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

PRINCIPAL INVESTIGATOR: Wolfdieter Springer, PhD

CONTRACTING ORGANIZATION: Mayo Clinic, Jacksonville, FL

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Wolfdieter Springer, PhD

E-Mail:

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4500 San Pablo Road
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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 will determine the regulation of mitophagy as well as the genetic architecture of EOPD.

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?**
     During the last reporting period, we have continued to focus on the miR-29 family that has emerged as our strongest lead. From RNAseq that we analyzed upon transfection with miR-29a, we first prioritized a number of target genes. With a stringent cut-off of and adjusted p-value lower than 0.01 we received 146 target genes. We further stratified the genes as follows: 1) we determined the overlap with the hits from a recent screen from Hoshino et al., 2) we determined the overlap with a manually curated mitophagy gene list that was built over the years by our lab and includes genes from previously published screens such as the one from Hasson et al.. Further we 3) manually checked the involvement of each of these genes by performing a manual literature search. The literature search was performed in order to find any genes involved with a) mitochondria, b) neuron/brain/neurodegeneration and/or c) ubiquitin/lysosome/autophagy/degradation. Using these 3 ways to prioritize the genes, we found that only one gene (ATG9A) was present with all 3 strategies. We further prioritized a several genes based on their function: VPS25, PTEN, SLC25A13, GRP37 as well as TIMM8B and considering that we need good quality tools (siRNA and antibodies) as well we decided to start to analyze VPS25, PTEN, GRP37 and ATG9A.
     We ordered siRNAs, antibodies and taqman probes for each of these four genes and started knock down experiments in HeLa cells. For the initial experiments we used qRT-PCR to verify successful knockdown and then continued with western blot to confirm reduction of the protein. We had tried different conditions to optimize the silencing and we found that the best condition was transfecting the siRNA on two consecutive days and incubating the cells for 48h after the

second transfection. For GPR37, we first couldn't achieve knockdown but succeeded after ordering an different siRNA for this gene.

After we established the silencing of all four target genes, we further characterized the downstream effects upon knockdown. We started by measuring the mRNA levels of PINK1 and PTEN and monitored the mRNA levels of the target gene itself as a control. We further treated siRNA transfected cells with CCCP to induce mitochondrial depolarization and prepared western blots. This confirmed that indeed siRNA silencing of ATG9A affected the PINK1-PRKN mitophagy response. Levels of pS65-Ub, which is PINK1 and PRKN-dependently generated, were significantly lower in cells treated with ATG9A siRNA compared to control after CCCP treatment (see figure 1A). There seemed to be a trend towards lower PINK1 protein levels in line with the qRT-PCR results, but this was not significant. For PTEN, we observed a slight increase of PINK1 protein, however the effects on pS65-Ub were less consistent among our replicate experiments and overall, there was no change. Inhibition of VPS25 or GPR37 did not reduce pS65-Ub levels and hence do not seem to be responsible for the observed effect of miRNA-29. Quantifications of Ubiquitin smears are inherently difficult, and we are planning to confirm the effects on pS65-Ub using our very sensitive and accurate in-house sandwich ELISA. In addition to measuring pS65-Ub, we were monitoring PRKN translocation upon silencing of all four candidate genes and found that only ATG9A reduces PRKN translocation and therefore mimicked the effect of miRNA-29a (see figure 1B). While it is too early to draw firm conclusions our data at this time point towards that ATG9A could indeed be the gene responsible for the observed effect of miRNA-29a. We have confirmed that miRNA-29a overexpression reduces the protein levels of ATG9A (see figure 1C). We plan to follow up on ATG9A as the potential main effector of the miRNA-29 in the next reporting period with additional cell biological analysis and assays.

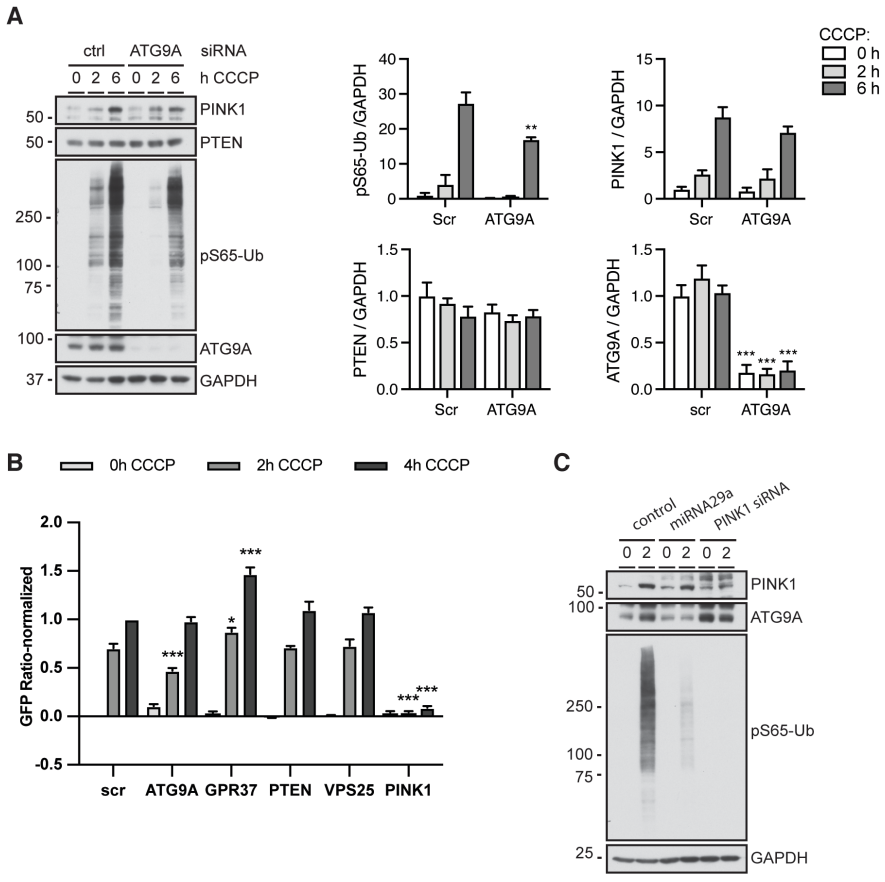


Figure 1 Silencing of ATG9A inhibits mitophagy. *Knockdown of ATG9A with siRNA inhibits the accumulation of pS65-Ub (A) and PRKN translocation (B). ATG9A protein levels are affected upon transfection of cells with miR29a.*

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

- **How were the results disseminated to communities of interest?**
  Nothing to Report.

- **What do you plan to do during the next reporting period to accomplish the goals?**
  We have confirmed that miRNA-29a regulates ATG9A RNA and protein levels and we have strong data that ATG9A could indeed be the effector of miRNA-29a with regards to mitophagy. However, we have not included miRNA-29b and -c much yet. Hence, we will also confirm that ATG9A is the effector of the entire miRNA-29 family. Furthermore, we will try to mechanistically understand the connection between ATG9A and PRKN. ATG9A was recently reported to bind to OPTN and to initiate de novo biogenesis of autophagic membranes on ubiquitin-coated damaged mitochondria. According to this, ATG9A should be downstream of PINK1 and PRKN in the mitophagy pathway. Hence conceptually is not quite clear how ATG9A could affect the translocation of PRKN to mitochondria and we want to analyze this further.
  In addition, we had reported our findings to Dr. Ross and received back a list with genetic variants in miRNA and the prioritized miRNA-29a target genes While there were no variants in miRNA29a, b, or c, there were a couple variants in the ATG9A gene. One non-synonymous coding variant in the ATG9A gene seems intriguing and it could have biologically interesting functions too. We want to analyze this variant further by expressing a mutant cDNA in cells and compare the response to the wild-type variant. The latter should be able to partially or fully restore the effect of miR-29 expression.

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 still experience delays caused by COVID-19 that affects staffing of support staff as well as supply chain and have enacted strategies to reduce the impact as good as possible by anticipating our needs ahead of time.

   - **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:**
   - **Publications, conference papers, and presentations**
     - **Journal publications.** With acknowledgement of federal support:

1. Hou, X., J.O. Watzlawik, F.C. Fiesel, and W. Springer, *Autophagy in Parkinson's Disease.* J Mol Biol, 2020. **432**(8): p. 2651-2672.

2. Soto-Beasley, A.I., R.L. Walton, R.R. Valentino, P.W. Hook, C. Labbe, M.G. Heckman, P.W. Johnson, L.A. Goff, R.J. Uitti, P.J. McLean, W. Springer, A.S. McCallion, Z.K. Wszolek, and O.A. Ross, *Screening non-MAPT genes of the Chr17q21 H1 haplotype in Parkinson's disease.* Parkinsonism Relat Disord, 2020. **78**: p. 138-144.

3. Hou, X., J.O. Watzlawik, C. Cook, C.-C. Liu, S.S. Kang, W.L. Lin, M. DeTure, M.G. Heckman, N.N. Diehl, F.S. Hanna Al-Shaikh, R.L. Walton, O.A. Ross, H.L. Melrose, N. Ertekin-Taner, G. Bu, L. Petrucelli, J.D. Fryer, M.E. Murray, D.W. Dickson, F.C. Fiesel, and W. Springer, *Mitophagy alterations in Alzheimer's disease are associated with granulovacuolar degeneration and early tau pathology.* Alzheimer's & Dementia: The Journal of the Alzheimer's Association, 2020 Oct 8;17(3):417-30. doi: 10.1002/alz.12198

4. Watzlawik, J.O., X. Hou, D. Fricova, C. Ramnarine, S.K. Barodia, T.F. Gendron, M.G. Heckman, M. DeTure, J. Siuda, Z.K. Wszolek, C.R. Scherzer, O.A. Ross, G. Bu, D.W. Dickson, M.S. Goldberg, F.C. Fiesel, and W. Springer, *Sensitive ELISA-based detection method for the mitophagy marker p-S65-Ub in human cells, autopsy brain, and blood samples.* Autophagy. 2020 Oct 28:1-16. doi: 10.1080/15548627.2020.1834712

5. Milanowski LM, Oshinaike O, Broadway BJ, Lindemann JA, Soto-Beasley AI, Walton RL, Hanna Al-Shaikh R, Strongosky AJ, Fiesel FC, Ross OA, Springer W, Ogun SA, Wszolek ZK. E*arly-Onset Parkinson Disease Screening in Patients From Nigeria.*Front Neurol. 2021 Jan 14;11:594927. doi: 10.3389/fneur.2020.594927

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

Name:	*Wolfdieter Springer*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2.5*
Contribution to Project:	*Together with the co-PI Dr. Ross, Dr. Springer has supervised the project, collected all regulatory material and ensured all necessary steps towards completion of the milestones*
Funding Support:	
Name:	Fabienne Fiesel, PhD
Project Role:	Co-investigator
Researcher Identifier (e.g. ORCID ID):	

Nearest person month worked:	2.0
Contribution to Project:	Dr. Fiesel has coordinated the analysis of the miRNA-29 in cells and trained, and supervised Ms. Markham.
Funding Support:	
Name:	Briana Markham, BSc
Project Role:	technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12.0
Contribution to Project:	Mr. Markham has performed experiments to validate the targets of miRNA-29a.
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?**

  Wolfdieter Springer, PhD
  Changes in Active Support:
  New
  Title: Determining PINK1 and PRKN enzyme activities in vivo
  Grant ID: 019046
  PD/PI: Springer
  Effort: 1.20
  Supporting Agency: Michael J Fox Foundation for Parkinson's Research
  Grants Officer: Allison Morris, Research Programs Officer
  Performance Period: **03/01/2021**-8/31/2022
  Funding Amount: (total costs)
  Goals: To measure pS65-Ub levels in vivo in WT as well PINK1 and PRKN hetero and homozygous mice with or without the POLG mutator background.

  Title: Biomarker for mitophagy pathway activation
  Grant ID: 019258
  PD/PI: Springer
  Effort: 1.25
  Supporting Agency: Michael J Fox Foundation for Parkinson's Research
  Grants Officer: Allison Morris, Research Programs Officer
  Performance Period: **12/01/2020**-5/31/2022
  Funding Amount: (total costs)
  Goals: To establish and validate a sensitive assay to detect pS65-Ub and activated Parkin in biofluids to interrogate their suitability as PD biomarkers

  Title: The Impact of Enhancing PINK1-PRKN Mitophagy on Healthy Aging
  Grant ID: N/A
  PD/PI: Springer
  Effort: 0.60

Supporting Agency: Mayo Clinic Robert and Arlene Kogod Center on Aging
Grants Officer: N/A
Performance Period: **06/01/2021**-05/31/2023
Funding Amount: (total costs)
Goals: To validate the effects of increasing mitophagy in a novel mouse model in vivo

Ended
Title: Selective autophagy in Alzheimer's disease and related dementias
Grant number: R56 AG062556
Committed Time: 2.40
Supporting Agency: National Institute of Aging
Contracting/Grants Officer: Austin Yang, Program Officer
Performance Period: 08/01/2019-**07/31/2021 (NCE)**
Level of Funding:(current annual direct costs)
Goals & Specific Aims: To elucidate the impact of tau on different arms of the autophagy system and to identify the contributions of specific autophagy impairments to AD pathogenesis
Role: PD/PI

Title: Functional assays to identify novel regulators of mitochondrial quality control
Grant Number: 15007
Committed Time 0.60
Supporting Agency: Michael J Fox Foundation for Parkinson's Research
Contracting/Grants Officer: Allison Morris, Research Programs Officer
Performance Period: 10/17/2017-**04/30/2021 (NCE)**
Level of Funding (current annual direct costs)
Goals & Specific Aims: To perform structure/function characterization of PINK1/Parkin variants identified from PPMI patients
Role: PD/PI

Title: Characterization of new Parkin activation mutants
Grant Number: 14681
Committed Time: 0.12
Supporting Agency: Michael J Fox Foundation for Parkinson's Research
Contracting/Grants Officer: Allison Morris, Research Programs Officer
Performance Period: 12/01/2017-**01/07/2021 (NCE)**
Level of Funding: (current annual direct costs)
Goals & Specific Aims: To identify and validate activating Parkin missense mutations
Role: PD/PI

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

8. **SPECIAL REPORTING REQUIREMENTS**
   - **COLLABORATIVE AWARDS:**
     For an update on Major Task 2 see report from the co-PI Dr. Ross.
   - **QUAD CHARTS:**
     See appendix
9. **APPENDICES:** *Quad chart*

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

PR160606

W81XWH-17-1-0248

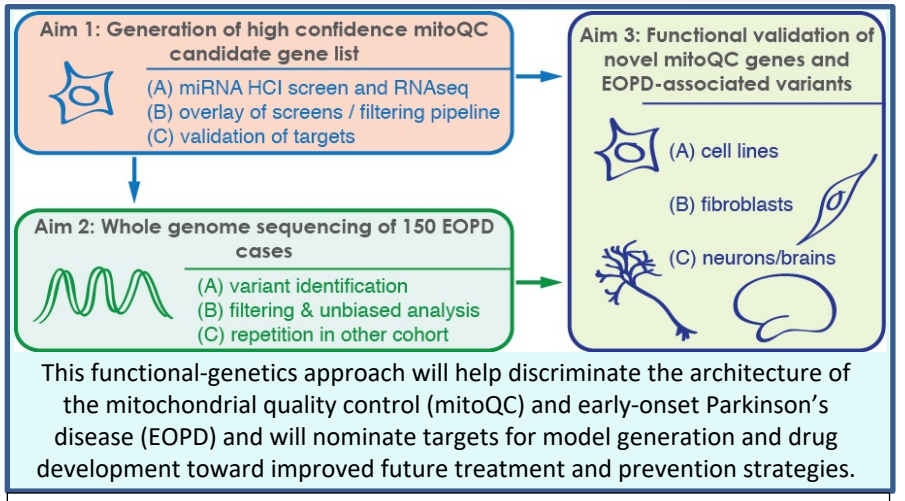
PI: Wolfdieter Springer, PhD **Org:** Mayo Clinic Jacksonville **Award Amount:** $1,176,917

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: Analysis and validation of miRNA screening hits. Analysis and validation of RNAseq data and miRNA targets.

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: (09/17/2021)

Goals/Milestones

CY17 Goal – miRNA screening & confirmation of hits

☑ Complete regulatory review and approval by HRPO

☑ Analysis of miRNA screen and validation of hits

CY18 Goals – Bioinformatic processing & functional validation

☑ Overlay of datasets and bioinformatic filtering

☑ Functional validation of miRNA-target predictions

CY19 Goal – Functional testing of genes/variants

☐Confirmation of mitoQC/EOPD genes & mechanisms

☐Validation of mitoQC/EOPD genes under endogenous conditions

CY20 Goal – Final validation in patients specimens

☐Validation of mitoQC/EOPD genes in dopaminergic neurons/brains

☑ **Comments/Challenges/Issues/Concerns**

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

Budget Expenditure to Date

Projected Expenditure:

Actual Expenditure: $1,052,766.81