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TITLE: How mtDNA Mutations Cause Mitochondrial Disease

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14. ABSTRACT Mutations in the mitochondrial genome (mtDNA) cause devastating diseases with a myriad of clinical features. Tissues with high energetic demands including the brain, liver, muscle, and heart are particularly vulnerable to mtDNA mutations. Majority of mtDNA diseases are caused by heteroplasmic mutations, Heteroplasmy refers to a state in which mutant mtDNA coexists with wildtype mtDNA in the same cell. Heteroplasmic mtDNA mutations cause disease then their level exceeds a critical threshold (typically 60-80%). While it is known that mutant mtDNA levels can vary between tissues, the underlying mechanisms are not known. In this project, we seek to develop C. elegans as a model system to study how mutant mtDNA levels can vary across different cell types. Below I detail the progress we have made on this project since the submission of the last progress report.					
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Table of Contents

	<u>Page</u>
INTRODUCTION.....	2
KEYWORDS.....	2
ACCOMPLISHMENTS.....	2-5
IMPACT.....	5
CHANGES/PROBLEMS.....	5-6
PRODUCTS.....	6
PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS.....	6
SPECIAL REPORTING REQUIREMENTS.....	6
APPENDICES.....	6

INTRODUCTION

Mutations in the mitochondrial genome (mtDNA) cause devastating diseases with a myriad of clinical features. Tissues with high energetic demands including the brain, liver, muscle, and heart are particularly vulnerable to mtDNA mutations. Majority of mtDNA diseases are caused by heteroplasmic mutations. Heteroplasmy refers to a state in which mutant mtDNA coexists with wildtype mtDNA in the same cell. Heteroplasmic mtDNA mutations cause disease then their level exceeds a critical threshold (typically 60-80%). While it is known that mutant mtDNA levels can vary between tissues, the underlying mechanisms are not known. In this project, we seek to develop *C. elegans* as a model system to study how mutant mtDNA levels can vary across different cell types. Below I detail the progress we have made on this project since the submission of the last progress report.

KEYWORDS

mtDNA, mitochondria, heteroplasmy, cell types, FACS, droplet digital PCR (ddPCR)

ACCOMPLISHMENTS

Specific Aim 1: Determine cell type specific differences in mutant mtDNA heteroplasmy levels

Subtask 1: Cross three heteroplasmic strains (uaDf5, mptDf2, mpt1) into transgenic lines in which specific cell types (neurons, muscles, epithelial like seam cells, phagocytic coelomocytes, and intestinal cells) are fluorescently labeled with GFP. Timeline: Months 1-2.

We were able to cross three heteroplasmic strains (uaDf5, mptDf2, and mptDf3) into transgenic lines in which neurons and muscles are fluorescently labeled with GFP. We decided on mptDf3 rather than mpt1 because mptDf3 behaved very differently than uaDf5 and mptDf2. More specifically, mptDf3 persists at low levels, compared to uaDf5 and mptDf2, which persist at relatively high levels. Consequently, we reasoned that mptDf3 might show different cell-type specific responses. However, we discovered that we could not maintain animals with mptDf3 when trying to grow large populations of worms needed for FACS sorting (**Figure 1**). Consequently, we decided to test another heteroplasmy, mpt2. We are currently in the process of crossing mpt2 into transgenic lines with neurons, muscles and intestinal cells labeled. Our crosses to generate the strains we need for the FACS experiments take much longer than initially anticipated because we also need to cross in a *glp-1* mutation. Presence of this *glp-1* mutation, which allows us to grow animals without the germline in a temperature sensitive manner, is necessary because we discovered that is important to successfully isolate cells for FACS.

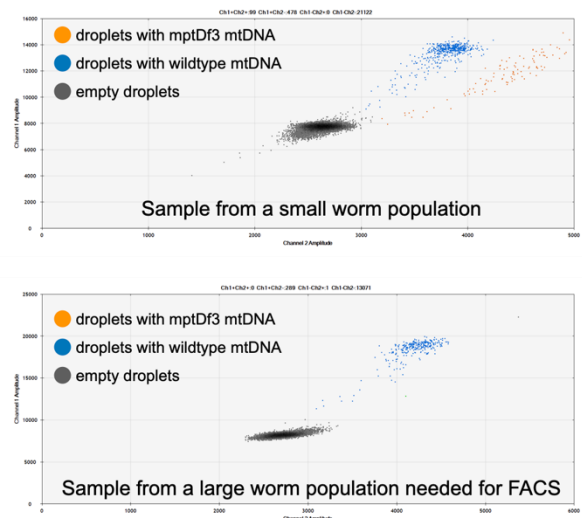


Figure 1. mtDNA heteroplasmic mutant mptDf3 is lost in large worm populations that are needed for FACS.

The *glp-1* mutation and the transgene that GFP-labels intestinal cells (*ges-1p::GFP*) are located on the same chromosome. Consequently, we could not cross these. To overcome this, we used

CRISPR to introduce a point mutation in *glp-1* in the animals that already carried the *ges-1p::GFP* transgene. We were successful in obtaining the CRISPR-generated *glp-1* mutants and are now in the process of crossing these into the different mtDNA heteroplasmies.

Subtask 2: Perform fluorescence activated cell sorting (FACS) across 4 developmental stages (L1, L2, L3, and L4) and different ages of adulthood (Day 1, Day 4, and Day 10). Timeline: Months 3-7.

We have successfully performed FACS on L1 and D1 adults that carry *uaDf5* and *mptDf2* heteroplasmies. We were able to do this from neurons and muscles. We also performed preliminary experiments on older animals (Day 8). However, we discovered that they have very low mtDNA copy number, which precludes us from being able to accurately measure heteroplasmy frequency (**Figure 2**). Consequently, we will confine our future experiments to just two time points L1 and D1.

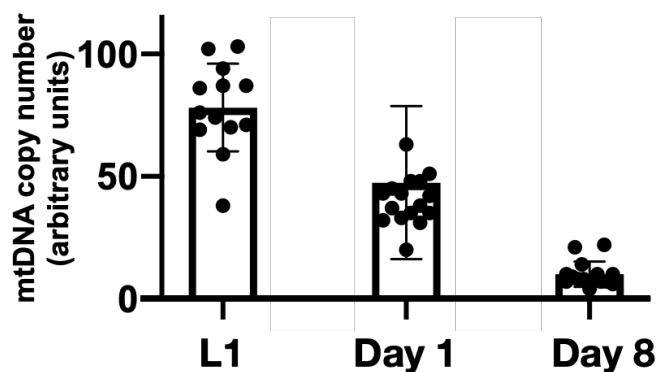


Figure 2. mtDNA copy number in Day 8 old adults is too low to allow for accurate measurement of heteroplasmy frequency.

While we have had good success in using FACS to isolate muscle and neurons, we have encountered difficulties in isolating intestinal cells at the L1 stage. The expression of the *ges-1* promoter that is used to drive GFP expression in the intestinal cells is sensitive to starvation, a step that is necessary to acquire large number of synchronized L1 stage animals (**Figure 3**). Thus, we now plan to perform FACS on L2 stage animals, which we predict will have recovered their GFP expression after being on food for a few hours.

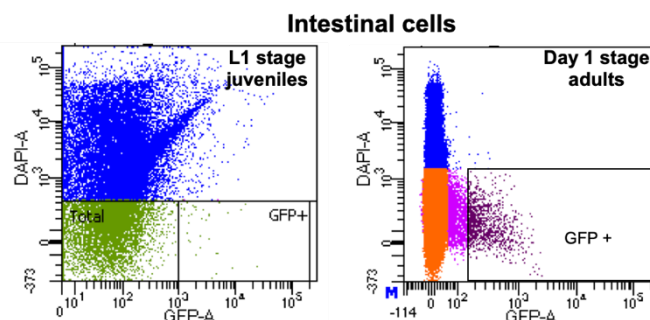


Figure 3. GFP signal is high in intestinal cells of Day 1 stage adults but not in L1 stage juveniles. Consequently, very few GFP-positive cells can be recovered from L1 stage animals.

Our FACS sorting efforts came to a standstill due to the COVID pandemic at the end of March of 2020. We have recently begun performing FACS again but in a very limited capacity. Part of the difficulty in performing FACS is that we are not allowed to enter the FACS facility for safety purposes. Instead, we are required to hand off our samples to the FACS core technician, who then performs the FACS. However, this prevents us from communicating with the FACS technician when they are setting the gates, which is crucial for a successful FACS run. We are therefore proceeding cautiously and carefully with our FACS experiments in the near future.

Subtask 3: Perform droplet digital PCR (ddPCR) on the flow sorted GFP positive and negative fractions to determine mutant mtDNA levels across cell types. Timeline: Months 8-9.

We reported in our last progress report that we encountered significant technical hurdles to get a working ddPCR protocol, but we were able to successfully troubleshoot these and develop a robust protocol. Indeed, we have been able to use this protocol to run and successfully collect ddPCR data from our samples. Our efforts slowed down substantially during the Spring and

Summer due to the COVID pandemic, but we have returned to performing ddPCR in a limited capacity since the beginning of the Fall semester.

Milestone(s) achieved: Determined how heteroplasmy dynamics change during development and aging across different cell types for 3 different mutant mtDNA.

We have accomplished 2/3 of the work we had set out to. Specifically, have collected data from 2 different heteroplasmy from 2 different cell types. We are on route to collect data for these 2 heteroplasmy from one additional cell type, and from one additional heteroplasmy from 3 different cell types.

Specific Aim 2: Investigate mitochondrial mechanisms that regulate cell type specific differences in mtDNA heteroplasmy levels.

Subtask 1: Cross heteroplasmic strains with GFP labeled cells generated in Aim 1 into different mutant backgrounds that affect mitophagy (pink-1 and parkin mutants), mitochondrial biogenesis (atfs-1 mutant), and fusion/fission (fzo-1 and drp-1 mutants). Timeline: Months 3-4.

A manuscript was published on a preprint server BioRxiv, which investigated the impact of *pink-1* and *parkin* mutants on heteroplasmy dynamics in a cell type specific manner. Consequently, rather than repeat these experiments, we decided to focus our efforts on characterizing impact of other mutations on heteroplasmy levels. We also discovered that the *fzo-1* and *drp-1* mutant animals with heteroplasmy are too unhealthy to be able to successfully grow in large populations. Taken together, we have decided to focus on asking how loss of AMPK affects heteroplasmy levels. AMPK is an important sensor of ATP levels, with wide-ranging effects. We crossed *ampk* mutants with *uaDf5* heteroplasmy into transgenic animals expressing GFP in neurons or muscles. Interestingly, our data show that loss of AMPK differentially affects *uaDf5* heteroplasmy levels in muscles versus neurons (**Figure 4**).

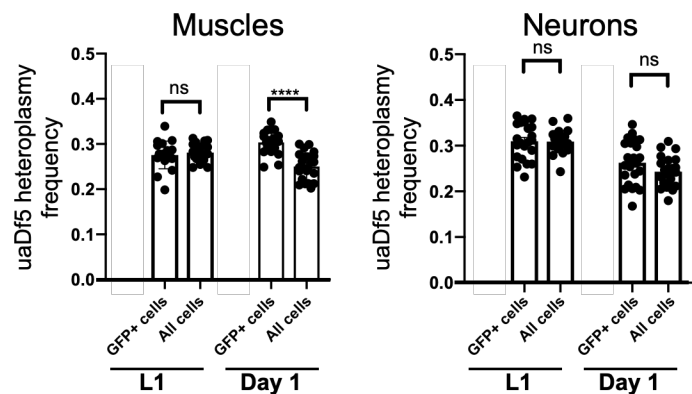


Figure 4. *uaDf5* heteroplasmy frequency in muscle cells (GFP+ cells) of *ampk* mutants starts out as being the same as in the rest of the somatic cells (All cells) in L1 stage animals. However, muscle cells have greater *uaDf5* mutant mtDNA levels in Day 1 old adult animals compared to rest of the somatic cells. In contrast, the *uaDf5* heteroplasmy levels in neurons of *ampk* mutants is not significantly different at either the L1 or the Day 1 stage of adulthood.

Subtask 2: As in Aim 1 subtask 2, perform FACS across developmental stages and different ages of adulthood from the different mutants. Timeline: Months 10-14.

We were able to successfully perform FACS before COVID-forced shutdown on *ampk* mutants with *uaDf5* heteroplasmy from muscles and neurons from 2 different developmental stages.

Subtask 3: As in Aim 1 subtask 3, perform ddPCR on the flow sorted GFP positive and negative fractions to determine mutant mtDNA levels across cell types. Timeline: Months 15-16.

We were able to perform ddPCR on *ampk* mutants samples that we had collected as part of Subtask 3. These data are shown in **Figure 4**.

Subtask 4: Write manuscript describing findings from the study on cell type specific heteroplasmy dynamics. Timeline: Months 17-18.

While the scope of the work has been slightly reduced due to the COVID pandemic, our plans to write a manuscript describing our findings from this project remain unchanged. We plan to publish one paper from this work. It will represent an important milestone in the study of cell type specific differences in mutant mtDNA levels. Our goal is to submit a manuscript for publication at the end of the funding period (January 2021).

Milestone(s) achieved: Determined the role that mitochondrial physiology (mitophagy, biogenesis, and fusion/fission) plays in regulation of cell type specific heteroplasmy dynamics.

Our original milestone of determining the role of mitochondrial physiology in the regulation of heteroplasmic mutant mtDNA dynamics in cell type specific manner remains unchanged.

IMPACT

We are on route to demonstrating that it is possible to study the regulation of mutant mtDNA heteroplasmy levels in cell type specific manner in *C. elegans*. Our pipeline demonstrates proof of concept. It can be applied to measure mutant mtDNA levels from any cell type that can be labeled with GFP. While our work is done using *C. elegans* as a model system, theoretically, our pipeline can be used in other systems including mammalian systems. Additionally, we observe that loss of AMPK differentially impacts mutant mtDNA levels in neurons versus muscles. These data suggest that it is possible to manipulate mtDNA levels in a cell-type specific manner by altering cell signaling pathways. These findings open the door to exploring whether modulation of AMPK can be used as a potential therapeutic target for the treatment of mitochondrial diseases caused by heteroplasmic mtDNA mutations.

CHANGES/PROBLEMS

We were able to overcome most of the challenges and problems in this project in the first year, which were highlighted in last year's annual progress report. This year, we encountered a few obstacles but these were not technical in nature, and do not represent insurmountable hurdles. Specifically, we discovered that one of the heteroplasmic mutations, *mptDf3*, is prone to getting lost from worms that are required to be grown in large populations for FACS. Fortunately, we have other heteroplasmic mutations and we have decided to utilize pursue our studies with mutant mtDNA variant called *mpt2*. Second, we discovered that *glp-1* mutation and the transgene that labels intestinal cells are both located on the same chromosome, precluding our ability to genetically cross them together. We were able to overcome this challenge by using CRISPR to introduce *glp-1* mutation in the background that already containing the transgene *ges-1p::GFP*, which labels intestinal cells. Third, the *ges-1p::GFP* is sensitive to starvation conditions and hence not amenable to sort intestinal cells from L1 stage animals. Thus, we plan to instead sort intestinal cells from the L2 stage. Finally, we could not carry forward with our original proposal to investigate impact of loss of *drp-1* and *fzo-1* on cell-type specific heteroplasmy levels because these animals are too unhealthy to grow in large numbers. We therefore shifted our efforts to a different target, namely AMPK. We have collected promising data, showing that loss of AMPK differentially affects heteroplasmy levels in muscles versus neurons in an age-dependent manner. We anticipate being able to submit a manuscript reporting our findings by the end of the grant period in the beginning of 2021.

Perhaps the great challenge we encountered this year was the inability to work on this project at full capacity due to the COVID pandemic. Fortunately however, we had troubleshooted most of

the technical hurdles in the prior year, and are therefore able to move forward in a limited capacity with our original work plans.

PRODUCTS

2020 Seminar, Genome Sciences Department, University of Washington, Seattle.

2020 Seminar, Department of Biology, University of Pennsylvania, Philadelphia.

Conference attendance and presentations cancelled due to COVID pandemic.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name: Nikita Tsyba

Project role: Graduate student

Nearest person month worked: 12

Contribution to the project: Mr. Tsyba has performed all the experiments for this project. He has worked on optimizing the protocol to isolate cells from *C. elegans* to be used for FACS. He also developed ddPCR protocol to quantify mutant mtDNA levels from flow sorted cells.

Name: Benjamin Saunders

Project role: Research Assistant I

Nearest person month worked: 6

Contribution to the project: Mr. Saunders is aiding Mr. Tsyba in their efforts to carry out fluorescence activated cell sorting (FACS) coupled droplet digital PCR (ddPCR) in *C. elegans* and downstream analysis of the data. Duties performed by Mr. Saunders include maintaining worm stocks, cleaning bacterial contamination from worm strains, growing large scale worm cultures, performing PCR to verify presence of heteroplasmic mutant mtDNA and qualitatively checking mutant mtDNA levels. Additional duties include preparing worm plates, making aliquots of reagents, preparing media, autoclaving, dishwashing, and maintenance of relevant equipment.

SPECIAL REPORTING REQUIREMENTS

Nothing to report

Appendices

Nothing to report