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14. ABSTRACT

Mitochondrial dysfunction is associated with several chronic, deployment-associated conditions in Veterans. *In situ* cryo-electron tomography (cryo-ET) imaging is a new 3D imaging approach that we have used to study patient cells with unprecedented detail. Our goal is to test the feasibility of cryo-ET to visualize mitochondrial structural changes in patient cells. We hypothesize *in situ* cryo-ET will resolve structural changes in the individual mitochondrial respiratory complexes and organization of these complexes into higher-order supercomplexes. We have now obtained primary fibroblast cells from patients with distinct mitochondrial complex I mutations including ND6, ACAD9, and NDUFV1 subunits and optimized growing these mutant cells on EM grids. We characterized effects of these mutations on mitochondrial morphology and dynamics in living patient cells. *In situ* cryo-ET revealed disruptions of mitochondrial inner membranes and crista morphology distinct to each mutant. Our results suggest these respiratory complex subunits are key regulators of overall mitochondrial structure and function and their disruption directly alters their function to produce profound human disease.

15. SUBJECT TERMS
None listed.

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1. INTRODUCTION

Mitochondrial diseases are often caused by mutations that disrupt structures of the mitochondrial respiratory complexes, significantly impairing mitochondrial function. Substantial numbers of people are also affected by mitochondrial dysfunction associated with prevalent diseases including diabetes, Alzheimer's disease, and Parkinson's disease. The combined impact of these mitochondrial impairments is enormous. Mitochondrial dysfunction is suspected in a variety of chronic, deployment-associated conditions in Veterans or in association with Gulf War Illness (GWI). For example, exposure to neurotoxicants including pesticides (*e.g.*, Agent Orange, permethrin, paraquat) and airborne hazards in military theater have been negatively associated with Veterans' health post-deployment. Yet, the precise mechanisms remain poorly understood. Our ability to accurately diagnose disturbances in mitochondrial function remains profoundly limited. Current diagnostic approaches test mitochondrial function (*i.e.*, respiration) and cannot resolve specific disease-causing mitochondrial structural defects despite evidence of broader mitochondrial dysfunction. Such approaches are often invasive and may alter the appearance of mitochondria, hindering accurate diagnosis. Thus, there is great clinical need for more accurate diagnostic approaches since better diagnosis will translate to improved patient outcomes. *In situ* cryo-electron tomography (cryo-ET) imaging is a new three-dimensional (3D) imaging approach to visualize disease-induced changes in mitochondrial structure directly in primary human patient cells for the first time. The unprecedented resolution provided by cryo-ET can visualize alterations in respiratory complex structures unique to the disease-causing mutations and/or acquired mitochondrial defects. We previously used *in situ* cryo-ET to identify profound structural changes in mitochondria within primary fibroblasts from a patient with Leigh Syndrome (LS), a debilitating mitochondrial disease. The structural alterations, produced by a novel mutation, have not been previously identified via conventional imaging, illustrating the power of this approach. We therefore hypothesize that: (**A**) *In situ* cryo-ET will visualize distinct structural changes both in the individual mitochondrial respiratory complexes and organization of the complexes into larger, higher-order assemblies termed supercomplexes. These visualized altered structures will explain the loss of functional efficiency and metabolic flux in complexes as evidenced in mitochondrial disease mutations, GWI, or exposures to pesticides and PB. (**B**) Drugs that improve respiratory chain function can correct structural abnormalities of supercomplex organization, providing the basis for novel therapies. To test these hypotheses, we aim to do the following: (**1**) To determine the effects of disease-causing mutations on individual mitochondrial respiratory complexes and higher-order supercomplex organization *in situ*; and (**2**) To identify effects of GWI and associated neurotoxicants on respiratory chain complex structure and higher-order organization *in situ* in patient cells. We have established new experimental systems where we have grown primary cells taken from patients directly on EM grids followed by imaging of the mitochondria within these cells via *in situ* cryo-ET to resolve disease-induced changes to mitochondrial structure and morphology. We have also developed novel drugs like JP4-039 that improve mitochondrial function in affected patient cells potentially through their actions on mitochondrial respiratory complex structure. In the short term, we expect that our proposed *in situ* cryo-EM studies will reveal mitochondrial structural alterations that disrupt mitochondrial respiratory complex structures in response to GWI or neurotoxicant exposures affecting Veterans. This may form the basis for new, highly accurate and non-invasive diagnostic approaches for mitochondrial disease. In the longer term, our studies will serve as a foundation for future work that leads to new, highly targeted personalized therapies for active military, Veterans and the general population that are directed at correcting structural changes responsible for mitochondrial dysfunction.

2. KEYWORDS

Keywords relevant to the work proposed here include:

1. Mitochondrial disease
2. Cryo-electron tomography
3. Respiratory complex
4. Supercomplex
5. Pesticide
6. Gulf War Illness

3. ACCOMPLISHMENTS

- **What were the major goals of the project?**
  The major goals of the project are as follows:

1) Sample preparation and imaging acquisition of three-dimensional tomograms from mitochondrial disease patient and control cells.
2) Analysis of imaged mitochondria and respiratory complexes from well-defined mitochondrial disease mutation-containing patient and control fibroblasts.
3) Determine whether the respective mitochondrial disease-causing mutations alter the 3D structures of the mitochondrial respiratory complexes *in situ.*
4) Determine whether improved respiratory chain function in patient cells following treatment with JP4-039 is via the drug's ability to correct respiratory complex structural abnormalities.
5) Visualize mitochondrial respiratory complex structure and supercomplex organization in healthy primary human fibroblasts after pesticide or pyridostigmine bromide (PB) treatment.
6) Determine whether JP4-039 corrects mitochondrial structural abnormalities in GWI or pesticide/PB-treated control cells.

- **What was accomplished under these goals?**
  During the reporting period for Year 2 of this award, we conducted studies to address major goals of the project as follows:

I. Sample preparation and imaging acquisition of three-dimensional tomograms from mitochondrial disease patient and control cells.

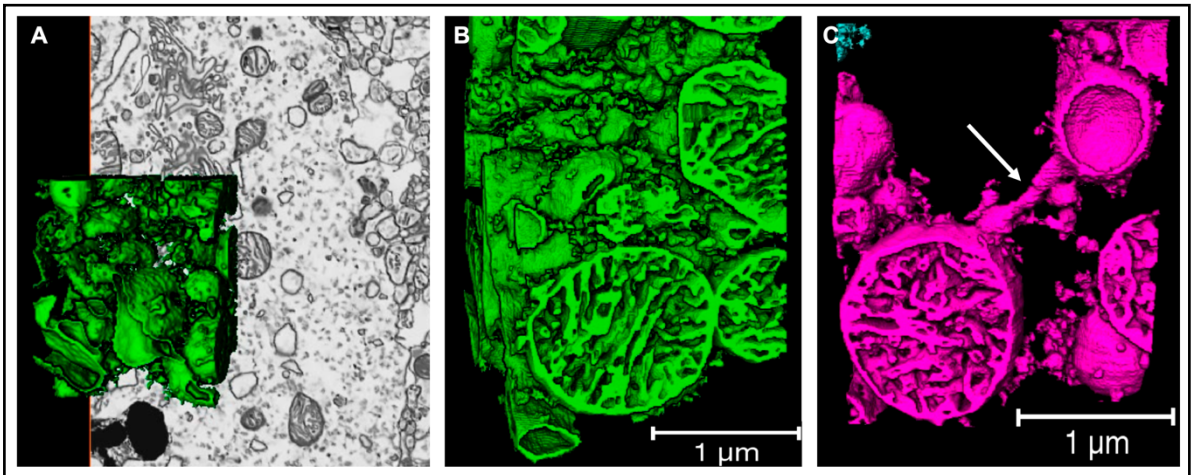

Fig. 1. Dual beam focused ion beam/scanning electron microscopy (FiB/SEM) three-dimensional imaging of mitochondria in human brain. FiB/SEM of postmortem brain tissue acquired from the cortical region of an unaffected subject reveal numerous mitochondria. **(A)** Overview of an area within the cell body of a cortical neuron reveals numerous mitochondria (segmented in green). **(B)** Segmentation of mitochondria from the Panel A inset reveals extensive cristae membranes; scale bar = 1 um. **(C)** 3D reconstruction of mitochondria reveals nanotubes (marked by white arrow) connecting mitochondria to other vesicular structures within neurons of intact cortical brain tissue; scale bar = 1 um).

- We recently adapted serial focused ion beam/scanning electron microscopy (FiB/SEM) technology to cryogenic conditions, enabling us to generate large 3D volumes of cells in their near-native states (Zhu *et al.*, Structure 2021). We have continued optimizing the FiB/SEM approach with the aim of translating this technology directly to the tissues of individuals with primary mitochondrial disease, including the brain – one of the most adversely impacted tissues by mitochondrial disease. As a next step, we have successfully established imaging approaches for acquiring and visualizing large three-dimensional (3D) volumes of brain imaging data using a cutting-edge dual beam FiB/SEM microscope (Fig. 1). As a result, our FiB/SEM data has enabled us to reconstruct the complete 3D architecture of neuronal mitochondria directly in human brain (Figs 1A, B). In the process, we have discovered that mitochondria communicate with one another as well as other organelles including endoplasmic reticulum via fine nanotubes

(Fig. 1C). Though communications between mitochondria and other organelles have been demonstrated biochemically, our new imaging data enables us to structurally characterize these mitochondrial connections at nanometer resolution for the first time. Such detail will be especially important for elucidating the structural basis for mitochondrial dysfunction directly in the tissues of individuals affected by mitochondrial disease.

- Using the FiB/SEM approach, we have also discovered distinct physical interactions between mitochondria and the Golgi apparatus which have not been described prior to this work (Fig. 2). Our 3D reconstructions of mitochondria in brain cortical tissue revealed that mitochondria which appeared to be near cisternae of the Golgi apparatus (Fig. 2A) are fully enveloped by Golgi cisternae (Fig 2B). Indeed, we discovered that every Golgi subcompartment (*e.g.*, cis, medial, trans) makes numerous discrete interactions with the mitochondria nested within the whole stack. We also find smaller satellite mitochondria on the periphery of the Golgi stacks making additional connections between the organelles. These findings provide a putative bioenergetic mechanism by which the mitochondria contacting the Golgi apparatus fuel the pH gradient across the Golgi cisternae that is required for posttranslational processing of new proteins. Future work will examine the functional relevance of these mitochondrial-Golgi contacts in the context of mitochondrial disease by imaging these contacts directly in the cells and tissues of affected subjects.

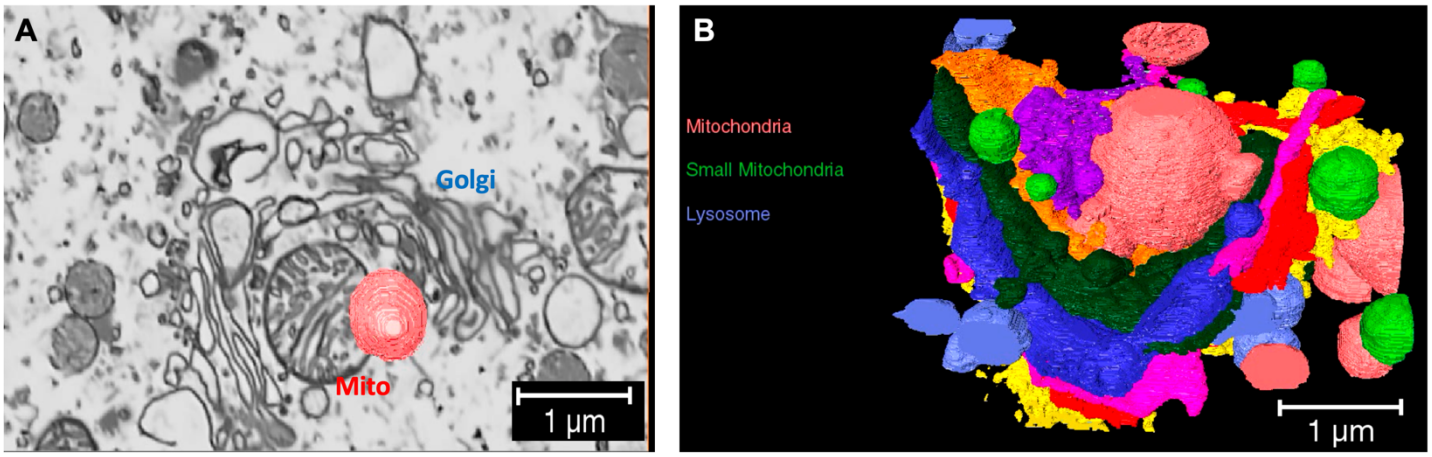

Fig. 2. Dual beam FiB/SEM reveals novel interactions between mitochondria and the Golgi apparatus in human brain. (A) Representative FiB/SEM image from a stack of serial micrographs recorded from the cell body of a cortical neuron from postmortem cortical brain tissue of an unaffected individual reveals several mitochondria in close proximity or in direct contact with cisternae of the Golgi apparatus. One of the mitochondria with direct contacts has been segmented (in pink). Scale bar = 1 um. **(B)** Segmentation of the 3D stack in Panel A reveals several large mitochondria (in pink) entirely enveloped within the Golgi apparatus making numerous physical contacts with cis (in orange), medial (in dark green) and trans (in dark blue) Golgi compartments. Additional smaller satellite mitochondria (in light green) make further contacts with the Golgi periphery including the trans Golgi network (in yellow). Putative lysosomal vesicles (in light blue) are also evident in the periphery of the Golgi stacks. Scale bar = 1 um.

II. Analysis of imaged mitochondria and respiratory complexes from well-defined mitochondrial disease mutation-containing patient and control fibroblasts.

- Building on our work investigating complex V mutant cells, we have made significant progress in visualizing mitochondria affected by mitochondrial disease-causing mutations in additional respiratory complexes including complex I directly in the cells of patients as well as in unaffected controls. As noted above, we focused on the following mitochondrial disease-causing complex I mutants:
  - **ND6 mutant:** ND6 is a mitochondrial chromosome-encoded structural subunit of mitochondrial respiratory complex I embedded in the inner mitochondrial membrane.
  - **NDUFV1 mutant:** NDUFV1 is a nuclear-encoded subunit of respiratory complex I that forms part of the NADH binding domain that extends into the mitochondrial matrix.
  - **ACAD9 mutant:** ACAD9 is a respiratory complex I assembly factor, and mutations within

this protein lead to a complete deficiency of fully assembled respiratory complex I.

We characterized the metabolic impacts of our respiratory complex I mutants at the cellular level by measuring the growth rates of the primary patient cells and cells acquired from several unaffected individuals of differing ages (ages 3, 22, and 40 years) (Fig. 3). We found differences in growth rate among the controls, with the fastest rate in cells from a 22-year-old unaffected male, followed by the cells from a 3-year-old unaffected male. Interestingly, the cells from the unaffected 40-year-old male grew at a considerably slower rate versus the other unaffected subjects, suggesting an age-related effect on cellular bioenergetics (Fig. 3). Among the patient cells, the ACAD9 and NDUFV1 mutant cells grew at the slowest rates while the ND6 cells exhibited growth rates that almost matched the cells from the younger 3- and 22-year-old unaffected individuals (Fig. 3). Our results indicate that ACAD9 and NDUFV1 mutations produce profound energetic defects as reflected in slower growth rates. In contrast, ND6 mutant cells may be able to compensate for the energy demands required for maintaining cell growth.

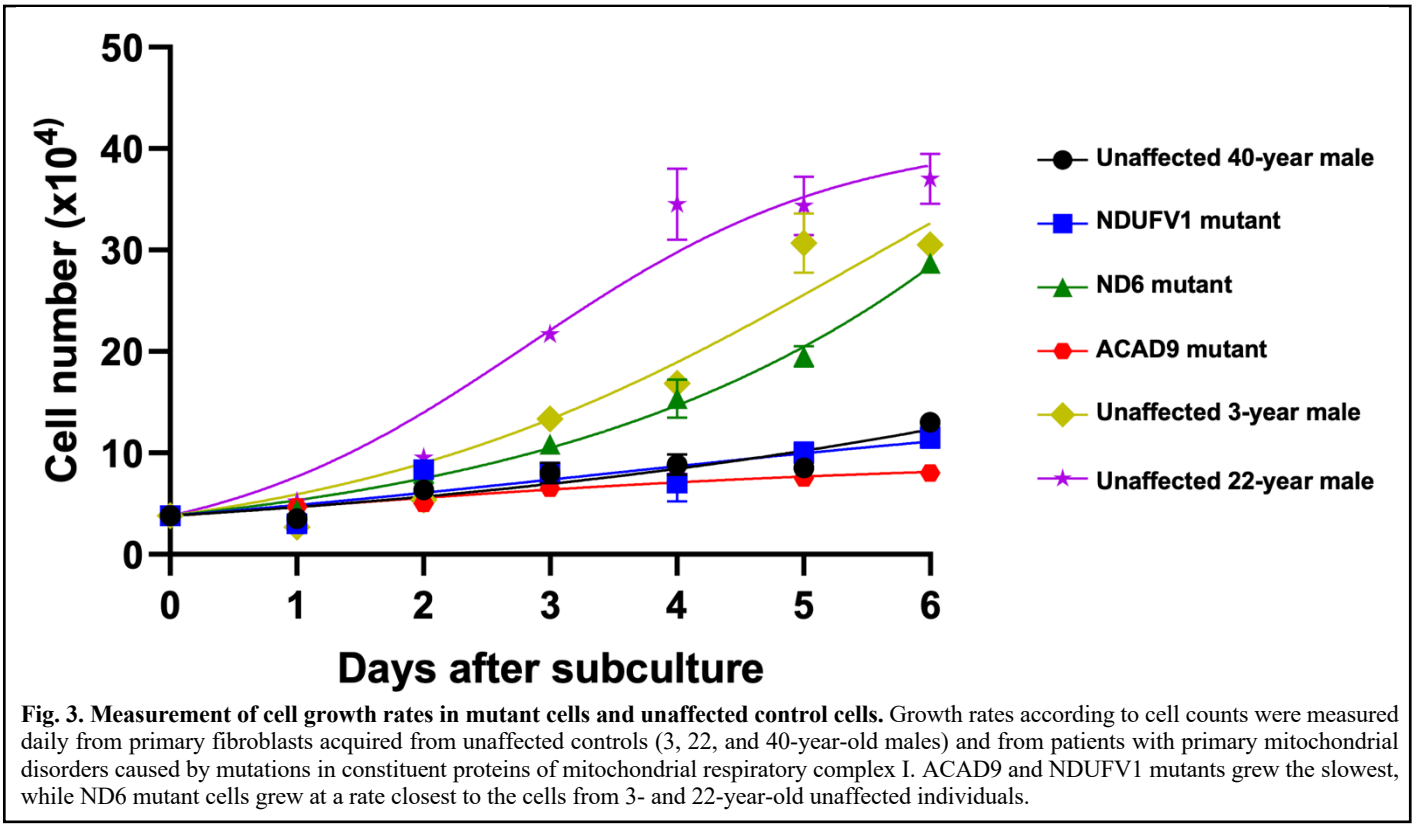

Fig. 3. Measurement of cell growth rates in mutant cells and unaffected control cells. Growth rates according to cell counts were measured daily from primary fibroblasts acquired from unaffected controls (3, 22, and 40-year-old males) and from patients with primary mitochondrial disorders caused by mutations in constituent proteins of mitochondrial respiratory complex I. ACAD9 and NDUFV1 mutants grew the slowest, while ND6 mutant cells grew at a rate closest to the cells from 3- and 22-year-old unaffected individuals.

- We conducted additional analyses of the NDUFV1, ACAD9, and ND6 mutant patient cells by examining the impact of these respective respiratory complex I mutations on mitochondrial turnover. Analysis of mitochondrial turnover significantly contributes to our understanding of the pathogenesis produced by mitochondrial disease since mitochondrial dysfunction produced by disease-causing mutations elicits a cellular compensatory response characterized by a combination of mitophagy and mitochondrial biogenesis. Loss of these compensatory processes results in the accumulation of damaged mitochondria which generate increased levels of cytotoxic reactive oxygen species and disrupt calcium homeostasis that ultimately trigger cell death. Therefore, to study mitochondrial turnover in our mutant patient cells, we employed mitoTimer, a genetically-

encoded fluorescent biosensor whose emitted fluorescence shifts from green to red in response to mitochondrial turnover. For example, a higher red/green ratio is found in older mitochondria which indicates less turnover. Alternatively, a lower red/green ratio signifies the presence of younger mitochondria because of increased turnover and mitochondrial biogenesis. Consequently, we used live-cell confocal imaging of the respective control and mutant cells expressing mitoTimer, finding that cells from patients with NDUFV1 and ACAD9 mutations had the lowest red/green ratios compared to the unaffected control cells, while ND6 did not significantly differ from our unaffected control cells (Fig. 4). Our results therefore indicate that cells with disease-causing NDUFV1 or ACAD9 mutations in respiratory complex I can compensate for these bioenergetic mutations by increasing rates of mitochondrial turnover to generate greater numbers of mitochondria within cells. Future work will examine effects of mitochondrial turnover on levels of reactive oxygen species in the respective mutant cells.

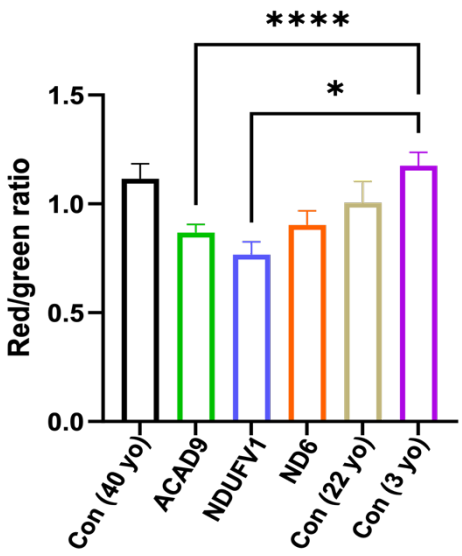

Fig. 4. Quantification of live-cell confocal imaging of mitochondrial turnover in primary fibroblast cells of mitochondrial disease patients and unaffected controls. MitoTimer, a reporter of mitochondrial turnover was expressed in NDUFV1, ACAD9, and ND6 mutant patient primary fibroblast cells as well as in cells from unaffected controls of differing ages. Compared to control cells from a 3-year-old unaffected male, there was significantly greater mitochondrial turnover in ACAD9 ($p<0.0001$) and NDUFV1 ($p<0.01$) mutant cells, as indicated by a lower red/green ratio. Data is represented a mean ± SEM.

III. Determine whether the respective mitochondrial disease-causing mutations alter the 3D structures of the mitochondrial respiratory complexes *in situ.*

- We have continued developing *in situ* cryo-ET methodologies, taking advantage of cryo-ET's much higher resolution to directly visualize effects of the disease-causing mutations on mitochondrial structure. Specifically, we have refined methods to segment the membranes of mitochondria imaged within our three-dimensional cryo-tomograms. As proof-of-principle, we succeeded in finely detailed segmentation of mitochondrial membranes imaged within the primary fibroblast cells acquired from unaffected subjects (Fig. 5). Our membrane segmentation approach allows us to accurately measure numerous aspects of mitochondrial ultrastructure including inner membrane surface area and volume, cristae length and cristae tip angles that can reveal the structural perturbations produced by disease-causing mutations. We have also applied our segmentation to the mutant cells. We have segmented

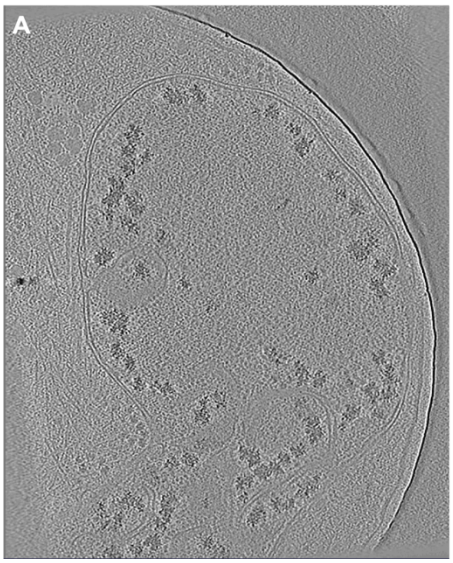

Fig. 5. Segmentation of mitochondrial structures in primary fibroblast cells from an unaffected individual. (A) Representative 2D slice from a cryo-ET tomogram acquired from a primary fibroblast derived from an unaffected 3-year-old male individual featuring a mitochondrion. **(B)** Segmentation of the mitochondrion within the cryo-electron tomogram clearly demonstrated salient ultrastructural features including outer mitochondrial membranes (in orange), as well as inner mitochondrial membranes (in dark blue) which form extensive cristae. Segmentation also enabled labeling of the volume within cristae (in light blue).

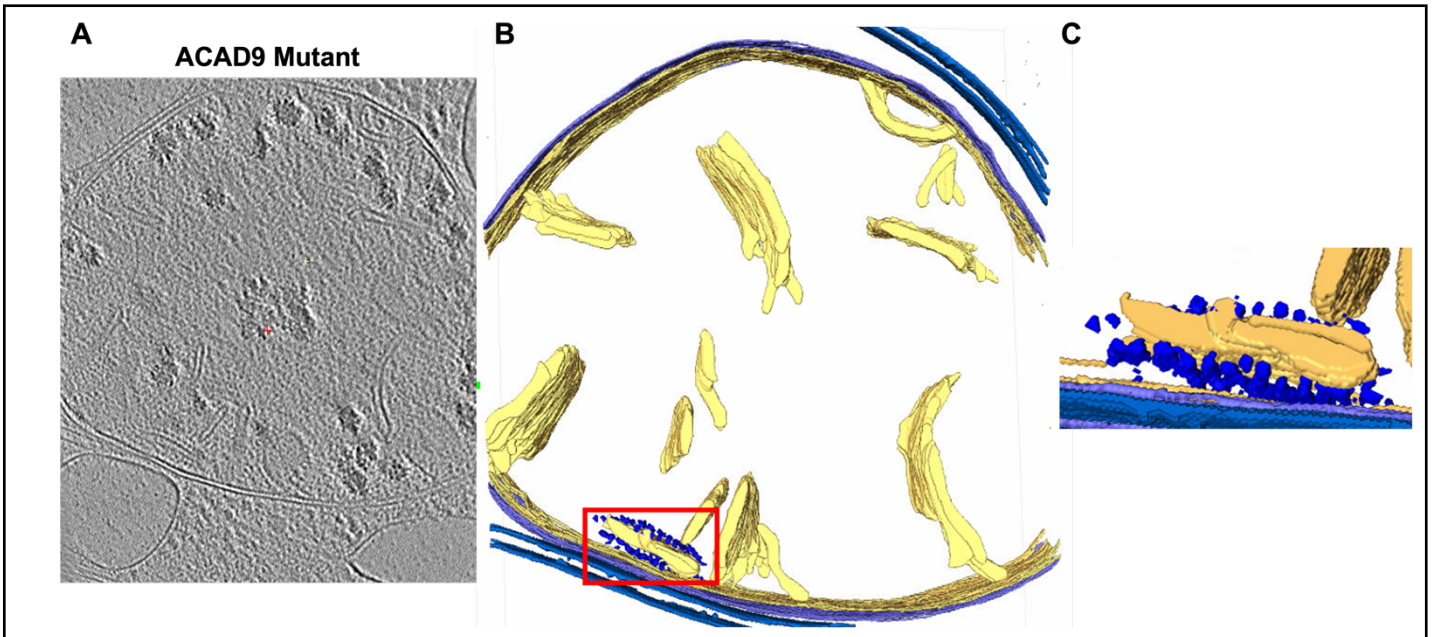

Fig. 6. Segmentation of mitochondrial structures in ACAD9 mutant primary patient fibroblast cells. (A) Representative 2D slice from a cryo-ET tomogram featuring a mitochondrion. The cryo-tomogram was acquired from a primary fibroblast derived from a patient with a mitochondrial disease-causing mutation in ACAD9. **(B)** Segmentation of 2D cryo-ET images of the mitochondrion in Panel A revealed smaller, less complex organization of cristae (in light yellow); outer mitochondrial membranes (in blue) and inner membranes (in dark yellow) remained intact. **(B)** ND6 mutant mitochondria also have large clusters of calcium phosphate granules (in yellow). **(C)** Enlarged image within the highlighted mitochondrial region from Panel B (in red). 3D segmentation of this region clearly shows a regular array of respiratory complexes (in dark blue) organized along a crista membrane.

mitochondria affected by a disease-causing ACAD9 mutation (Fig. 6). Our *in situ* cryo-ET imaging has provided sufficient detail to segment not only the cristae membranes (Fig. 6B), but also the respiratory complexes that are localized to the cristae (Fig. 6C). Next steps will be to measure disease-induced changes to the mitochondrial ultrastructure using these segmentation approaches.

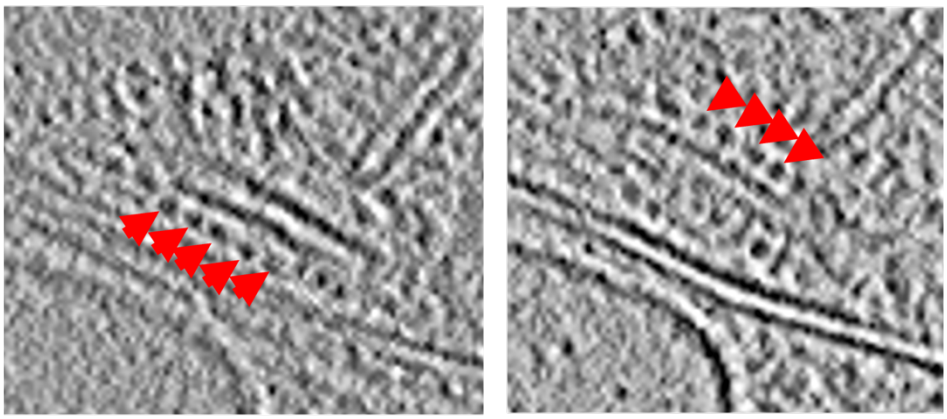

Fig. 7. Identification of mitochondrial respiratory complex structures on cristae within cryo-tomograms of primary fibroblast cells. Representative 2D tomographic slices from a 3D cryo-ET tomogram acquired from a primary fibroblast derived from an unaffected 3-year-old male. Both panels demonstrate a regular array of respiratory complexes anchored within cristae membrane with the distinctive shape of respiratory complex V (indicated by red arrowheads).

- In parallel to our analyses of mitochondrial membrane ultrastructure, we have continued developing subtomogram averaging approaches to resolve the structures of the individual respiratory complexes as well as the higher-order supercomplexes *in situ* within the patient and control mitochondria. To achieve this, we have been optimizing our *in situ* cryo-ET imaging parameters to best resolve respiratory complexes on the cristae membranes. Consequently, we succeeded in clearly visualizing regular arrays of respiratory complexes including the distinctive respiratory complex V on cristae (Fig. 7). Additionally, we have continued working with collaborator, Dr. Min Xu (Carnegie Mellon University), to test machine learning approaches for semi-automated membrane segmentation within cryo-tomograms. We have also been working with Dr. Xu to establish novel template-free structural pattern mining pipelines to facilitate unbiased detection of mitochondrial proteins and complexes extracted from our cryo-ET images.

IV. Determine whether improved respiratory chain function in patient cells following treatment with JP4-039 is via the drug's ability to correct respiratory complex structural abnormalities. We have comprehensively metabolically characterized the respective patient cells containing mitochondrial disease-causing mutations (*e.g.*, ND6, ACAD9, NDUFV1 mutants) and compared these cells to control cells

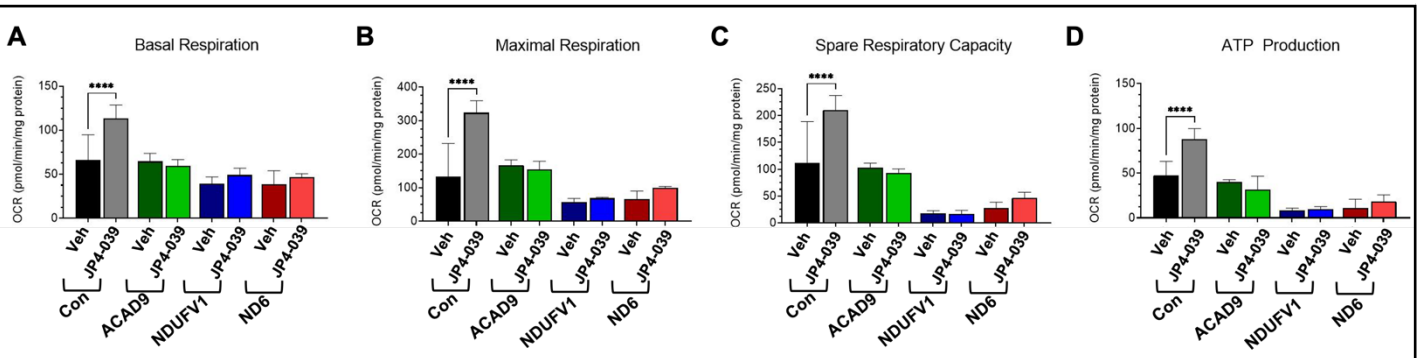

Fig. 8. Effects of JP4-039 on mitochondrial respiration in primary cells from mitochondrial disease patients and unaffected controls. Primary fibroblast cells from patients with disease-causing mutations in mitochondrial respiratory complex I proteins ACAD9, NDUFV1, or ND6 as well as cells from an unaffected subject were treated with antioxidant compound JP4-039 and compared to vehicle. Seahorse metabolic analysis measuring: **(A)** basal respiration; **(B)** maximal respiration; **(C)** spare respiratory capacity; and **(D)** ATP production revealed significant JP4-039-induced increases in all measures tested for the unaffected control cells. In contrast, JP4-039 had no significant effect on mitochondrial respiration resulting in ATP synthesis compared to vehicle ($p>0.05$).

acquired from an unaffected subject (Fig 8). Using Seahorse metabolic analyses, we found that treatment of the control cells with JP4-039 significantly increased all measures of mitochondrial respiration including basal and maximal respiration as well as spare respiratory capacity ($p<0.0001$). Consistent with this, JP4-039 boosted ATP production in the control cells. In contrast, we discovered that JP4-039 did not significantly improve respiratory function or ATP production in any of the patient cells compared to the vehicle control. Interestingly, when we measured proton leak which indicates the remaining basal respiration not coupled to ATP production, we found that JP4-039-treated significantly boosted this respiratory activity in NDUFV1 mutants compared to vehicle ($p<0.001$; Fig 9); there were no significant effects of JP4-039 on the other mutants or in the control ($p>0.05$) (Fig 9). Together, these data suggest that the structural abnormalities to mitochondrial respiratory complex I produced by the respective disease-causing mutations are sufficiently significant to prevent the JP4-039 compound from markedly boosting overall mitochondrial ATP production. On the other hand, JP4-039's effects on basal respiration in the NDUFV1 mutant outside of ATP production (*e.g.*, proton leak) suggests that that this molecule can have therapeutic value by positively impacting mitochondria function in the context of specific mitochondrial defects. Future work can explore the development of compounds that specifically target and repair the structural disruptions produced by each of the mutations studied here.

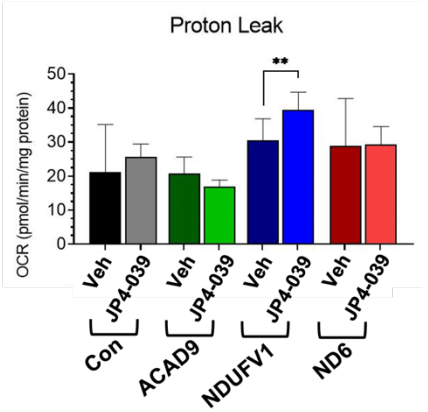

Fig. 9. Impact of JP4-039 on mitochondrial proton leak in mitochondrial disease patient cells. Seahorse measurement of proton leak from primary cells of patients with mitochondrial disease-causing mutations in ACAD9, NDUFV1, or ND6 versus cells from an unaffected control. JP4-039 significantly raised proton leak in NDUFV1 mutant cells ($p<0.001$), while all other cells demonstrated no significant effects versus vehicle ($p>0.05$).

V. Visualize mitochondrial respiratory complex structure and supercomplex organization in healthy primary human fibroblasts after pesticide or pyridostigmine bromide treatment.

- We intend on co-administering pesticide and pyridostigmine in the next 6-8 months to our healthy human fibroblasts followed by cryo-ET imaging to resolve effects of the drugs on respiratory complex structure and organization.

VI. Determine whether JP4-039 corrects mitochondrial structural abnormalities in GWI or pesticide/ pyridostigmine bromide-treated control cells.

- Our recent progress in resolving effects of the mitochondrial disease-causing mutations on mitochondrial structure and morphology in patient cells will lay the groundwork for visualizing effects of JP4-039 directly on the respiratory complex structures via cryo-ET. We expect to conduct these experiments in the next 12 months.

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

- **How were the results disseminated to communities of interest?**
  Our results have been disseminated to communities of interest at local, national, and international scientific meetings. These meetings include the United States Army Research Lab conference on Soldier Protection Against Evolving Threats (2021) and at Carnegie Mellon University's Division of Biological Science Seminar Series (2022). Additionally, results were presented as an invited speaker at the International Symposium on Endoplasmic Reticulum hosted at the University of Paris (Paris, France, 2021). Collectively, presenting recent findings stemming from this project were instrumental in advancing the idea that *in situ* cryo-ET offers a new approach towards better understanding and treating mitochondrial diseases by identifying how these illnesses alter mitochondrial structure and morphology. In presenting this work during talks, and abstracts, our findings were broadly disseminated to a broad scientific audience whose expertise spans multiple disciplines including neuroscience, cell biology, biophysics, and clinical medicine. Furthermore, in addition to our recent publication of our initial results from this project in *Structure*, a high-impact journal of structural biology, we presently have three manuscripts under preparation based on work resulting from this award. We expect to submit these manuscripts in the next 6-12 months.

- **What do you plan to do during the next reporting period to accomplish the goals?**

I. Sample preparation and imaging acquisition of three-dimensional tomograms from mitochondrial disease patient and control cells.

- We will continue culturing the primary human fibroblasts acquired from patients with well-defined mitochondrial diseases including those in complex I. In the next phase of studies, the respective fibroblast cells will be pre-treated with either JP4-039 or vehicle control followed by immediate plunge-freezing and vitrification of the samples.
- Once frozen, we will continue imaging the respective EM grids by *in situ* cryo-ET to obtain three-dimensional cryo-tomograms for further analysis of the patient and control cells.

II. Imaging Analysis of imaged mitochondria and respiratory complexes from well-defined mitochondrial disease mutation-containing patient and control fibroblasts.

- We will continue working with consultant Dr. Min Xu to develop new computational approaches using machine learning to recognize 3D respiratory complex structures *in situ* in mitochondria within patient and control cells. Structures from our data sets will be compared to high resolution structures of the respiratory complexes. These approaches will include auto-picking algorithms to accelerate the recognition and selection of the protein components of the mitochondrial respiratory complexes.
- We will continue segmenting structures within disease-altered complexes as well as the membrane organization (*i.e.*, cristae, inner and outer membranes) and then place the segmented structures back into tomograms to determine spatial relationships between interacting proteins and membranes.
- We will continue to focus on structural determination of supercomplex organization within the patient and control cell mitochondria.

2. **Determine whether the respective mitochondrial disease-causing mutations alter the 3D structures of the mitochondrial respiratory complexes *in situ.***
    - We will continue developing our computational pipelines to enable accurate particle-picking and subtomogram extraction to better resolve the respiratory complex structures directly affected by disease-causing mutations. Such automated, reference-free approaches as we are developing will ultimately replace laborious manual particle-picking and eliminate potential bias by human pickers. Additionally, as part of our collaborative efforts with Dr. Xu, we will further continue improving feature discrimination to optimize recognition of distinct protein confirmations/interactions by training an autoencoder model to denoise raw tomographic images and build a feature map featuring distortion-free enhanced images optimized for protein recognition. In parallel, we will continue optimizing the data acquisition parameters (*e.g.*, tilt schemes on the cryo-TEM microscope, total electron dose per tilt, use of a phase plate) to further improve the potential resolution of the complexes within the tomograms.

3. **Determine whether improved respiratory chain function in patient cells following treatment with JP4-039 is via the drug's ability to correct respiratory complex structural abnormalities.**
    - We will image patient and control fibroblasts with JP4-039 via *in situ* cryo-ET, to ascertain the effects of the drug directly on the 3D structures of the affected respiratory complexes at subnanometer resolution.

4. **Visualize respiratory complex structure and supercomplex organization in healthy primary human fibroblasts after pesticide or pyridostigmine bromide treatment.**
    - Once we complete acquiring 3D tomographic data of patient and control human fibroblasts in the next 6-8 months, we will treat primary cells healthy controls with the pesticide paraquat. In parallel, we will treat these healthy control cells with the pesticide rotenone which specifically targets respiratory complex I. Use of the rotenone will allow us to compare and contrast paraquat's effects specifically on the structural organization of complex I as well as on higher order supercomplex organization. We will also treat a set of the control cells with pyridostigmine followed by their cryo-ET imaging. Finally, we plan on ascertaining whether these respective toxicant exposures produce structural differences different from those found in the patient cell lines both in terms of the individual respiratory complex 3D structures, as well as on the supercomplexes. In the event that the resolution is insufficiently high *in situ*, we will image purified mitochondria from treated cells to decrease background from the crowded cell environment and thus better resolve the affected complexes.

5. **Determine whether JP4-039 corrects mitochondrial structural abnormalities in GWI or pesticide and pyridostigmine-treated control cells.**
    - In the coming months, we will apply JP4-039 to our GWI samples as well as to healthy control fibroblasts pre-treated with either paraquat or pyridostigmine, followed by plunge-freezing and cryo-ET imaging. This will be followed by subtomogram averaging to determine whether JP4-039 improves the structural alterations evident in the respiratory complexes. We will then compare the respiratory complex structures in JP4-039-treated cells versus those treated with vehicle.

4. IMPACT

- **What was the impact on the development of the principal discipline(s) of the project?**

Our results have been widely disseminated to communities of interest at local, national, and international scientific meetings. These meetings have included the Annual Meeting of the American Society of Cell Biology (2020), Annual American Physical Society Meeting (2021), International Symposium on Endoplasmic Reticulum (2021), and the United States Army Research Lab Conference on Soldier Protection

Against Evolving Threats (2021). Additionally, results have been presented as an invited speaker at Weill Cornell Medical College's Department of Biochemistry (2020) as well as an invited speaker at Carnegie Mellon University's Division of Biological Science's Seminar Series (2022). Collectively, presenting recent findings stemming from this project were instrumental in advancing the idea that *in situ* cryo-ET offers a new approach towards better understanding and treating mitochondrial diseases by identifying how these illnesses alter mitochondrial structure and morphology. This has begun generating considerable interest within the greater scientific community.

- **What was the impact on other disciplines?**
  In presenting results from this project, our findings have been broadly disseminated to a diverse scientific audience whose expertise spans multiple disciplines including neuroscience, cell biology, microscopy, and clinical medicine. In the longer-term, appealing to a broader audience fosters new knowledge that leads to development of new, highly targeted therapies for active military, Veterans, beneficiaries, and the general population that are directed at correcting structural changes responsible for mitochondrial dysfunction. Ultimately, such a development could significantly reduce serious morbidity and mortality from mitochondrial diseases and its resulting neurological and cardiovascular consequences. Since current treatments only address disease symptoms, our project's development of methods to directly visualize mitochondrial disease-causing mutations and their effects on the mitochondrial architecture may produce new personalized interventions to treat underlying causes unique to each patient, significantly improving patient care for mitochondrial disorders. Such treatments may also boost mitochondrial function in healthy individuals and thus could be used as tools to increase fitness of active military personnel especially in combat situations. Moreover, our personalized approaches to visualizing mitochondrial disorders can lead to fundamental insights into the mechanisms for development of these illnesses and may even be applied to the study of other diseases.

- **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

There have been no changes in the scope of work since the last reporting periods and therefore the SOW remains the same as originally defined.

6. PRODUCTS

- **Publications, conference papers, and presentations**

Journal publications
We have 3 manuscripts in preparation based on the work resulting from this award and expect to submit them in the next 6-12 months.

Books or other non-periodical, one-time publications
Nothing to report.

Other publications, conference papers, and presentations
Data based on the studies originally proposed for this award were presented at the following meetings:

1. Ribosome-Associated Vesicles: a dynamic sub-compartment of the endoplasmic reticulum. Invited talk presented at the International Symposium on Endoplasmic Reticulum (2021), University of Paris, Paris, France
2. Mitochondrial cryo-electron tomography. Invited talk presented at the United States Army Research Lab Conference on Soldier Protection Against Evolving Threats (2021), Pittsburgh, PA

Additionally, Dr. Freyberg was an invited speaker at the following seminars where he presented the work produced from this funded work:

1. Invited speaker, Division of Biological Sciences seminar series, Carnegie Mellon University, Pittsburgh, PA; 2022

- **Website(s) or other Internet site(s)**
  Nothing to Report.

- **Technologies or techniques**
  Nothing to Report.

- **Inventions, patent applications, and/or licenses**
  Nothing to Report.

- **Other Products**
  Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

• Name:	Zachary Freyberg M.D., Ph.D.
• Project Role:	Principal Investigator
• Researcher Identifier (*e.g.* ORCID ID):	ORCID ID: 0000-0001-6460-0118
• Nearest person month worked:	1.8
• Contribution to Project:	Dr. Freyberg has designed and analyzed all experimental data concerning both the live-imaging and *in situ* cryo-ET microscopy of mitochondria from cells taken from patients with mitochondrial disease and associated controls.
• Funding Support:	DoD Peer Reviewed Medical Research Program Discovery Award (PR192466); PA Tobacco Formula Fund, NIH/NIA R21 Award (R21AG068607), NIH NIDA CEBRA Award (R21DA052419), NIH/NIGMS R01 (R01GM134020), NIH/NIDDK R01 (R01DK109907), NIH/NHLBI/NIA R01 supplement (R01HL150432 Supplement)

• Name:	Jill Glausier, Ph.D.
• Project Role:	Co-Investigator
• Researcher Identifier (*e.g.* ORCID ID):	ORCID ID: 0000-0001-9838-3414
• Nearest person month worked:	0.6

• Contribution to Project:	Dr. Glausier has analyzed the tomographic data to successfully build three-dimensional maps of the imaged cells. She will use her expertise in mitochondrial assays to validate the structural findings concerning complex organization with biochemical assays on patient mitochondria including blue native gels and clinical electron transport chain analysis.
• Funding Support:	DoD Peer Reviewed Medical Research Program Investigator-Initiated Research (PR192466); NIH/NIDA (N0175N95019C00047), NIH/NIDA (DA051390), NIH/NIMH (R21MH125012)

• Name:	James Conway, Ph.D.
• Project Role:	Co-Investigator
• Researcher Identifier (*e.g.* ORCID ID):	ORCID ID: 0000-0002-6581-4748
• Nearest person month worked:	0.6
• Contribution to Project:	Dr. Conway has provided technical expertise and assistance with imaging and data analysis.
• Funding Support:	DoD Peer Reviewed Medical Research Program Investigator-Initiated Research (PR141292), NIH/NIAID (R01AI089803)

• Name:	Jiying Ning, Ph.D.
• Project Role:	Research Associate
• Researcher Identifier (*e.g.* ORCID ID):	N/A
• Nearest person month worked:	3.3
• Contribution to Project:	Dr. Ning has maintained the patient and control primary human cells (*e.g.*, primary fibroblasts) used in the study as well as optimized the sample preparation methodologies.
• Funding Support:	Department of Defense Peer Reviewed Medical Research Program Discovery Award (PR192466), PA Tobacco Formula Fund, NIH/NIGMS R01 (R01GM134020), NIH/NIDDK R01 (R01DK109907)

• Name:	Alexander Makhov, Ph.D.
• Project Role:	Facility Manager
• Researcher Identifier (*e.g.* ORCID ID):	N/A
• Nearest person month worked:	0.6
• Contribution to Project:	Dr. Makhov has assisted with data collection for the proposed cryo-electron microscopy and tomography data. He maintains the cryo-electron microscopes in an ongoing manner and conduct microscope repairs.
• Funding Support:	Department of Defense Peer Reviewed Medical Research Program Discovery Award (PR192466)

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

There has been no change in the active other support of the PI or senior/key personnel since the last reporting period.

- **What other organizations were involved as partners?**

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

- **Collaborative Awards**

Not Applicable.

9. APPENDICES

Not Applicable.