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

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E-Mail:

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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?**
  Towards subtask 1.2 of the previous progress report (functional miRNA screening hit validation) we have received the analyzed data from the screening facility. Overall, the hit confirmation, in particular among the miRNA inhibitors was excellent and only 10 out of 50 miRNAs did not reconfirm to the extent they were expected. As mentioned previously, there was a change in instrumentation and the lack of full confirmation might be attributed to a slight variation in image analysis or due to the delivery of a chemically different library. As a next step, we had received the re-screening plates at our facility and independently confirmed the results with our own high content imager and analysis package. Upon manual quality inspection we noticed though that some of the positive hits might come up because of their overall effect on cellular morphology. In these cases, PRKN translocation might be affected indirectly and as result of global cellular response towards cytotoxicity. Using our own set of images, we have therefore manually scored the reconfirmation plates and generated a high confidence list of miRNAs where the cellular shape was not altered and hence the effect on PRKN translocation should be a primary effect of the induced gene repression. We have shared this list with Dr. Ross for analysis of genetic variants in EOPD cases and controls. Since the same phenomenon could have been present during the original whole genome screening of miRNA we are assessing possibilities to re-analyze the original screening data for a high confidence hit list. We have received all 32,000 images from the facility and are testing possibilities to arrange an automated way to score these for the cell shape in order to enrich for primary PRKN translocation affecting miRNAs.

In the meantime we continued to work on the miRNA-target relationship (subtask 1.3). We mainly employed three computational algorithms to predict miRNA target relationship: TargetScan, miRTarBase and mirDIP. TargetScan is one of the first algorithms for predicting targets in vertebrates and widely used. mirTarBase contains experimentally validated targets. miRDIP is a quite novel database that integrates the data of 30 different miRNA target prediction algorithms. It computes an integrative score based on predictions obtained across all 30 algorithms. To evaluate the miRNA target prediction confidence or mirDIP, we generated a training set based on 15 miRNAs under the assumption that the predicted targets of these miRNAs would be enriched for 100 known genes related to PRKN translocation. However, no statistically significant enrichment was detected, most likely because too many targets per miRNAs are predicted. As an alternative to database mining, we explored publicly available datasets to tighten the miRNA/target relationships. We chose to analyze those samples from the Broad Institute's Cancer Cell Line Encyclopedia that had expression values for mRNA sequencing and miRNA Nanostring quantification. We selected two different cell line groups - Neural cell lines (N=64) and gynecological cell lines (N=75). Within each of these groups, we performed correlation analysis between all available miRNA probes (734 probes) and all entries of the mRNA expression data (56202 transcripts). We prioritized identifying miRNA29a and -b as training data set since these have been validated in house. We selected those genes with the top 5% negative correlation values for each of the two miRNAs and found the overlap between these gene sets. To identify any potential biological impacts associated, we performed non-direction KEGG pathway analysis to identify those pathways with over-representation for predicted gene targets. In order to guide these efforts we will be using the RNAseq data that we have already obtained for miRNA29a and -b and other high confidence miRNAs that we are currently processing (subtask 1.4). The final analysis and merging of the data is still ongoing.

For subtask 3.1 we have selected siRNAs against a few preliminary target genes that were picked based on high confidence experimental miRNA/target relationship data and their biological data. We have also designed primers in order to verify successful knockdown of these. Currently we are in the process of optimizing siRNA transfection into the cells in multi-well plates in order to do High Content imaging for PRKN translocation.

- **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?**
  Data from the RNAseq experiments will be used to overlay with the correlation data sets and/or the public database miRNA/target relationships in order to narrow the target list down. In addition, we will continue with our efforts to validated the preliminary candidates in cell culture. We have already coded a method called rank-rank hypergeometric overlay (RRHO) to identify genes whose miRNA correlations and z-scores could together predict an impact on PRKN protein translocation for each of the different miRNAs and are awaiting full RNAseq data analysis to make this approach viable to confidently identify targets of the corresponding transfected miRNAs within our cells. From then we will proceed to functionally testing more of the high confidence targets and as a second steps variants found in patients that we will obtain from Dr. Ross' work.

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**
     As mentioned above, looking at the re-screening plates ourselves, we noticed that perhaps some miRNAs may result in false positive signal to their effect on cell shape. For now we have manually curated a short-list of miRNAs from the rescreen (hit validation), but have also requested and received all images from the initial genome-wide screen. Although this was not anticipated, we are exploring ways to automatically process these files in order to eliminate other potential false positives.

     We initially thought to rely on miRNA-target predictions for the most part, but now realized the limitations of those predictions. Thus, we incorporated other publicly available large-scale, experimental datasets for miRNA-gene expression correlation analyses. We are further combining this with the RRHO approach and enriching the data through our own RNAseq output. By now we have established optimal conditions for larger scale miRNA transfections that allow both bulk RNA extraction while controlling for effects on PRKN translocation high content imaging, and have expanded this to additional high confidence miRNAs. We are confident that this combinatorial approach will help filter high confidence target genes for subsequent functional validation.

   - **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:	*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 screens in consultation with biostatisticians and bioinformaticians and has supervised the hit validation and cellular re-testing, RNAseq experiments and candidate gene testing.
Funding Support:	
Name:	Xu Hou, PhD
Project Role:	Postdoctoral fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3.0
Contribution to Project:	Dr. Xu has performed work relating to the cellular testing of miRNAs hits.
Funding Support:	
Name:	Marie Jenny Bredenberg, MSc
Project Role:	technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6.0
Contribution to Project:	Mrs. Bredenberg has supported the cell-based work.
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: Molecular Mechanisms of PINK1-PRKN directed mitochondrial quality control
Grant number: RF1 NS085070 (renewal)
Committed Time: 2.40
Supporting Agency: National Institute of Neurological Disorders and Stroke
Contracting/Grants Officer: Beth-Anne Sieber, Program Officer
Performance Period: **09/01/2019**-05/31/2024
Level of Funding: $360,754 (current annual direct costs)
Goals & Specific Aims: To elucidate the modifying roles of heterozygous PINK1-PRKN mutations in Parkinson's disease and related Lewy body disorders
Role: PD/PI

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**-07/31/2020
Level of Funding: $538,868 (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: Mitochondrial Sirtuin 3 in Parkinson's disease
Grant number: R01 NS110085
Committed Time: 1.20
Supporting Agency: National Institute of Neurological Disorders and Stroke
Contracting/Grants Officer: Beth-Anne Sieber, Program Officer
Performance Period: **06/01/2019**-05/30/2024
Level of Funding: $304,468 (current annual direct costs)
Goals & Specific Aims: To validate a role for SIRT3 in alpha-synuclein mediated mitochondrial dysfunction in PD and to establish SIRT3 as a novel target for therapeutic intervention.
Role: Co-PI

Title: Validation of novel, selective autophagy biomarkers in Alzheimer's disease
Grant number: 9AZ10
Committed Time: 1.20
Supporting Agency: Florida State Department of Health
Contracting/Grants Officer: Rachelle Saint-Fort, Research Grant Manager
Performance Period: **02/01/2019**-01/31/2020
Level of Funding: $75,810 (current annual direct costs)
Goals & Specific Aims: To validate potential biomarkers in body fluids from patients with Alzheimer's disease
Role: PD/PI

Ended
None.

- **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*

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PR160606
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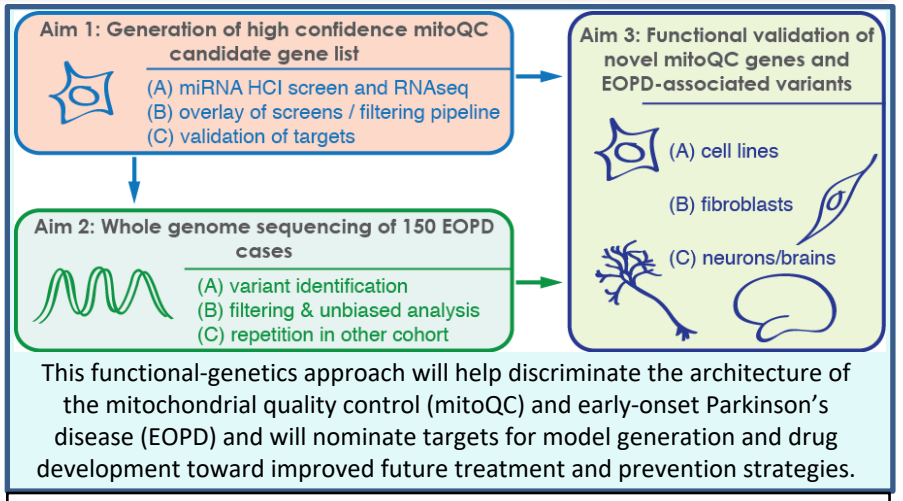
PI: Wolfdieter Springer, PhD **Org:** Mayo Clinic Jacksonville **Award Amount:** $1,176,917

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.

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: (09/11/2019)

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

Budget Expenditure to Date
Projected Expenditure:
Actual Expenditure: 542,577.99