AWARD NUMBER: W81XWH-17-1-0140

TITLE:

Engineering a Minimal Competency Machinery for Mitochondria

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REPORT DATE:

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command

Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
March 2019	Final Report	1 Jun 2017 - 30 Nov 2018

4. TITLE AND SUBTITLE
Engineering a Minimal Competency Machinery for Mitochondria

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-17-1-0140

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

In our study, we will first engineer nucleic acid uptake for the organelle by introducing bacterial competency components targeted to the appropriate sub compartment of the mitochondrion. Using evolutionarily-related Gram-negative bacterial species as model systems, we will identify the minimal competency unit for the mitochondrion. We will then perform mitochondrial genome editing by introducing small guide RNAs and exogenous DNA sequences to the organelle for use by the CRISPR/Cas9 system. Together, we expect to develop a technique allowing for mitochondrial genome editing, which will lay the foundation for the future development of animals harboring human pathogenic mitochondrial DNA mutations. Having relevant and faithful disease models will provide new insights into the pathophysiology of disease, as well as the development of therapeutics.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified		19b. TELEPHONE NUMBER *(include area code)*

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

This research addresses the FY16 PRMRP Topic Area "Mitochondrial Disease." Mutations in the mitochondrial genome result in progressive and untreatable diseases in humans. A major barrier to developing accurate models is our complete inability to engineer precise mutations in the mitochondrial genome. This fundamental gap is due to the lack of a method to introduce nucleic acid templates into the organelle. Bacteria, the evolutionary ancestors of mitochondria, have evolved machinery for the import and recombination of exogenous DNA, termed "competency." We hypothesize that the introduction of bacterial "competency" components to the mitochondrion may be sufficient to allow for regulated entry of DNA/RNA species into the organelle. In our study, we will first engineer nucleic acid uptake for the organelle by introducing bacterial competency components targeted to the appropriate sub compartment of the mitochondrion. Using evolutionarily-related Gram-negative bacterial species as model systems, we will identify the minimal competency unit for the mitochondrion. We will then perform mitochondrial genome editing by introducing small guide RNAs and exogenous DNA sequences to the organelle for use by the CRISPR/Cas9 system.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Mitochondrial DNA, competency, comEC, comF

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Task	Timeline	% completion
Major Task 1: Specific Aim 1A		
Subtask 1: Synthesis of expression constructs.	June – Aug 2017	100
Subtask 2: Testing of expression constructs for localization	Sep – Nov 2017	100
Major Task 2: Specific Aim 1B		
Subtask 1: Testing competency constructs for DNA import	Dec 2017 – May 2018	100
Major Task 3: Specific Aim 2A		
Subtask 1: Testing Cas9-nuclease function	June – Aug 2018	100
Subtask 2: Testing Cas9-nuclesae function (heteroplasmy)	June - Aug 2018	100
Major Task 4: Specific Aim 2B		
Subtask 1: Testing for Cas9-correction of mtDNA mutations.	Sep – Nov 2018	100
Subtask 2: Testing for Cas9-creation of mtDNA mutations.	Sep – Nov 2018	100

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

1.) Major activities:

-Identification of bacterial competency genes from > 50 species.
-Design and construction of bacterial competency genes for mammalian expression and mitochondrial localization.
-Testing of bacterial competency genes for expression and proper localization in mammalian cell lines.
-Testing of bacterial competency genes for import of exogenous nucleic acids.
-Construction of mitochondrial localized Cas9 constructs (mitoCas9).
-Expression and localization of mitochondrial localized Cas9 constructs.
-Testing of bacterial competency genes to mediate mitoCas9-dependent nuclease function.
-Testing of bacterial competency genes to mediate mitoCas9-dependent mtDNA editing.

2) Specific Objectives:

-Construction of codon-optimized constructs for mammalian expression of bacterial competency genes.
-Testing engineered bacterial competency genes for expression.
-Testing engineered bacterial competency genes for nucleic acid uptake into mitochondria.
-Construction of mitoCas9-expressing cell lines.
-Testing engineered bacterial competency genes for mitoCas9-dependent nuclease activity.
-Testing engineered bacterial competency genes for mitoCas9-dependent editing.

3) Significant results or key outcomes or conclusions:

A) Design and construction of competency constructs:

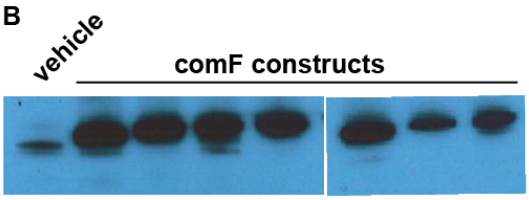
Through literature and homology searches, we identified deposited protein sequences for competency proteins from ~50 Gram-negative bacterial species (Fig. 1A). Highly-similar sequences (>90% identity) were de-duplicated. After automated codon optimization and manual confirmation, we synthesized and cloned constructs encoding components of the nucleic acid import machinery, specifically comEC and comF proteins. We created a modular cloning strategy using site-specific endonucleases which allowed us to attach multiple mitochondrial targeting sequences in parallel, using pcDNA3.1 expression as a backbone. C-terminal myc tags were added to all constructs to aid in localization by immunofluorescence. Construct identity was verified by restriction digest and Sanger sequencing. With this, we have created a library of codon-optimized and mitochondrial targeted bacterial nucleic acid channels for testing in mammalian cells.

B) Testing of bacterial competency genes for expression and proper localization in mammalian cell lines.

We varied transfection conditions to optimize expression of comF proteins in HEK293 cells. Lipofectamine-mediated transfection was sufficient to generate robust expression in >90% of tested constructs (Fig. 1B). We then used immunofluorescent staining to verify mitochondrial localization (Fig. 1C). ~50% of constructs demonstrated robust mitochondrial localization.

A

Species name	Chromosome Accession
Staphylococcus aureus Mu50	BA000017.4
Bacillus licheniformis DSM 13	AE017333.1
Bacillus subtilis 168	AL009126.3
Bacillus amyloliquefaciens FZB42	CP000560.1
Lactobacillus sakei 23K	CR936503.1
Leuconostoc carnosum JB16	CP003851.1
Streptococcus mutans UA159	AE014133.2
Streptococcus thermophilus	CP000419.1
Streptococcus salivarius JIM8777	FR873482.1
Streptococcus infantarius CJ18	CP003295.1
Streptococcus macedonicus ACA-DC	HE613569.1
Streptococcus oralis Uo5	FR720602.1
Streptococcus pneumoniae R6	AE007317.1
Streptococcus mitis B6	FN568063.1
Streptococcus intermedius JTH08	AP010969.1
Streptococcus anginosus SK1138	
Streptococcus cristatus	
Streptococcus sanguinis SK36	CP000387.1
Streptococcus gordonii Challis	CP000725.1
Streptomyces virginiae[b] *(spp.) S*	CP002993.1
Thermosynechococcus elongatus BP-	BA000039.2
Synechocystis spp. PCC6803	BA000022.2
Synechococcus elongatus PCC 6301	AP008231.1
Chlorobium limicola DSM 245	CP001097.1
Chlorobium tepidum TLS	E006470.1;AL646053.
Deinococcus radiodurans R1	AE000513;AE001825
Thermus thermophilus HB27	AE017221.1
Ralstonia solanacearum GMI1000	AL646052.1
Neisseria meningitidis MC58	AE002098.2
Neisseria gonorrhoeae FA 1090	AE004969.1
Kingella kingae ATCC 23330	
Kingella denitrificans ATCC 3339	
Xylella fastidiosa M12	CP000941.1
Legionella pneumophila Philadelph	AE017354.1
Pseudomonas fluorescens Pf0-1	CP000094.2
Pseudomonas stutzeri A1501	CP000304.1
Azotobacter vinelandii DJ; ATCC B	CP001157.1
Pseudomonas mendocina ymp	P000680.1;AE003853.
Vibrio fischeri ES114	000020.2; CP000021
Vibrio cholerae N16961	AE003852.1
Vibrio vulnificus CMCP6	016795.3 ;AE016796
Vibrio spp. EX25	001805.1; CP001806
Escherichia coli[c] *K-12*	U00096.2
Gallibacterium anatis UMN179	CP002667.1
Actinobacillus suis H91-0380	CP003875.1
Actinobacillus pleuropneumoniae L	CP000569.1
Haemophilus parasuis SH0165	CP001321.1
Haemophilus influenzae Rd KW20	L42023.1
Haemophilus parainfluenzae T3T1	FQ312002.1
Aggregatibacter aphrophilus NJ870	CP001607.1
Aggregatibacter actinomycetemcomi	CP001733.1



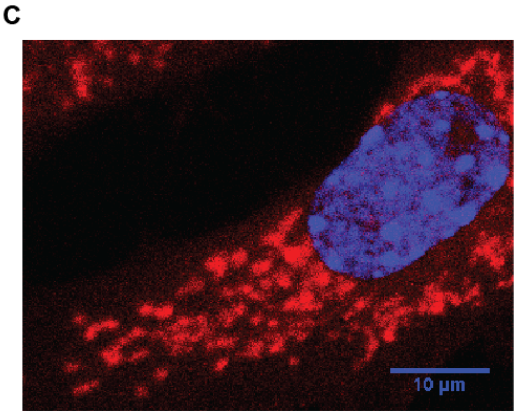


Figure 1: A) List of Gram-negative baceterial species with identifiable competency components. B) Western blot testing expression of comF constructs after transfection into HEK293 cells reveals robust expression in most constructs. Detection: α-myc (clone 9E10). C) Immuno-fluorescence detection of comF expressing construct in HEK293 cells reveals mitochondrial localization. Blue: DAPI; Red: α-myc (clone 9E10).

comEC proteins displayed much poorer expression levels in HEK293 cells and we were unable to detect expressed and targeted protein by western blot (Fig. 2A). Unlike comF proteins which were targeted to the mitochondrial matrix, comEC constructs were designed for integration into the mitochondrial inner membrane (since comEC proteins comprise the physical protein channel which mediates nucleic acid transfer across a membrane). Unlike matrix-targeting sequences, mitochondrial inner membrane targeting sequences are not particularly well characterized in the literature, and their efficiency in targeting orthologous proteins is not particularly well-established.

We therefore re-designed a series of expression vectors containing 8-distinct inner membrane targeting sequences from known mitochondrial inner membrane proteins (including Opa1, AIF, Oxa1L, Yme1L, Oma1). Our ~50 comEC constructs were transferred into each of these 8 vectors (~400 constructs total), and re-tested for expression. Of these, only a small subset of ~1% displayed detectable low expression consistent with expected size (Fig. 2B). Immunofluorescence was unable to detect expression of these construct in stained cells. We screened these constructs for their ability to uptake Cy3-labeled oligonucleotides, and unfortunately were not able to detect any significant mitochondrial uptake (Fig. 2C).

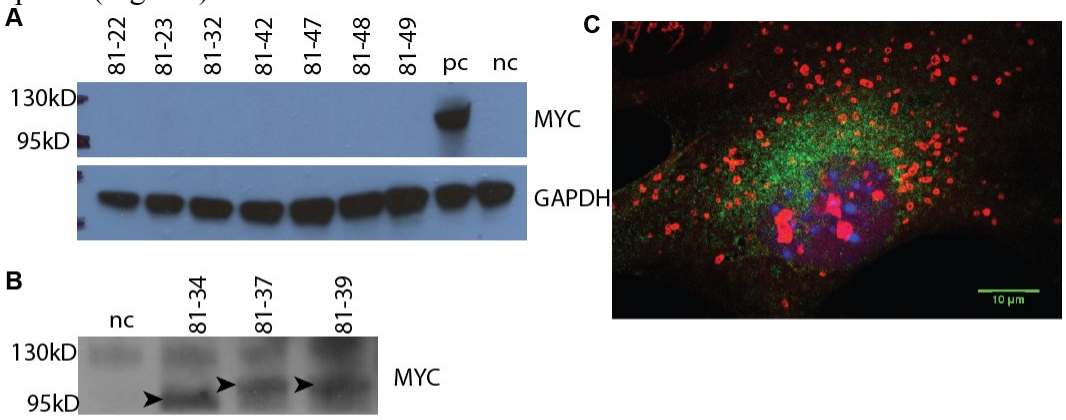


Figure 2: A) Expression screening by Western blot of a subset of comEC targeted constucts (81-xx). p.c., positive control myc-construct. n.c., negative control. GAPDH staining shown as a loading control. Detection: α-myc (clone 9E10). B) Western blot expression levels of comEC constructs displaying low expression levels. Arrowheads point to prtn bands of expected molecular weight. n.c., negative control. C) Immunofluorescence detection of nucleic acid (Cy3; green) uptake into mitochodria (MitoTracker Red; red). Blue: DAPI.

C) Creating MTS-Cas9 expressing cell lines.

We carried on with experiments to address Cas9-activity in mitochondria, under the hypothesis that the above low-expressing comEC constructs may mediate small levels of nucleic acid uptake. To achieve this, we first created a mitochondrial-targeted Cas9 (MTS-Cas9) construct. A mitochondrial targeting sequencing from Cox8a was appended to the 5'-end of the *S.pyogenes* Cas9 cDNA, and the resulting fragment was cloned into a retroviral mammalian expression vector (pQCXIP). Transient transfection experiments revealed robust expression in HEK293 cell lines (Fig. 3A). Retroviral particles for MTS-Cas9 expression were used to transduce HEK293 cells followed by puromycin selection. Immunofluorescent analysis revealed clear mitochondrial localization of Cas9 in the stably transduced cell line (Fig. 3B).

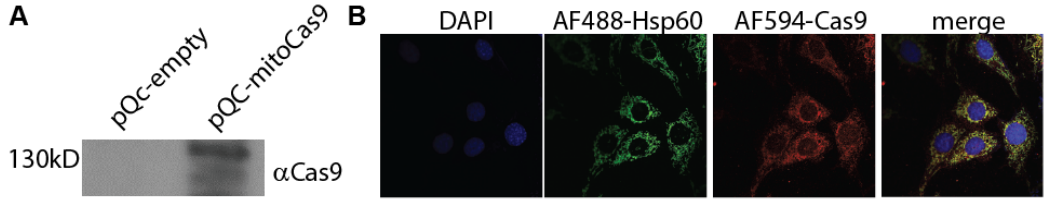


Figure 3: A) Expression levels of Cas9 (detected by western blot) for HEK293 cells transfected with the indicated construct. B) Immunolocalization of mitoCas9. Mitochondria are visualized by staining against Hsp60 (green). DAPI, nuclei. AF488: Alexafluor 488. AF594: Alexafluor 594.

D) Testing bacterial competency genes for MTS-Cas9-dependent nuclease activity.

We first designed small guide RNAs (sgRNAs) targeting multiple regions of the mitochondrial genome and tested their ability to mediate target cleavage. To test sgRNA efficiency, we cloned target regions (e.g., from Cox2) into the 5'region of a BFP (blue fluorescent protein) expressing construct, and co-transfected with U6 promoter driven sgRNA constructs into Cas9-expressing HEK293 cells (Fig. 4A). In this setup, cleavage of the target sequence will disrupt BFP expression, while YFP expression marks transfected cells. Image analysis confirmed that BFP expression was selectively inhibited in experiments using mito-targeted sgRNAs (but not non-targeting sgRNAs) (Fig. 4B).

We next transfected sgRNA constructs plus our bacterial competency constructs into mitoCas9 expressing cell lines, and selected transfected cells by FACS analysis (Fig. 4C). In this experiment, mitoCas9 cleavage activity on the genome should be detected by depletion of mitochondrial DNA content (measured by qPCR). As a positive control, a mitochondrial targeted nuclease (EcoRI) was transfected, which reliably depleted mtDNA levels by >500 fold (Fig. 4D). We transfected HEK293T cells expressing either Cas9 or MTS-Cas9 with bacterial competency constructs and COX2-targeted sgRNAs, and measured mtDNA levels by qPCR (Fig. 4E). Our results did not indicate any mtDNA-directed nuclease activity, indicating that the comEC constructs do not aid sgRNA uptake and stimulatd Cas9 activity in the mitochondrion.

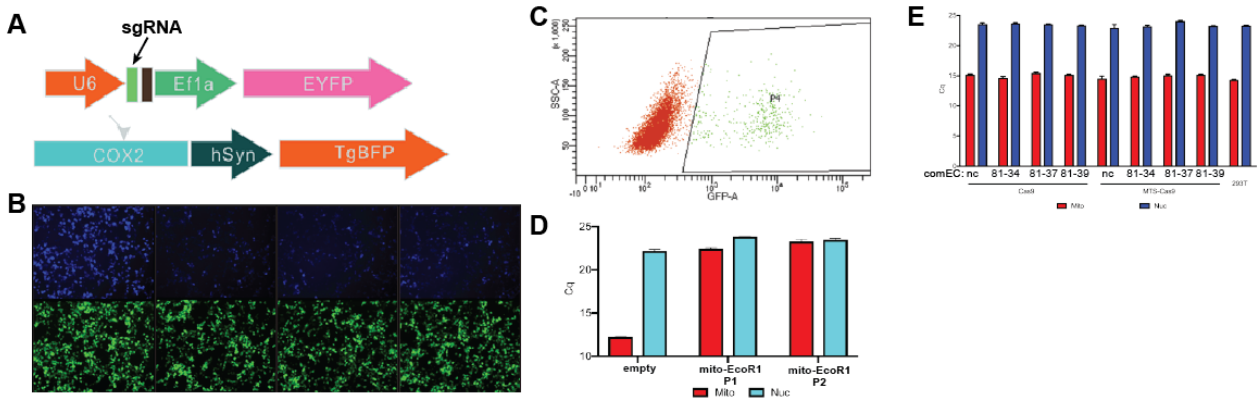


Figure 4: A) Construct design to test sgRNA - target efficiency in vivo. U6-promoter driven sgRNA plasmids are co-transfected with a BFP expressing plasmid containing the 5'-targeting region. In the presence of Cas9, successful clevage of the target region should result in decreased BFP expression. B) BFP (blue) and YFP (green) images of Cas9-expressing HEK293T cells transfected with nt (non-targeting) or mito-targeted (COX2, ND1, ND4) sgRNAs and target regions. C) FACS analysis to sort GFP+ cells (indicative of transfection with sgRNA expressing plasmids). D) Mito genome and nucleaer (Nuc) genome levels in cells transfected with the indicated constructs, post-FACS. The y-axis (Cq) represents the threshold cycle number from qPCR analysis; lower Cq values represent higher genome copy number. (E) Same as (D), but in Cas9 or MTS-Cas9 expressing cells transfected with the indicated competency construct plus a COX2-targeted sgRNA.

We also tested the ability of our competency constructs to mediate heteroplasmy shifts; in this scenario, MTS-Cas9 nuclease activity on a particular mtDNA haplotype should mediate a relative shift in heteroplasmy (via depletion of the targeted haplotype). We first designed a droplet digital PCR (ddPCR) assay targeting wild-type mtDNA (…GCCC**T**GGC) and the NARP ATP6 mutant (T8993G: GCCC**G**GGC) which creates a novel PAM site for SpCas9 function. Fluorescently quenched probes were designed and synthesized to specifically detect each DNA species, and were validated by mixing pure mitochondrial genomes in various ratios (Fig. 5A,B).

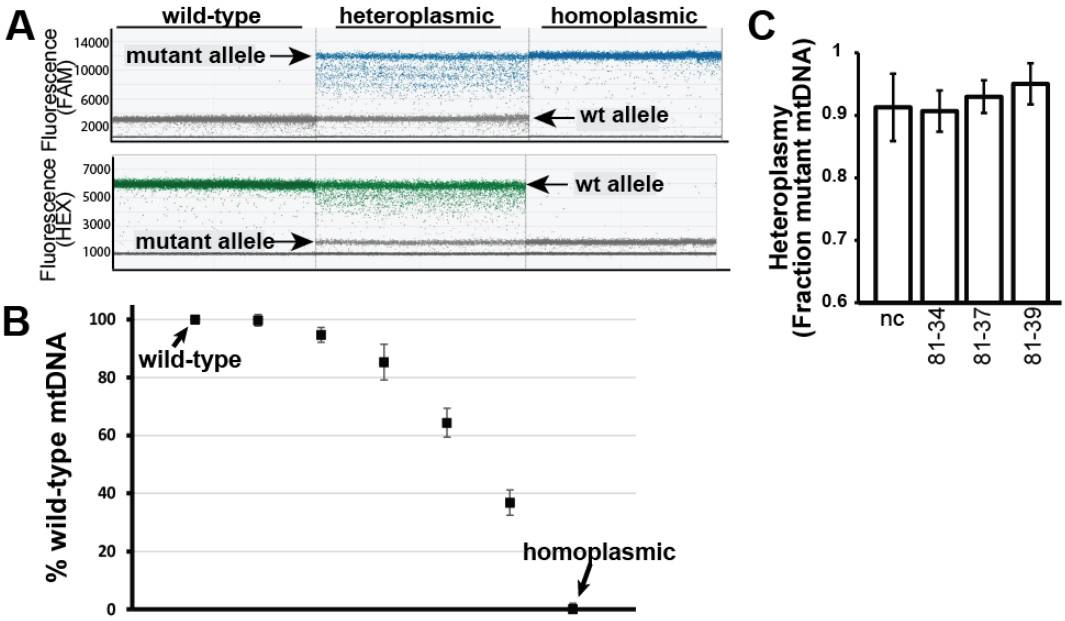


Figure 5. A) Representative ddPCR profiles measuring wild-type (HEX fluorescence) and ATP6 T893G mutant (FAM fluoresence) using target FAM/HEX-labeled oligonucleotide probes. Counting of positvie droplets within each profile allos accurate quantitation of heterplasmy in a genomic DNA sample. B) ddPCR allows quantitation over a large range of heteroplasmy levels. Data is shown from heteroplasmic samples for the ATP6 T8993G mutation. C) Heteroplasmy levels from heteroplasmic T8993G mutant cells transfected with MTS-Cas9, T8893G-targeted sgRNA, and the indicated competency construct or negative control (nc). No significant changes in heteroplasmy levels were detected. Experiments performed in triplicate; error bars indicate standard deviations.

We obtained heteroplasmic NARP cells containing ~90% levels of mutant T8993G DNA from the Coriell repository and transfected them with mito-Cas9, T8993G-targeted sgRNA, and the indicated competency constructs (Fig. 5C). Heteroplasmy levels were measured by ddPCR following FACS sorting (for transfected cells) and 48hrs of culture. Cells transfected with our construct retained ~90% heteroplasmic levels, and were not significantly different from control transfected cells. Thus, we do not see evidence of heteroplasmy shifts mediated by mito-Cas9 and our designed competency constructs.

E) Testing bacterial competency constructs for MTS-Cas9 dependent genome editing.

We first attempted to correct pathogenic mutations in homoplasmic cell lines. We obtained 143B cell lines homoplasmic for the NARP T8993G mutation, and transfected them with mito-Cas9, T8993G-targeted sgRNA, competency constructs, as well as a linear ssDNA spanning the gRNA targeting region but containing wild-type sequence. Transfected cells were selected based on YFP fluorescence (as described earlier), and cultured in either glucose or galactose-containing media. Since homoplasmic NARP mutant cells are incompetent for growth in galactose-media, we surmised that that this condition will select for cells with partial mitochondrial function, and therefore enrich for low-frequency editing events. Unfortunately, we were unable to recover transfected cells following selection in galactose media, indicating that no appreciable editing events had occurred. Consistent with this, sequencing of unselected transfected cells (i.e., cultured in glucose-media) did not reveal any evidence of editing events (Fig. 6A).

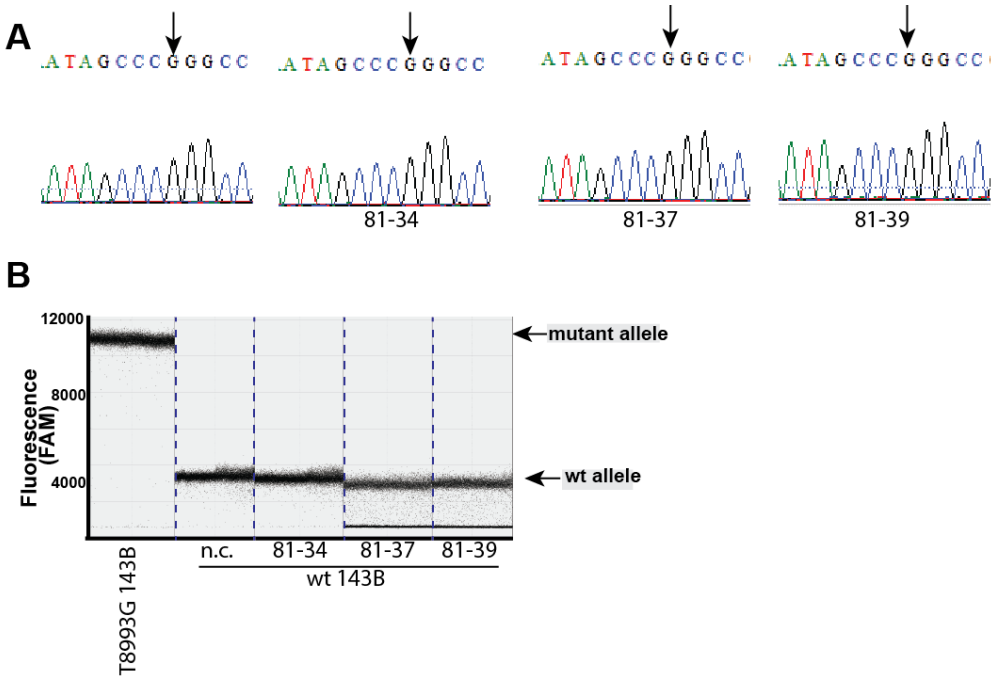


Figure 6. A) Sanger sequencing of the ATP6 gene from homoplasmic T8993G mutant 143B cells transfected with MTS-Cas9, T8993G-targeted sgRNA, wt ATP6 ssDNA, and the indicated competency construct or negative control (n.c.). The arrow indicates position 8993. B) ddPCR profiles of wild-type 143B cells transfected with MTS-Cas9, ATP6-targeted sgRNA, T8993G ssDNA, and the indicated competency construct or negative control (n.c.). Profile from T8993G cells are shown as a positive control.

We also attempted to create do novo pathogenic mutations at multiple sites in a wild-type mitochondrial genome. In these experiments, we transfected wild-type 143B cells with MTS-Cas9, mtDNA-targeted sgRNAs, our competency constructs, as well as linear ssDNA containing known human pathogenic mtDNA mutations (including ATP6 T8993G, and ND1 G3460A). Transfected cells were selected by FACS sorting, followed by 48 hours of culture. Since no selection scheme is available for mutant mtDNA haplotypes, we utilized ddPCR assays which have reported sensitivity rates on the range of 1:10,000. We did not observe detection of editing events in either controls or cells transfected with our competency constructs (Fig. 6B).

E) Summary/Discussion.

As a high-risk discovery award, it was certainly possible that our ultimate goal of achieving nucleic acid uptake into mitochondria would not be successful as initially outlined. We successfully synthesized and cloned competency constructs from all known competent bacterial species for expression and localization to mammalian mitochondria. While many constructs exhibited robust expression in mammalian cells, we found that expression of the comEC family was particularly difficult on the human mitochondrial inner membrane. The comEC family encodes the critical machinery of competency, namely the protein channel which mediate nucleic acid transfer across membranes. Through optimization and screening of inner membrane targeting sequences, we identified a few constructs that displayed low expression levels and carried forward with these sequences for functional experiments. However, we were unable to detect nucleic acid uptake mediated by the identified constructs, and we found no evidence of mitoCas9 activity in numerous assays and selection schemes testing nuclease activity, heteroplasmy shifts or mtDNA editing. We suspect that the lack of functional success is due to the difficult nature of reconstituting comEC channels on the mitochondrial inner membrane, and this is primarily reflected by the poor expression levels obtained in the heterologous mammalian system. Thus, future efforts will need to focus on the identification and engineering of a well-expressing comEC construct which can efficiently mediate nucleic acid uptake.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Professional development:
Participation in conferences/seminars:

Seminar series:

1.) Green Center Supergroup meeting (UT Southwestern Medical Center), invited oral presentation, June 2018.

2.) World Muscle Society meeting, invited oral presentation (June, 2018).

Training activities:

1) Development of high-sensitivity assays to detect mtDNA editing events, including quantitative PCR, FACS analysis and droplet digital PCR assay. Staff in the Mishra laboratory were trained to devleop and perform these assays.

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Seminar series:

1.) Green Center Supergroup meeting (UT Southwestern Medical Center), invited oral presentation, June 2018.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Nothing to Report (Final Report).

4. **IMPACT:** D*escribe 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?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Import of nucleic acids into the mitochondrion has been a long standing challenge to biologists, and remained unsolved for decades, despite numerous creative approaches. In this project, we tested the idea that bacterial DNA uptake machinery could be translocated to human mitochondria. We were able to synthesize constructs for competency machinery from known bacterial species, as well as develop sensitive assays to detect mitochondrial genome editing. Unfortunately, we found that the key components of the bacterial competency machinery were not well expressed or functional in human mitochondria, suggesting that further engineering of this machinery will be necessary to ultimately be successful.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report.

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

As a high-risk discovery award, it was certainly possible that our ultimate goal of achieving nucleic acid uptake into mitochondria would not be successful as outlined. Particularly, we found that expression of comEC bacterial proteins was particularly difficult on the human mitochondrial inner membrane, and we were unable to detect nucleic acid uptake despite synthesizing, cloning and screening >500 constructs. While a few constructs displayed low levels of expression, they were unable to promote mitoCas9-based activity within the mitochondria. Thus, future efforts along this line of research will primarily focus on identification and engineering of a well-expressing functional comEC construct.

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

N/A.

Significant changes in use or care of vertebrate animals

N/A.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Nothing to report.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to report.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report.

- **Other Products**

  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*

Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name: Prashant Mishra
Project Role: PI
Researcher Identifier:
Nearest Person month worked: 1.8
Contribution to Project: Dr. Mishra has performed work in project planning, design and construction of competency constructs, analysis of data, and oversight of the project.

Name: HongLyn Chen
Project Role: Research Assistant
Research Identifier:
Nearest Person month worked: 3.2
Contribution to Project: Mrs. Chen has performed work in performing experiments, developing reagents, cloning and cell culture maintenance.

Name:Bogdan Bordieanu
Project Role: Research Assistant
Research Identifier:
Nearest Person month worked: 11.2
Contribution to Project: Mr. Bordieanu has performed work in designing, constructing, cloning, and testing competency constructs.

Name: Xun Wang
Project Role: Post-doctoral fellow
Research Identifier:
Nearest Person month worked: 9
Contribution to Project: Dr. Wang has performed work in designing, constructing, cloning, and testing competency constructs.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

New active grants:
Multi-Investigator Research Grant (Morrison, Mishra, DeBerardinis) 8/30/2018 – 8/29/2022
Cancer Prevention Research Institute of Texas (CPRIT)
$367,000 Annual Direct Cost; 2.4 calendar months.
Title: Metabolic enablers of melanoma progression.
Role: Co-PI
The goal of this project is to investigate correlations between mtDNA haplotypes and metastatic efficiency in human melanomas.

New Innovator Award (1DP2ES030449-01) 9/30/2018 - 9/29/2023
NIH – National Institute of Environmental Health Sciences (NIEHS)
$300,000 Annual Direct Cost; 3.0 calendar months.
Title: Engineering faithful animal models of mitochondrial disease.
Role: PI
The goal of this project is to create animal models of mitochondrial disease.

RO1 (1R01AR073217-01A1) 9/20/2018 – 8/30/2023
NIH – National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS)
$225,000 Annual Direct Cost; 2.4 calendar months.
Title: Deciphering the role of amino acid transporters in mitochondrial myopathy.
Role: PI
The goal of this project is to understand the role of amino acid transport and metabolism in the development of mitochondrial myopathies.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*