AWARD NUMBER: W81XWH-17-1-0185

TITLE: Analysis of toxicant induced translational control through codon-usage bias in lung cancer

PRINCIPAL INVESTIGATOR: Ahmed Fazly

CONTRACTING ORGANIZATION: Massachusetts Institute of Technology
Cambridge, MA 02139

REPORT DATE: October 2018

TYPE OF REPORT: Final

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
October 2018	Final	1 Jul 2017 - 30 Jun 2018

4. TITLE AND SUBTITLE

Analysis of toxicant induced translational control through codon-usage bias in lung cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-17-1-0185

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Ahmed Fazly and Peter Dedon

E-Mail: pcdedon@mit.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

MASSACHUSETTS INSTITUTE OF TECHNOLOGY
77 MASSACHUSETTS AVE
CAMBRIDGE MA 02139-4301

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

The goal of this project was to test the idea that human cells respond to stress using a translational control mechanism involving changes in the levels of dozens of modified nucleosides in tRNAs, which causes selective translation of codon-biased survival genes. Here we report that exposure of human cells to oxidative stressors, including arsenic, rapidly increases the level of the wobble modification queuosine (Q) and one of its glycosylated derivatives, galactosyl-Q (gal-Q). Exposure to alkylating agents and, ironically, ionizing radiation did not alter Q or gal-Q. Proteomics analyses revealed that proteins up-regulated by arsenic were derived from genes enriched in the tyrosine codon TAC, which is read by tRNA with the anticodon (gal-Q)UA. Among up-regulated proteins from TAC-enriched genes were those involved in glycolysis, which is consistent with the fact that arsenic uncouples oxidative phosphorylation in mitochondria. These results support a model in which cells respond to arsenic exposure by reprogramming the tRNA pool to selectively translate mRNAs from families of codon-biased genes needed to survive arsenic toxicity. These results have implications for lung cancer cells, which depend upon glycolysis as a result of the Warburg effect in tumor cells. We are now analyzing tRNA modifications and protein levels in lung cancer cells to test the idea that cancer cells are in a permanent state of codon-biased translation favoring glycolysis pathways.

15. SUBJECT TERMS
Epitranscriptomics, tRNA modifications, cancer cells, glycolysis, genomics, codon usage, translation biology, mass spectrometry

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)*

TABLE OF CONTENTS

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

All cells respond to stress by changes in signal transduction, transcription and post-translational modification of proteins, which integrate to a change in phenotype. Recently, we discovered a new mechanism of translational control in the response of yeast and bacteria to stress, in which stresses induce reprogramming of dozens of modified ribonucleosides in tRNA, which causes selective translation of codon-biased mRNAs from families of stress-response genes. This project tested the role of this mechanism in human cells for the first time, focusing on the cellular response to exposure to stresses associated with chemotherapy (e.g., alkylating agents) and tumorigenesis (e.g., oxidizing agents). Toxicant-induced changes in the levels of tRNA modifications was quantified using our RNA modification analysis platform and these changes were then linked to stress-induced changes in the proteome using mass spectrometry-based proteomics and computational genomics.

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

Epitranscriptomics, tRNA modifications, cancer cells, glycolysis, genomics, codon usage, translation biology, mass spectrometry

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.

Major Task 1: I*dentify toxicant-induced changes in the levels of tRNA modifications in human normal and lung cancer cells.* *Determine dosing conditions for stressors (Month 1). RNA modification analysis by mass spectrometry (Months 2-3). Milestone(s) Achieved: Identification of stress-critical tRNA modifications.* These goals were achieved for human tumor cells exposed to 4 oxidizing agents (hydrogen peroxide, t-butylhydroperoxide, arsenite, γ-radiation) and 4 alkylating agents (methylmethanesulfonate, ethylmethanesulfonate, i-propylmethane-sulfonate, N-nitorosodiethylamine). Completed October 2017.

Major Task 2: ***Mapping the location of stress-altered tRNA wobble modifications.*** *Affinity purification of specific tRNA (Month 4). Analysis of RNA modifications in purified tRNAs by mass spectrometry (Months 5-6). Milestone(s) Achieved: Mapping stress-critical tRNA modifications to specific tRNAs*. These goals were not achieved due to time constraints. However, specific tRNAs with stress-altered wobble modifications were identified by comparative genomics and the identities are now being validated as planned.

Major Task 3: ***Linking tRNA modifications to stress-induced changes in the proteome.*** *Proteomic analysis of cells exposed to stressors (Months 7-12). Milestone(s) Achieved: Linking tRNA modification changes to codon-biased translation of survival proteins.* This goal was achieved for human tumor cells exposed to arsenic, with important discoveries about the queuosine modification controlling translation of codon-biased mRNAs for proteins needed to survive arsenic toxicity. Completed June 2018.

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.

As a first-of-its-kind study in human cells, the goal of this project was to test the idea that tumor cells respond to stress using a translational control mechanism involving changes in the levels of dozens of modified nucleosides in tRNAs, which causes selective translation of codon-biased survival genes. We note at the outset that initial attempts to perform these studies with cultured human lung cancer cell lines were not successful due to repeated problems with different lots of serum from an otherwise reliable vendor. Due to the time constraints of the grant, we decided to pursue the studies in human liver cancer cells (HepG2). The results of studies, the first ever performed in human cells, will be applied to human lung cancer cells to test what we expect to be a generalized model in all types of human cancer, with variations reflecting the different tumor types and environments.

To test this translational control model, we first established toxicity dose-response relationships conditions for HepG2 cells exposed to 4 oxidizing agents (hydrogen peroxide, H_2O_2; t-butylhydroperoxide, TBOOIH; arsenite, $NaAsO_2$; γ-radiation, RAD) and 4 alkylating agents (methylmethanesulfonate, MMS; ethylmethanesulfonate, EMS; i-propylmethanesulfonate, IMS; N-nitorosodiethylamine, NDEA). The determination of equitoxic doses for each agent was essential for comparing the tRNA modification and proteome changes for each agent.

Having established dose-response relationships for the 8 toxicants, we next proceeded to apply our mass spectrometry platform to quantify the changes in the levels of 25 modified nucleosides in tRNA isolated from HepG2 cells 24 hours after a 2-hour exposure to LD_{20} doses of the toxicants. **Figure 1** shows a heat map from an unstructured hierarchical clustering of log_2 fold-change data for changes (relative to untreated cells) in the levels of the 25 modified ribonucleosides (rows) as a function of the toxicant (columns). The results show segregation of the oxidants from the alkylating agents, which reveals class-specific changes in the patterns of tRNA modifications. Such patterns also suggest similar mechanisms of cell response to the two different toxicant classes, which is expected. The most striking change involves the wobble tRNA modification, queuosine (Q), which showed large increases for three of the four oxidants but not the alkylating agents. Ironically, ionizing radiation did not alter Q levels and caused the smallest changes in other tRNA modification levels of all of the toxicants. This latter result is consistent with our previous studies in yeast.

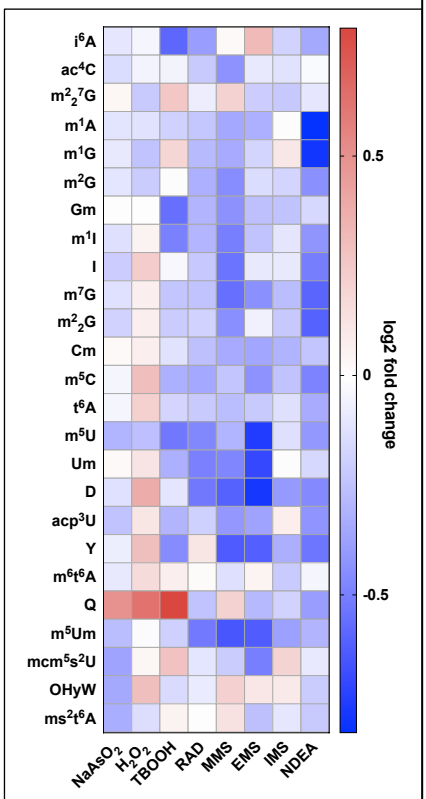

Figure 1. Toxicant-induced changes in the levels of 25 tRNA modifications in HepG2 cells.

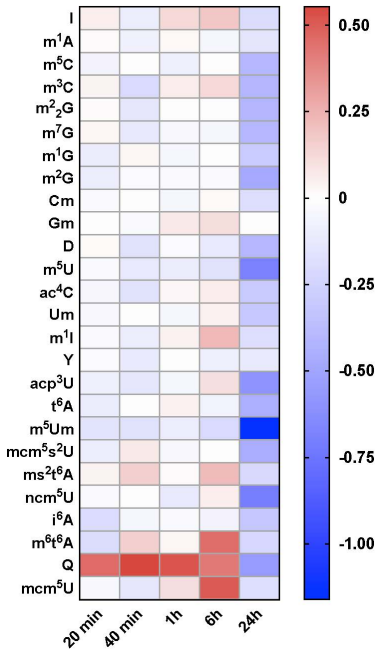

Figure 2. Time course for changes in 25 tRNA modifications in HepG2 cells exposed for 2 hours to an LD_{20} dose of arsenite.

To explore the kinetics of the tRNA reprogramming, we analyzed tRNA modification changes as a function of time after exposure to arsenite. As shown in **Figure 2**, Q is elevated as early as 20 min after a 2-hour exposure to $NaAsO_2$ and reaches a maximum at approximately 40-60 min.

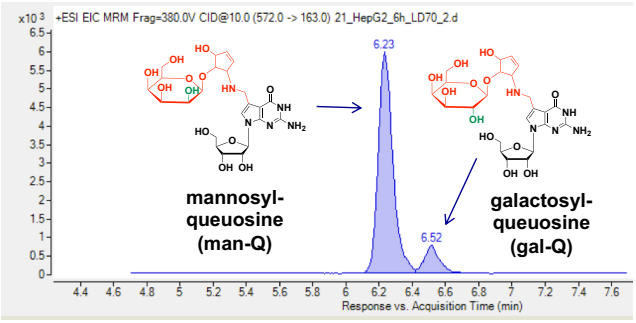

Figure 3. Mass spectrometric analysis revealed glycosylated Q derivatives gal-Q and man-Q, with a 1.4-fold increase in gal-Q following arsenite exposure.

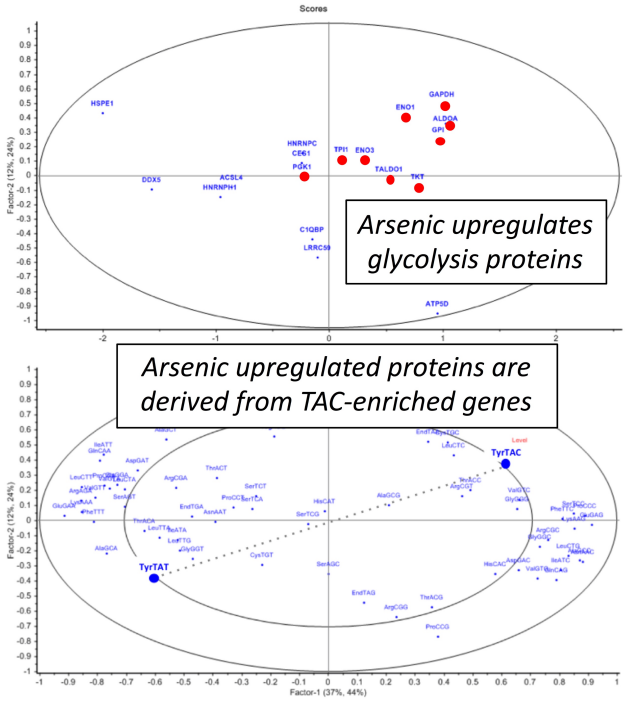

Figure 4. Principal component analysis (PCA) of codon usage patterns in proteins up- and down-regulated by arsenic exposure. ***Upper***: Scores plot of up- (red) and down-regulated proteins. ***Lower***: Loadings plot showing association of the Tyr-TAC codon with up-regulated proteins.

An important discovery was based on the observations of Thomas Carell's group in Germany. They had detected two glycosylated derivatives of Q: galactosyl-Q (gal-Q) and mannosyl-Q (man-Q) (**Figure 3**). While we do not yet have standards to validate the structural assignments, we observed signals and fragmentation patterns consistent with both gal-Q and man-Q. Importantly, the putative gal-Q increased 1.4-fold following arsenite exposure while the man-Q did not change. The reason that the gal-Q increase is important is that it is proposed to be the wobble modification of the (gal-Q)UA anticodon on the tyrosine tRNA that reads the TAC codon, which was found to be associated with arsenic exposure in proteomics studies.

Proteomics analysis of arsenic treated and untreated HepG2 cells was performed, with the arsenic-induced up- and down-regulated proteins analyzed for codon usage patterns of their parent genes and for biochemical pathways represented among the proteins. As shown in PCA loadings plot in **Figure 4**, the Tyr-TAC codon was found over-represented in proteins up-regulated by arsenic exposure, while its synonymous partner TAT was over-represented in down-regulated proteins. Among up-regulated proteins from TAC-enriched genes were those involved in glycolysis, which is consistent with the fact that arsenic uncouples oxidative phosphorylation in mitochondria.

These results support a model in which cells respond to arsenic exposure by reprogramming the tRNA pool to selectively translate mRNAs from families of codon-biased genes needed to survive arsenic toxicity. These results have implications for lung cancer cells, which depend upon glycolysis as a result of the Warburg effect in tumor cells. We are now analyzing tRNA modifications and protein levels in lung cancer cells to test the idea that cancer cells are in a permanent state of codon-biased translation favoring glycolysis pathways.

This work is being prepared for publication.

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.

Dr. Fazly, the postdoctoral scientist associated with this project, engaged in several professional development and training activities in the past year. In terms of training, his research supervisor, Prof. Peter Dedon, provided regular mentorship in experimental design, data interpretation, communication skills, and career opportunities. Dr. Fazly also participated in the MIT Postdoctoral Association in a variety of activities aimed at career networking, grant writing and communication skills.

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.

This work is being prepare for publication in a peer-reviewed journal and will be presented at an upcoming international conference.

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

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).

These studies will shed new light on the processes of carcinogenesis and tumorigenesis in humans. tRNA modification reprogramming and codon-biased translation as a stress response may be involved in both processes as the genes controlling tRNA modifications could become mutated to be expressed at inappropriate times in the normal cell. This could lead to uncontrolled cell growth of early stages of cancer and to inappropriate expression of genes for surviving tumorigenic stresses. The identification of aberrantly-expressed RNA modifying enzymes raises the possibility of new targets for cancer chemotherapy.

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.

The methods refined in these studies are now being used to study RNA modifications in all forms of life, including viruses, bacteria, parasites, yeast, rodents and humans. The observations made in these studies with human cells will be translated to other disciplines such as environmental health sciences for the exposure biology of the work, oncology for the potential of identifying new targets for cancer chemotherapy, and to other branches of medicine for the implications of the model system of tRNA modification reprogramming and codon-biased translation.

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.*

The methods used in these studies have been adopted widely by other scientists for the study of tRNA modifications and other RNA modifications as well as for the study of codon usage patterns.

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.*

The results of these studies will reveal potential new targets for cancer therapeutics. The tRNA-modifying enzymes found to be aberrantly expressed in tumor cells could be targeted for inhibition to render the tumor cells more susceptible for other forms of therapy.

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:*

Initial attempts to perform these studies with cultured human lung cancer cell lines were not successful due to repeated problems with different lots of serum from an otherwise reliable vendor. Due to the time constraints of the grant, we decided to pursue the studies in human liver cancer cells (HepG2). The results of the studies, the first ever performed in human cells, will be applied to human lung cancer cells to test what we expect to be a generalized model in all types of human cancer, with variations reflecting the different tumor types and environments.

Also, due to time constraints, we were unable to compare normal epithelial cells to cancer cells. However, such a comparison will be performed by another postdoctoral scientist in ongoing studies. Also, we will verify the glycosylated Q derivatives by mass spectrometry and then map their locations in the tRNA isoacceptors.

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.

Nothing to report

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

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

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).*

A manuscript describing this work is in preparation.

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 yet. The results will be presented at a major international conference in April 2019.

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.

The studies made use of existing mass spectrometry and informatics platforms for quantitative analysis of RNA modifications and codon usage patterns in the genome.

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.*

The proteomics data will be uploaded for public access to the Chorus database prior to publication of this work.

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:	Dr. Ahmed Fazly
Project Role:	Postdoctoral Scientist
Person months:	12 person months
Contribution:	Dr. Fazly performed dose-response response studies and quantified modified ribonucleosides by mass spectrometry. He also participated in the codon analysis.
Funding support:	This grant
Name:	Dr. Sabrina Huber
Project Role:	Postdoctoral Scientist
Person months:	12 person months
Contribution:	Worked with Dr. Fazly on all aspects of the project and performed analysis of glycosylated Q modifications.
Funding Support:	Postdoctoral fellowship from the Swiss government
Name:	Prof. Peter Dedon
Project Role:	Supervisor and mentor for Drs. Fazly and Huber
Person months:	1 person month
Contribution:	Worked with Drs. Fazly and Huber on experimental design, data interpretation, model building, and provided overall management of the grant.
Funding support:	MIT salary support, NIH and NSF grants, grant from the Singapore Government, a grant from the Israeli government

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.

Nothing to report

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.*