, Mandemakers W, Grochowska MM, et al. *LRP10* genetic variants in familial Parkinson's disease and dementia with Lewy bodies: a genome-wide linkage and sequencing study. *Lancet Neurol* 2018; published online June 7. http://dx.doi.org/10.1016/S1474-4422(18)30179-0.
3 Bu G. Apolipoprotein E and its receptors in Alzheimer's disease: pathways, pathogenesis and therapy. *Nat Rev Neurosci* 2009; **10:** 333–44.
4 Liu CC, Liu CC, Kanekiyo T, Xu H, Bu G. Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy. *Nat Rev Neurol* 2013; **9:** 106–18.
5 Zhang Y, Chen K, Sloan SA, et al. An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. *J Neurosci* 2014; **34:** 11929–47.
6 Boucher R, Larkin H, Brodeur J, Gagnon H, Theriault C, Lavoie C. Intracellular trafficking of LRP9 is dependent on two acidic cluster/dileucine motifs. *Histochem Cell Biol* 2008; **130:** 315–27.
7 Doray B, Knisely JM, Wartman L, Bu G, Kornfeld S. Identification of acidic dileucine signals in LRP9 that interact with both GGAs and AP-1/AP-2. *Traffic* 2008; **9:** 1551–62.
8 Brodeur J, Theriault C, Lessard-Beaudoin M, Marcil A, Dahan S, Lavoie C. LDLR-related protein 10 (LRP10) regulates amyloid precursor protein (APP) trafficking and processing: evidence for a role in Alzheimer's disease. *Mol Neurodegener* 2012; **7:** 31.
9 Abeliovich A, Gitler AD. Defects in trafficking bridge Parkinson's disease pathology and genetics. *Nature* 2016; **539:** 207–16.
10 Steinberg F, Gallon M, Winfield M, et al. A global analysis of SNX27-retromer assembly and cargo specificity reveals a function in glucose and metal ion transport. *Nat Cell Biol* 2013; **15:** 461–71.