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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

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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 top ranked miRNAs such as miR-29a and have extended our analysis to the other members of the same miRNA family including miR-29b and miR-29c. Transfection of miRNA-29 family members was performed and we used qPCR analysis to investigate the same transcripts that we were found changed in our RNAseq experiments. For all analyzed target genes, we observed robust downregulation with miR-29a as expected **(Figure 1)**. As a negative control we had used a commercial non-targeting miRNA mimic. Compared to miR-29a, transfection of miR-29b and miR-29c still led to downregulation of all selected target genes but the effect was weaker. We included two new target genes in our panel, AHR and ATG9A, that were predicted to be miR-29a specific, but also these seem to be regulated by miR-29b and miR-29c and thus all three members of the family.

  We had hoped to capitalize on the shared, but also distinct, predicted targets of the miRNA-29 family members, in order to further facilitate narrowing down and nominating individual target genes that perhaps are the true effectors responsible for the functional effect seen in the original Parkin translocation screen. However, our own experimental data suggest that the miRNA-target predictions are not particularly reliable at this point and speculate that most (if not all) target genes will be regulated by each of the three family members at least to some degree. On the other hand, this might indicate that the miRNA-29 family members have functionally redundant roles and globally regulate the same cellular processes but perhaps not to the same magnitude.

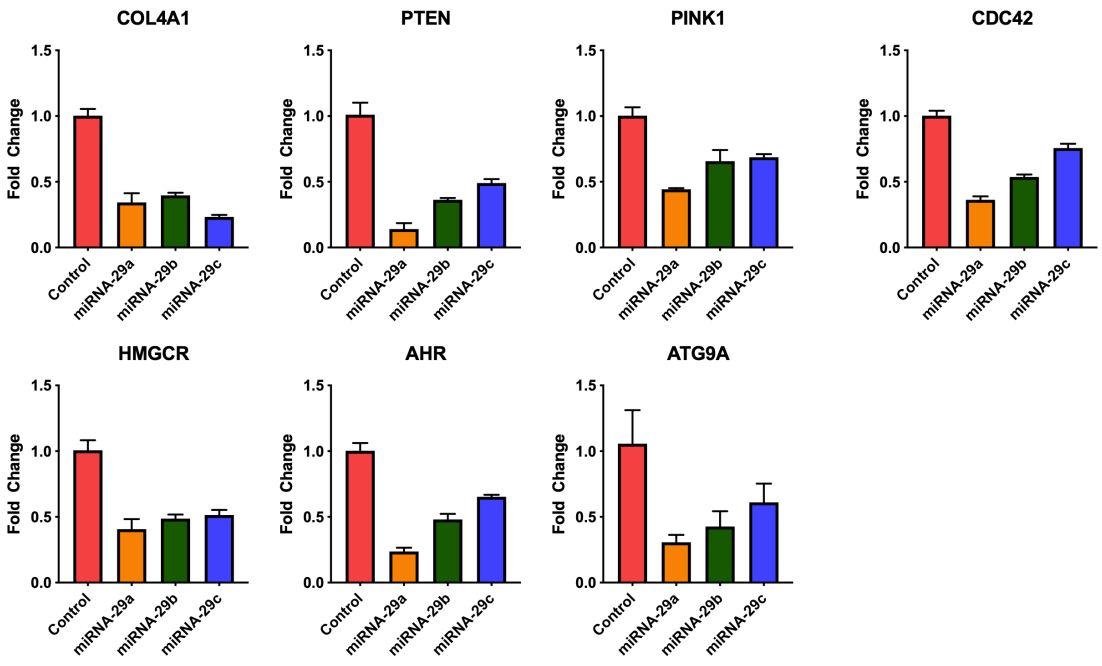


Figure 1: RT-qPCR of target genes upon transfection of control miRNA, miRNA-29a, -b, or -c.
HeLa cells were seeded at 200,000 cells/mL on a 6-well plate. The next day, cells were transfected using RNAiMax with 25nM of miRNA. After 24 hours, transfection media was removed and cells were harvested. RNA was prepared using the miRNeasy Kit from Qiagen. For qPCR, 1000ng of RNA was reverse transcribed using Transcriptor High Fidelity cDNA synthesis kit from Roche. 1:5 dilutions were used in triplicate with 0.25uL Taqman probe mix and 2.5uL iTaq Universal Probe Mix from Bio-Rad in a 5uL reaction. qPCR analyses were executed on a 384-well block on a LightCycler 480 system. Relative levels were calculated with RPL27 values as a housekeeping gene and normalized to the expression level of the FAM-labelled control transfected sample. Shown are bar graphs of the average of the triplicates for each sample. AHR and ATG9A are predicted miRNA-29a specific, all others should be affected by all miRNA-29 family members.

We therefore decided to continue characterizing a set of shared miRNA-29 family target genes that may play a role in the mitophagy pathway such as PTEN, ATG9A, and TMEM187. Some of the genes that are significantly downregulated upon miRNA-29 transfection are amongst those genes that have also been nominated in previous whole-genome wide siRNA screens for Parkin translocation or mitophagy, as we have hypothesized, and we will pay special attention to those candidates going forward.

Besides transcript levels of target genes, we have started analyzing how effects of miR-29a transfection translates to the respective protein level. One example here is PTEN, which is particularly interesting due to its name-lending connection with the ubiquitin kinase PINK1 (aka PTEN-INduced Kinase 1), but also the recent discovery that a long isoform of PTEN (termed PTEN-alpha or PTEN-long) could act as the ubiquitin phosphatase and could therefore regulate mitophagy by counteracting PINK1. We used antibodies against PTEN and PTEN-alpha and found that levels of both protein isoforms seem indeed somewhat reduced upon miRNA 29a transfection **(Figure 2)**.

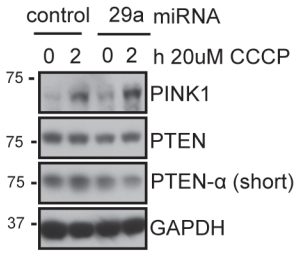


Figure 2: Western blot of PTEN and PTEN-alpha upon transfection of miRNA-29a and with and without mitochondrial stress.
HeLa cells were seeded at 200,000 cells/mL on a 6-well plate. The next day, cells were transfected using RNAiMax with 25nM of miRNA. After 24 hours, transfection media was removed and cells were treated without or with 20uM CCCP for 2h. Cells were harvested and lysed in RIPA buffer. 30ug of protein was loaded per lane on 8-16% gradient gels and transferred onto PVDF membranes, which were probed with antibodies of interest. GAPDH was used as loading control. Constistent with the RT-qPCR data there seems a slight decrease of PTEN (and PTEN-alpha) upon transfection of miRNA-29a. However, there is no clear decrease in PINK1 levels, they seem rather increased than decrease. Likewise the stabilization of PINK1 upon treatment with the mitochondrial stressor CCCP is not affected or rather upregulated in cells that have been transfected with miRNA-29a.

However, it is unclear at this point whether the observed (relatively subtle) reduction in PTEN protein (alone) would be sufficient to drive the inhibition of Parkin translocation as seen in the miRNA screen. In this context it is important to mention that the experimental conditions for the initial miRNA screen, the RNAseq, the re-validation on transcript level, and now the protein analysis were identical (i.e. 24 h transfection of the miRNA). While Parkin translocation was significantly impaired under these conditions and for example PTEN mRNA was significantly reduced, the resulting protein effect at this time point seem marginal though (i.e the PTEN protein has not yet been turned over to reflect the significant RNA changes). Thus it is uncertain whether the relatively large functional effect (on Parkin translocation) can be attributed to the relatively small loss of PTEN protein. However, as an enzyme, even small changes in PTEN protein could result in significant effect. Another possibility is that PTEN is not the main driver and we should be looking for candidate proteins that are less stable and in contrast have been significantly depleted after only 24 h miRNA transfection. Alternatively, no single gene is mediating the functional effect, but subtle, though simultaneous, reduction of several proteins in concert additively or synergistically impairs Parkin translocation. We consider all these possibly and will follow up one each accordingly.

One particular mechanism through which Parkin translocation is negatively impacted is through downregulation of PINK1 mRNA itself as we had earlier discovered for miR-27a and miR-27b family members and we will test that for all the miRNAs. Further we will confirm our preliminary finding on PTEN and PINK1 proteins levels by repeating the experiment multiple times and include quantification with statistical analysis, but will also expand to study the levels of phosphorylated ubiquitin. Similar to Parkin, ubiquitin is phosphorylated by PINK1 at a conserved serine residue (pSer65-Ub) and then acts as an allosteric activator of and receptor for Parkin on mitochondria. As such levels (and activities) of the ubiquitin kinase PINK1 and the ubiquitin phosphatase PTEN together likely affect the rate of Parkin activation/translocation. We now have a highly sensitive sandwich-type ELISA at hand that allows detection and relative quantification of minute amounts/differences in pSer65-Ub levels even at baseline (unstressed) conditions (Watzlawik *et al.,* Autophagy accepted). The assay can thereby discriminate between genotypes in cells and animal models and can distinguish between human control and diseased conditions (Hou *et al.,* Alzheimer's & Dementia in press).

- **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?**
  In addition to PINK1 and PTEN as well as the product/substrate pSer65-Ub, and depending on the availability of specific antibodies and tools, we will also analyze further miRNA candidate target genes on both the protein and enzymatic levels, such as ATG9A and others that have been nominated in the siRNA screens for Parkin translocation or mitophagy. As outline above and depending on the actual protein levels and enzymatic activities of the candidates after miRNA transfection, we will further consider the possibility of additive effects of multiple, rather than singular roles of proteins. In this context we might perform Ingenuity pathway analyses that may help us address this further. From our RNAseq data we have received a list of 147 transcripts that remained, upon correction for multiple testing, significantly altered upon expression of miRNA-29s in HeLa cells. Using GeneCards we have preliminarily curated a list of relevant genes that are involved in mitochondrial function,

lysosomal degradation and/or involved in neuronal function and neurodegeneration. From these functionally relevant genes (about 20 in each category, total of 60) we rank the top genes and order siRNAs against 3-5 genes. We will transfect these siRNAs and monitor Parkin translocation, PINK1 levels and pSer65-Ub levels. Going forward, we plan to apply the same strategy to other top ranked miRNAs. For each we will monitor PINK1 and pSer65-Ub levels upon mitochondrial stress as well. This will show if pSer65-Ub which is mainly driven by the PTEN-PINK1 pathway is the (main) effector that is driving the observed effect on Parkin translocation.

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**
  During the project we have encountered a variety of smaller problems that have added up to an overall delay but had been solved one by one. However during the previous months, we experienced a further significant delay in various research activities caused by COVID-19 and related closures of labs and cores. Although we still experience effects of the pandemic such as reduced staff density that are necessary to accommodate social distancing guidelines, by now most activities have resumed. The current 12 months no-cost-extension (until 08/15/2021) will be critical for us to catch up with our initial plan.

- **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. DOI: 10.1002/alz.12198. **in press**.

4. Watzlawik, J.O., X. Hou, D. Truban, 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. **accepted**.

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 miRNA/target gene/protein testing.
Funding Support:	
Name:	Caleb Hayes, MSc
Project Role:	technician
Researcher Identifier (e.g. ORCID ID):	

Nearest person month worked:	12.0
Contribution to Project:	Mr. Hayes 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: Validation of the 21st Century Rabbit anti-pS65 Ubiquitin antibody
  Grant number: 17399
  Committed Time: 0.60
  Supporting Agency: Michael J Fox Foundation for Parkinson's Research
  Contracting/Grants Officer: Allison Morris, Research Programs Officer
  Performance Period: **11/01/2019**-10/22/2021
  Level of Funding: $131,140 (current annual direct costs)
  Goals & Specific Aims: To characterize pS65 Ubiquitin Antibody Candidates from 21St Century/IPA
  Role: PD/PI

  Changes

  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: $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: 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 $125,000 (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: $25,000 (current annual direct costs)
Goals & Specific Aims: To identify and validate activating Parkin missense mutations
Role: PD/PI

Ended
Title: pS65-Ub as a biomarker for Parkinson's disease
Committed Time: 1.20
Supporting Agency: Michael J Fox Foundation for Parkinson's Research
Contracting/Grants Officer: Allison Morris, Research Programs Officer
Performance Period: 08/01/2018-**31/01/2020 (NCE)**
Level of Funding: $120,000 (current annual direct costs)
Goals & Specific Aims: To develop, optimize and validate a pS65-Ub biomarker assay
Role: PD/PI

Title: Validation of novel, selective autophagy biomarkers in Alzheimer's disease
Committed Time: 1.20
Supporting Agency: Ed and Ethel Moore Alzheimer's Disease Research Program
Contracting/Grants Officer: Rachelle Saint-Fort, Research Grant Manager
Performance Period: 02/28/2019-**02/29/2020**
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

- **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

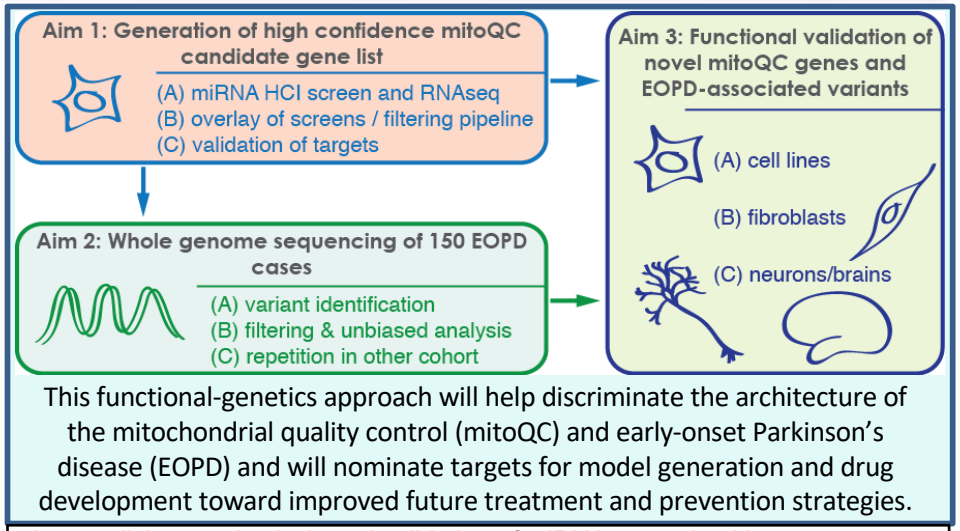
PI: Wolfdieter Springer, PhD **Org:** Mayo Clinic Jacksonville **Award Amount:** $1,176,917

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. Analysis and validation of target mRNAs.

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: (09/25/2020)

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. NCE until 08/14/2021.
- If off by more than one quarter in spending, comment here.

Budget Expenditure to Date
Projected Expenditure:
Actual Expenditure: $856,870