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14. ABSTRACT

A tumor cell's ability to modulate its metabolism influences multiple key aspects of a tumor's behavior, such as cellular signaling, differentiation and metastatic potential. Although the concept of targeting metabolic vulnerabilities in cancers has appeal from a therapeutic standpoint, delineation of critical vulnerabilities remains a barrier. *NF1* encodes neurofibromin, a GTPase activating protein that negatively regulates Ras signaling. *NF1* loss leads to activation of downstream Ras signaling effectors MEK and mTOR, which can be therapeutically targeted. *NF1* loss is also associated with metabolic dysregulation, however the molecules primarily responsible for metabolic dysregulation in *NF1*-mediated tumorigenesis remain poorly defined. Additionally, the role of metabolic reprogramming in the development of drug resistance (resistance to MEK inhibition-MEKi or mTOR inhibition-mTORi, for example) is also unknown. **Thus, a potentially groundbreaking but currently underdeveloped paradigm in the management of NF1 is identifying and treating disease on the basis of metabolic targets.** These studies will identify candidate molecules that are critical participants in tumor metabolic reprogramming and outline the conditions of metabolically-targeted strategies.

15. SUBJECT TERMS
CRISPRi, metabolism, NF1, MEK, mTOR

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

Because metabolic requirements impose physiologic constraints to growth, establishing the core pathways of metabolic dysregulation is likely to elucidate mechanisms by which *NF1* mutant cells circumvent barriers to proliferation, such as energy/nutrient limitation and hypoxia. **A potentially ground-breaking but currently underdeveloped paradigm in the management of NF1 is identifying and modifying disease development on the basis of metabolic targets.** Currently, tumor metabolism is measured diagnostically using PET-CT (1), although new approaches to non-invasively interrogate intra-tumoral metabolism includes magnetic resonance spectroscopy (MRS) (2). However, new clinical applications rooted in understanding disease metabolism might include nutritional strategies to modify or abrogate clinical features of the NF1 syndrome, metabolite-driven predictions of disease course and metabolism-optimized, combinatorial strategies to mitigate and treat NF1 neoplasms.

To study *NF1*-driven tumorigenesis, we previously mutagenized *Nf1* heterozygous mice with radiation to generate diverse *Nf1* null tumors. Our mouse models of *Nf1*-driven tumorigenesis implicate metabolic dysregulation as playing a central role in tumor pathogenesis (3). In addition, preliminary studies in drug-resistant *Nf1* mutant tumors indicate that resistance to mTOR or MEK inhibition is associated with distinctly contrasting metabolic profiles, suggesting that metabolic re-wiring is a component of therapeutic resistance. This proposal seeks to identify genes that modulate energy levels in *Nf1* mutant tumors to capitalize on therapeutically tractable vulnerabilities and develop metabolism-based diagnostic and therapeutic strategies. Metabolic approaches may potentiate a wide range of therapeutic approaches (10), including kinase inhibition that is currently used today. This provides a basis for determining how targeting metabolic vulnerabilities might cripple *Nf1* mutant tumors.

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

CRISPRi, metabolism, NF1, MEK, mTOR

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.

Hypothesis to be tested We hypothesize that *Nf1*-mutant tumors require metabolic reprogramming to develop resistance to molecular therapeutics, and that the involved genes/pathways represent tractable therapeutic targets. Using a live ATP-sensor that enables *in vivo* evaluation of cellular energy levels in individual cells, we will perform a CRISPRi-based functional genomics screen in *Nf1* mutant tumors to identify genes essential for energy production utilizing either glycolysis or respiration.

Specific Aims

Aim 1: To perform a CRISPRi-based functional genetics screen to identify genes essential for ATP production in *Nf1* mutant parental and drug-resistant tumor cells

Aim 2: To test bioenergetic targeting as a therapeutic strategy in *Nf1* mutant tumors
a. Determine if targeting bioenergetic pathways validated from the screen suppresses the growth of drug-resistant tumor cells.
b. Determine if bioenergetic targeting sensitizes *Nf1* mutant parental tumor cells to MEK or mTOR inhibition.

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.

Major Activities:
This reporting period we made progress in establishing our ATP-sensor, CRISPRi-based screen of metabolism genes in tumor cells and performed comprehensive sequencing analysis of *Nf1* mutant tumor cell lines and their drug-resistant derivatives as proposed (989 and 881). We successfully interrogated metabolic pathways and identified candidate genes for targeted investigation in *Nf1* mutant tumor cells.

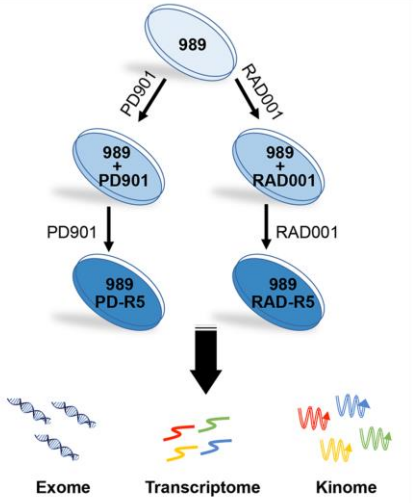
The objective of this proposal is to delineate metabolic vulnerabilities in *Nf1* mutant tumor cells that can be targeted for therapeutic purposes. Our aim is to investigate metabolic vulnerabilities in parental/drug-naïve tumor cells and drug-resistant tumor cells. To accomplish this, we utilized a CRISPRi-based approach coupled with sequencing analyses of well-characterized *Nf1*-mutant tumor cell lines and their drug-resistant derivatives (Figure 1).

Results:
CRISPRi-based genetic engineering of *Nf1* mutant tumor cells requires expression of dCas9, and a necessary step was that we generate 989 and mTORi or MEKi resistant derivatives (termed 989 RAD_R and 989 PD_R, respectively) expressing dCas9. These cells were transduced with a construct encoding BFP-dCas9 KRAB and high BFP-expressing cells were isolated by FACS (data not shown). These cells are now established and will be used for CRISPRi-based investigations in the next award period.

Acquired drug resistance can develop through multiple mechanisms, and to investigate this to identify metabolic genes and pathways that might be candidates for therapeutic manipulation, we augmented our studies of *Nf1* mutant and drug-resistant derivative cells with whole exome sequencing, RNA Seq and kinome analyses. Described below, these analyses identified multiple targets for *Nf1* tumors.

We first analyzed parental *Nf1* mutant tumor cell lines 989 and 881 lines with transcriptome and

Figure 1. *Nf1* mutant parental tumor cell line was exposed to acute and chronic MEKi (PD901) or mTORi (RAD001) to generate drug-resistant PD_R and RAD_R lines. Parental and drug resistant lines were analyzed by exome sequencing, RNA seq and kinome analysis.

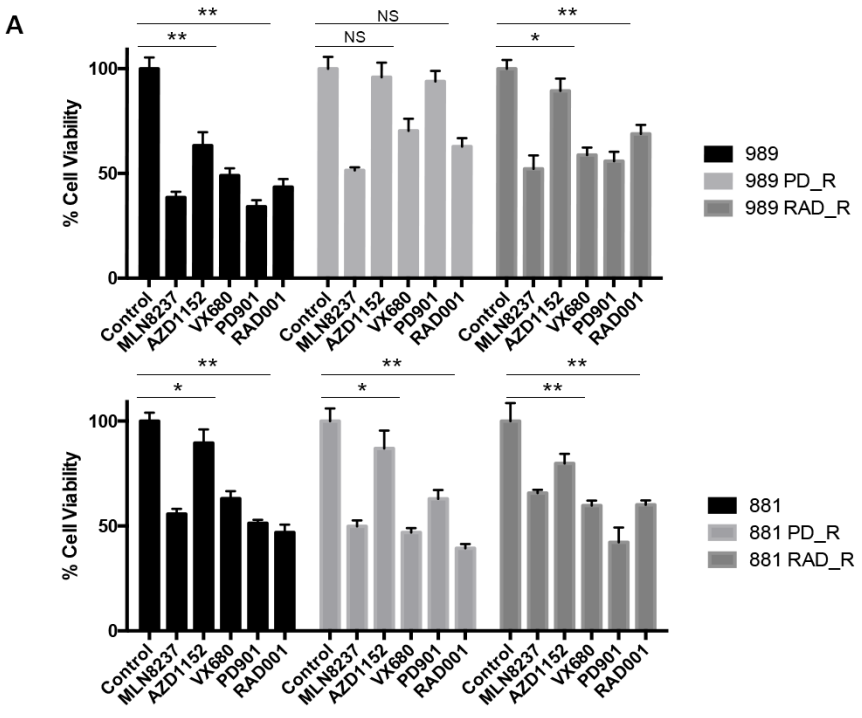


kinome profiling. These studies demonstrated that although these lines share *Nf1* loss and similar basal and stimulated PI3K/AKT and MAPK pathway activation, kinome-wide differences distinguish these lines.

Cytoscape software integrated with Genemania was used to visualize protein interaction networks composed of the kinomes of the 881 and 989 cell lines. Network analysis revealed that Aurora Kinase (AURKA) activity was among the common kinome features shared between these two cell lines (data not shown).

Based on these integrated transcriptome and kinome analyses, we tested whether AURKA identified by kinome analysis is functionally relevant to growth in our tumor cell lines. We assessed pharmacologic inhibition of AURKA and AURKB in these cells (Figure 2). The AURKA inhibitor MLN827, the AURKB inhibitor AZD1152 and the pan-AURK inhibitor VX680 each decreased phosphorylation levels of their respective targets and reduced growth of both the 989 and 881 cell lines, validating the identification of AURKA as a functionally relevant kinase that supports growth by both 989 and 881 *Nf1* mutant cell lines (Figure 2).

Figure 2. *Nf1* mutant tumor cells respond to Aurora Kinase inhibition A. Cell viability of parental and drug resistant cell lines treated with Aurora inhibitors MLN8237, AZD1152 or VX680 (PD901 and RAD001 shown for comparison) was measured by MTS assay after 72 hours of exposure (*P<0.05; **P<0.01; NS, not significant). B. Western blot analysis was performed to evaluate Aurora A, B and C phosphorylation in response to Aurora inhibitors MLN8237, AZD1152 or VX680.



We then assessed whether acquired resistance to either MEKi or mTORi also conferred resistance to AURKA inhibition. Drug resistant derivatives of 989 and 881 cell lines are denoted as the mTORi resistant 881 RAD_R and 989 RAD_R cell lines. MEKi

resistant cell lines are denoted as 881 PD_R and 989 PD_R. Both RAD_R cell lines and the 881 PD_R cell line responded to all three Aurora inhibitors (with AZD1152 producing the most modest response), which decreased cell growth and phosphorylation of AURKA, AURKB and H3 (Figure 2A/B).

We then performed kinome analysis of drug-resistant cells; 194 kinases were assayed in resistant and acutely treated 989 cells and compared to untreated parental 989 cells (Figure 3). The kinome profiles of the drug resistant 989 cells segregated from acutely treated cells, irrespective of the drug target, supporting the concept that targeted kinase inhibition is characterized by broader remodeling of kinome activity.

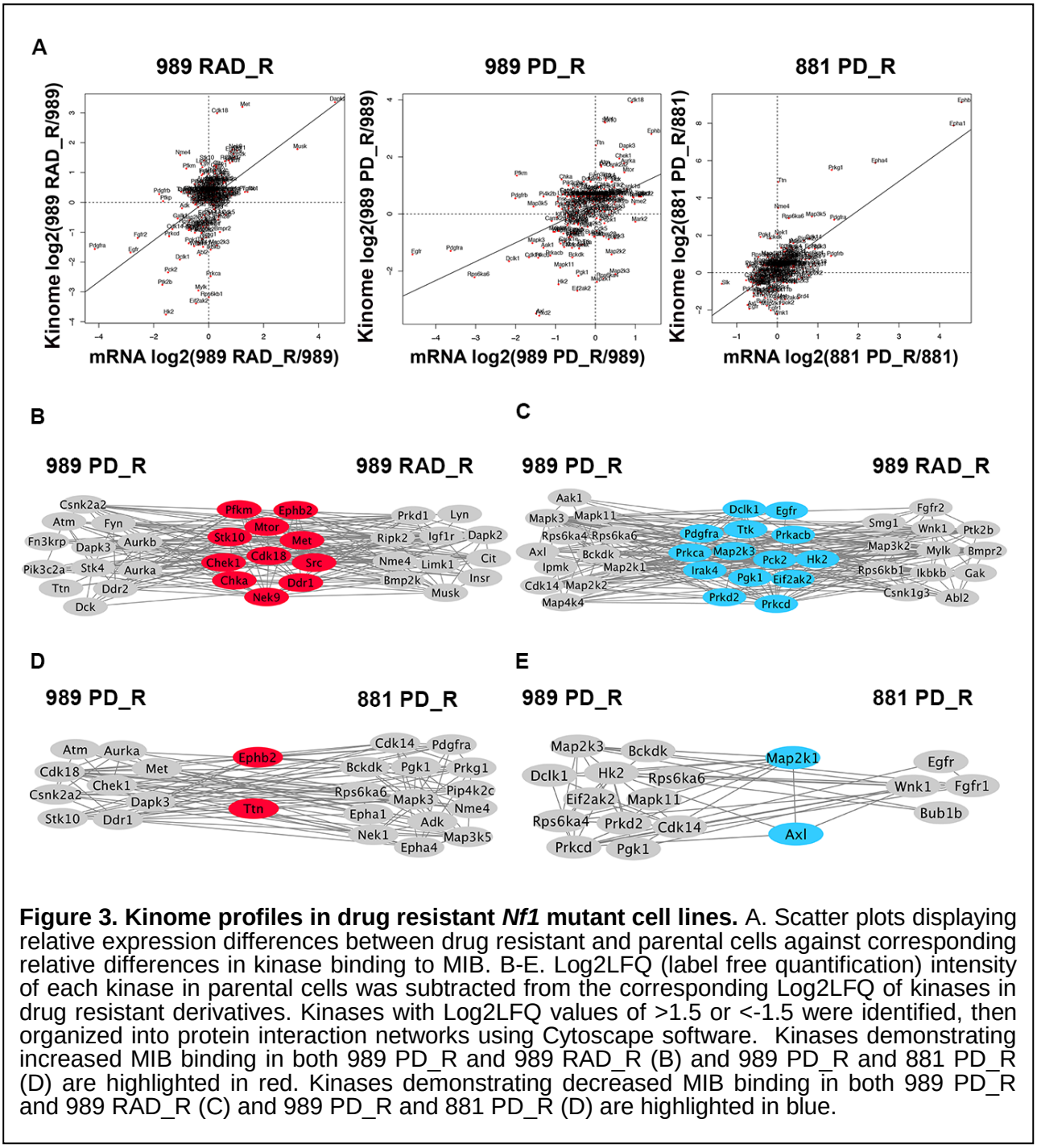


Figure 3. Kinome profiles in drug resistant *Nf1* mutant cell lines. A. Scatter plots displaying relative expression differences between drug resistant and parental cells against corresponding relative differences in kinase binding to MIB. B-E. Log2LFQ (label free quantification) intensity of each kinase in parental cells was subtracted from the corresponding Log2LFQ of kinases in drug resistant derivatives. Kinases with Log2LFQ values of >1.5 or <-1.5 were identified, then organized into protein interaction networks using Cytoscape software. Kinases demonstrating increased MIB binding in both 989 PD_R and 989 RAD_R (B) and 989 PD_R and 881 PD_R (D) are highlighted in red. Kinases demonstrating decreased MIB binding in both 989 PD_R and 989 RAD_R (C) and 989 PD_R and 881 PD_R (D) are highlighted in blue.

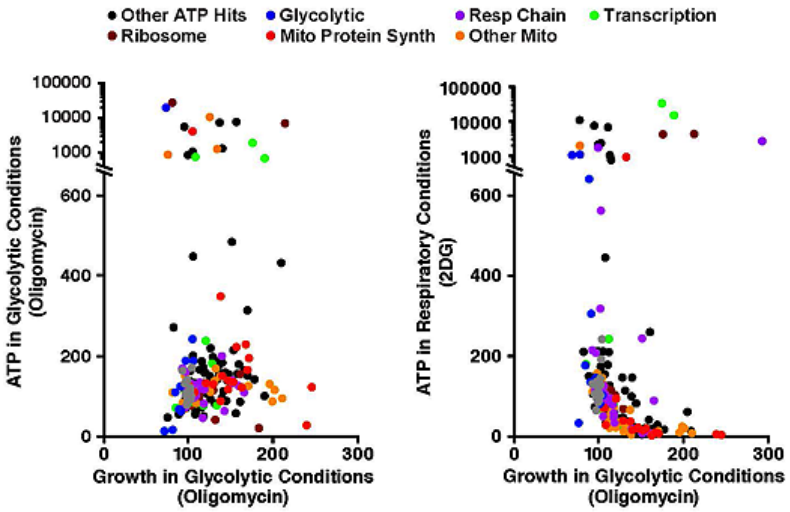
We then sought to identify common, shared alterations in specific metabolic kinases present in drug resistant cells (Figure 3); these included HK2 (Hexokinase 2), which catalyzes the first essential step of glucose metabolism, the conversion of the substrate glucose into glucose-6-phosphate (7) and the mitochondrial enzyme PCK2 (Phosphoenolpyruvate Carboxykinase 2), which catalyzes the conversion of oxaloacetate

to phosphoenolpyruvate in the presence of guanosine triphosphate (GTP) (8, 9).

CRISPRi-based growth and ATP screen

We performed a genome-wide ATP-FRET-based CRISPRi screen in human leukemia cells (manuscript submitted) and then developed a mini-CRISPRi library targeting the top ATP-modulating genes. To test the effects of changing ATP levels on growth in a solid tumor cell line, we generated HCC827 human lung cancer cells expressing dCas9-KRAB, and transduced them with the mini-library enriched in CRISPRi respiratory hits. We then grew cells in either basal, glycolytic (5 μM oligo) or respiratory (10 mM 2DG and 1.5 mM pyruvate) conditions, and determined the impact of each sgRNA on growth relative to the non-targeting guides.

Figure 4. High ATP hits increase growth through ATP-dependent and independent mechanisms. HCC827 cells expressing the CRISPRi mini-library were grown for 3 days in glycolytic conditions (5 μM oligo), and the fold-impact of each sgRNA on growth (mean readcount, normalized to nontargeting controls) plotted versus ATP level measured in parallel experiments.



This experiment validated the top ATP modulating hits and allowed us to refine the list of candidate genes for further study in the next funding period. Under respiratory conditions, knockdown of most mitochondrial ribosomal and other mitochondrial proteins that decrease mitochondrial-derived ATP produced a significant albeit modest negative impact on cell growth (Figure 4). This suggests that the tumor cells require mitochondrial-derived ATP under these conditions, although they maintained sufficient ATP to support growth even when mitochondrial-derived ATP was limited, likely because sufficient glucose (11.1 mM) was metabolized despite competitive inhibition by 10 mM 2DG.

Collectively, these results support the concept that cellular ATP availability is growth-limiting, providing a strong rationale for targeting ATP-modulating genes for cancer therapeutics. These findings also point to connections between respiratory and glycolytic metabolism, as silencing mitochondrial genes, which increases glycolysis-derived ATP (Figure 4), also strongly and almost uniformly promoted tumor growth under glycolytic conditions. This relationship implies that silencing or inhibiting one metabolic mechanism (glycolytic versus respiratory) must be coordinated with the appropriate substrate setting for optimal effect. This discovery informs our future preclinical studies.

Conclusion/next plans

In the next phase of the award we are focusing efforts on testing in *Nf1*-mutant tumor cells (both parental and drug resistant) the anti-tumor growth effects of individual candidate molecules in implicated by our data to date. These individual molecules, functioning in glycolytic or respiratory metabolism, represent potentially important pathways for tumor growth that will be investigated.

We have one manuscript in preparation that will report the above described analysis of *Nf1* mutant tumor cells and drug-resistant derivatives and expect to be submitting this for review in the next funding period.

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.

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.

In the next reporting period we will continue our proposed work to validate candidate metabolism modulating genes in Nf1 mutant cells, to confirm and compare their relative effectiveness at modulating cellular ATP and tumor cell growth.

To accomplish this, we will express individual sgRNAs targeting the candidate genes discussed in this report into *Nf1* mutant tumor cells expressing dCas9 (these were generated during this reporting period). After confirming that gene expression is significantly reduced (CRISPRi), *in vitro* and *in vivo* experiments are planned to test the function of these candidate genes and compare their effects on ATP and growth to non-targeting control.

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.

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.

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).*

  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: Jean Nakamura
Project Role: PI
ORCID ID: 0000-0002-2097-9223
Nearest person month worked: 3
Contribution to Project: Dr. Nakamura has directed the project, trained Dr. Nakaoka who is performing work on the project, and has analyzed data.
Funding Support: The Hagar Family Foundation

Name: Hiroki Nakaoka
Project Role: Post-doctoral Fellow
Nearest person month worked: 6
Contribution to Project: Dr. Nakaoka has performed CRISPRi-based experiments central to this proposal, including generating the relevant dCas9-KRAB expressing cells and optimizing the CRISPRi protocol for this proposal.
Funding Support: The Hagar Family Foundation

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.

I am a collaborator on a recent award from the John Templeton Foundation (subcontract to UCSF from Johns Hopkins University was finalized in August 2019). My effort on this award is 10% and there is no scientific overlap with the present award nor does this compromise my effort.

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.

REFERENCES

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