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TITLE: **Engineering a Minimal Competency Machinery for Mitochondria**

PRINCIPAL INVESTIGATOR: **Prashant Mishra**

CONTRACTING ORGANIZATION: **University of Texas Southwestern Medical Center**
Dallas, TX 75390

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6. AUTHOR(S)

Prashant Mishra

E-Mail: prashant.mishra@utsouthwestern.edu

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

University of Texas
Southwestern Medical Center
5323 Harry Hines Blvd.
Dallas, TX 75390

8. PERFORMING ORGANIZATION REPORT NUMBER

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U.S. Army Medical Research and Materiel Command
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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

None provided

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	15	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

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).*

mitochondria DNA, competency, comEC, comEA, 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: Construction of expression constructs.	June – Aug 2017	100
Subtask 2: Testing of expresion constructs for localization	Sep – Nov 2017	100
Major Task 2: Specific Aim 1B		
Subtask 1: Testing competency constructs for sufficiency	Dec 2017 – May 2018	80
Major Task 3: Specific Aim 2A		
Subtask 1: Testing Cas9-nuclease function (NHEJ)	June – Aug 2018	0
Subtask 2: Testing Cas9-nuclesae function (heteroplasmy)	June - Aug 2018	0
Major Task 4: Specific Aim 2B		
Subtask 1: Precise correction of mtDNA mutations.	Sep – Nov 2018	0
Subtask 2: Precise creation of mtDNA mutations.	Sep – Nov 2018	0

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.

2) Specific Objectives:

-Construction of codon-optimized constructs for mamallian expression of bacterial competency genes.
-Identification of engineered bacterial competency genes which target to mitochondria.
-Identification of engineered bacterial competency genes which mediate nucleic acid uptake into mitochondria.

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 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 (8) 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 mitochondrially 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 and conEC proteins in HEK293 cells. Lipofectamine-mediated transfection was sufficient to generate robust expression in >905% of tested constructs (Fig. 1B). We then used immunofluorescent staining to verify mitochondrial localization (Fig. 1C). ~50% of constructs demonstrated robust mitochondrial localization.

C) Testing of bacterial competency genes for ability to uptake nucleic acids.
Using Cy3-labeled DNA probes, we have set up a 96-well format screen to assess mitochondrial uptake of nucleic acids. A 24-hour co-transfection of Cy3-DNA (using a non-targeting sequence in the human genome) and competency expression construct, is followed by fixation, staining of mitochondria by MitoTracker Red, and fluorescent imaging. This method allows us to rapidly screen competency constructs for uptake ability into the organelle. To date, no single constructs have yet shown uptake ability (Figure 1D).

4) Other achievements:
Nothing to report.

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° *(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° *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

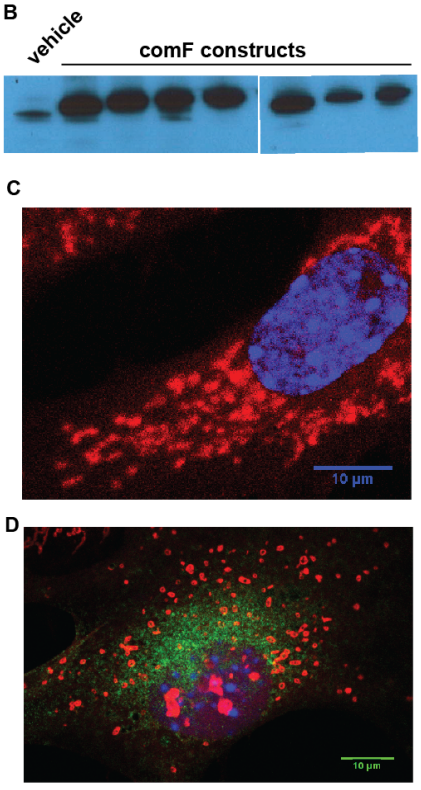

Figure 1: A) List of Gram-negative baceterial species with identifiable competency components. B) Western blot testing expression of comF constructs after transfection into HEK293 cells reveals robust expression in most constructs. Detection: α-myc (clone 9E10). C) Immuno-fluorescence detection of comF expressin construct in HEK293 cells reveals mitochondrial localization. Blue: DAPI; Red: α-myc (clone 9E10). D) Immunofluorescence detection of nucleic acid (Cy3; green) uptake in to mitochondria (MitoTracker Red; red). Blue: DAPI.

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.

Nothing to Report.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

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.

Nothing to Report.

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.

Our current activity is to continue to screen combinations of competency genes to identify conditions which allow for efficient uptake of nucleic acid into mitochondria. While no single construct has shown uptake ability thus far, we suspect that combinations of comEC and comF constructs may synergize to reconstitute nucleic acid uptake. We have developed a 96-well format assay that will be utilized to rapidly screen comF/comEC combinations for uptake ability into organelles. Achieving this objective is critical to the overall success of this project and proceeding to the next tasks.
In parallel, we will begin co-transfection into stable cell lines expressing mito-Cas9 to begin screening for nuclease activity, and heteroplasmy shifts (Major Task 3). This method is amenable to functional selection (by galactose selection) followed by heteroplasmy measurements to document Cas9-nuclease activity on the mitochondrial genome. We have developed droplet digital PCR methods to quantitate heteroplasmy changes.

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).

Nothing to Report for this period.

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 for this period.

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 for this period.

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 for this period.

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:*

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

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.

Currently, we have not identified a condition which allows uptake of nucleic acids into the mitochondrion, despite screening known bacterial competency genes. We plan to continue by 1) screening combinations of competency genes and 2) using galactose selection methods in the presence of Cas9 to select for rare uptake events (as outlined in the original grant proposal).

Changes that had a significant impact on expenditures
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: 1.2
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 peforming experiments, developing reagents, cloning and cell culture maintenance.

Name:Bogdan Bordieanu
Project Role: Research Assistant
Research Identifier:
Nearest Person month worked: 6.4
Contribution to Project: Mr. Bordieanu has performed work in designing, construcing, cloning, and testing competency constructs.

Name: Xun Wang
Project Role: Post-doctoral fellow
Research Identifier:
Nearest Person month worked: 6
Contribution to Project: Dr. Wang has performed work in designing, construcing, 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 grant:
UMDF PI-17-0828 (Mishra) 09/01/2017-08/31/2019 1.2 Calendar
United Mitochondrial Disease Foundation (UMDF) $50,000 Annual Direct Cost
Identification of SLC family members as predictive biomarkers for mitochondrial disease.
The goal of this project is to investigate the correlation between expression of SLC family members and mitochondrial disease.
Role: PI

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: *None*

QUAD CHARTS: *None*

9. APPENDICES: *None*