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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

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1. INTRODUCTION:

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 (4), 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:

CRISPRi, metabolism, NF1, MEK, mTOR

3. ACCOMPLISHMENTS:

What were the major goals of the project?

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.

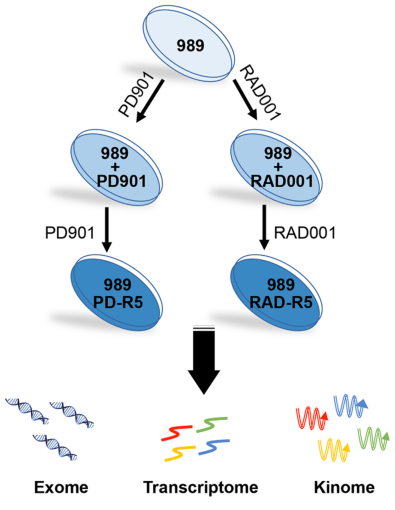
What was accomplished under these goals?

Major Activities:
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).

During the award period, we succeeded 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. These earlier accomplishments were published in *Molecular Cancer Therapeutics* in 2020.

Our proposed activities focused on analyzing the metabolic (glycolytic and respiratory) adaptations that accompany acquired drug resistance, which is described below in Results. To summarize, using the *Nf1* mutant cell lines we established and subsequently derived into drug-resistant variants (resistant to either MEKi or mTORi) as experimental systems to interrogate, we analyzed the metabolic dependencies of parental and drug resistant cells to glycolytic, respiratory metabolic inhibition (Aim 2) and found that acquired drug-resistance is indeed associated with metabolic consequences that differentiate resistant derivatives from the parental cells from which they were derived. These data suggest that metabolic re-wiring does occur as a component of acquired resistance to MEKi or mTORi, and has implications for anticipated metabolic consequences of these therapies in patients.

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.



Results:
For the execution of Aim 1, 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 important experimental

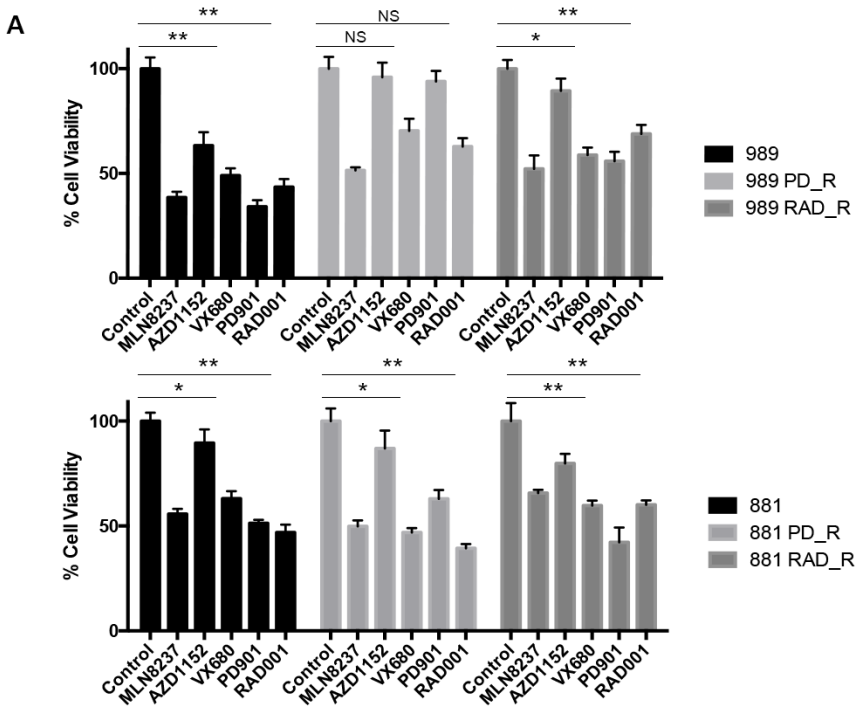
resources that we will continue to use for on-going CRISPR-based metabolic engineering of *Nf1* mutant tumor cells.

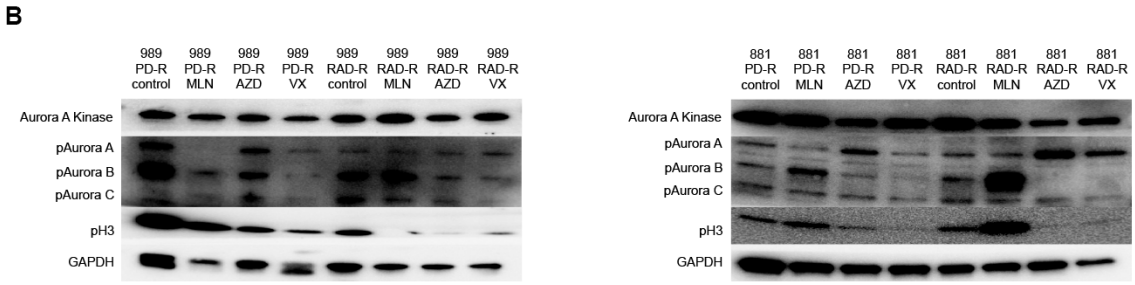
Acquired drug resistance can develop through multiple mechanisms, and to investigate this to identify metabolic genes and pathways that might be candidates for therapeutic manipulation (Aim 2), we augmented our studies of *Nf1* mutant and drug-resistant derivative cells with whole exome sequencing, RNA Seq and kinome analyses. Briefly described below, these analyses identified multiple targets for *Nf1* tumors. This work was recently published in *Molecular Cancer Therapeutics* (5).

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

Integrated transcriptome and kinome analyses (5) implicated candidate molecules that might be uniquely important and allow drug-resistant tumor cells to maintain their growth. We then tested whether these candidate molecules might be necessary for tumor cell growth. One candidate we tested was AURKA, which was particularly notable on kinome analysis and is pharmacologically targetable. We tested whether AURKA 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

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.





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).

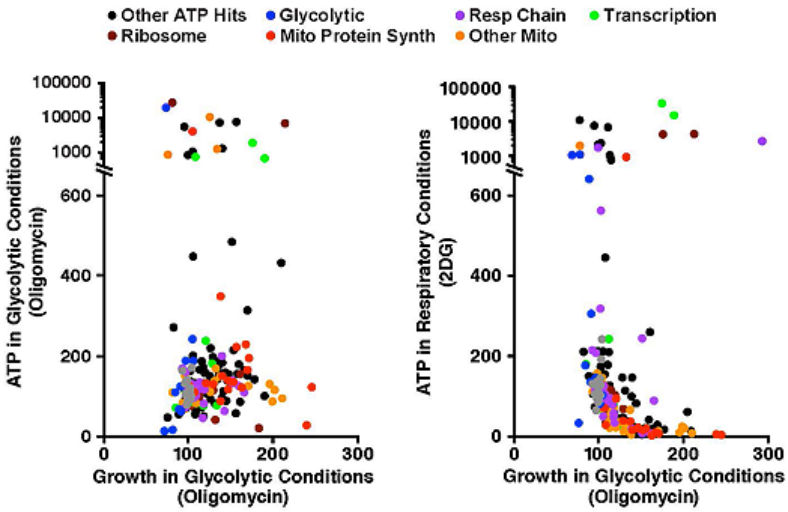
We then assessed whether acquired resistance to either MEKi or mTORi also conferred resistance to AURKA inhibition. Drug resistant derivatives of 989 and 881 cell lines are denoted as the mTORi resistant 881 RAD_R and 989 RAD_R cell lines. MEKi resistant cell lines are denoted as 881 PD_R and 989 PD_R. 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 sought to identify common, shared alterations in specific metabolic kinases present in drug resistant cells; these included HK2 (Hexokinase 2), which catalyzes the first essential step of glucose metabolism, the conversion of the substrate glucose into glucose-6-phosphate (6) and the mitochondrial enzyme PCK2 (Phosphoenolpyruvate Carboxykinase 2), which catalyzes the conversion of oxaloacetate to phosphoenolpyruvate in the presence of guanosine triphosphate (GTP) (7, 8).

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 3. 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 3). 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 3), 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.

Acquired drug-resistant *Nf1* mutant tumor cells demonstrate altered glycolytic and respiratory metabolism when compared to parental cells

Cancer cells adapt their metabolism and re-program their metabolism to meet different growth conditions, and the central hypothesis of this proposal is that acquired drug resistance invokes a change in metabolic function. To evaluate this, we utilized our *Nf1* mutant tumor cell paradigm to specifically test whether drug-resistant cells, compared to their parental source cells, respond differently to targeted disruption of either glycolytic or respiratory function. Cells from each line were seeded into 96 well plates. Then,cells were treated with 2-deoxyglucose, oligomycin, phenformin or glucose restriction (glucose-free media) over 72 hours. Cellular viability was measured following incubation 72H in treatment media using the MTS tetrazolium assay (Promega G9241).

Following glycolytic inhibition, 989 RAD resistant cells showed relatively higher resistance to glycolytic inhibition using 2DG (Figure 4A) and through glucose restriction (Figure 4B) as indicated by its higher cellular proliferation compared to 989 parental lines. This may indicate that the mechanisms of PD resistance may be associated with the alterations in the glycolytic reliance of 989 cells, allowing 989 PD resistant cells to continue proliferation despite the presence of 2-deoxyglucose, or limited amounts of glucose. In contrast, 881 PD resistant cells showed higher sensitivity to glycolytic inhibition using 2-deoxyglucose compared to 881 parental lines (Figure 4C), although similar results were not found through glucose deprivation (Figure 4D).

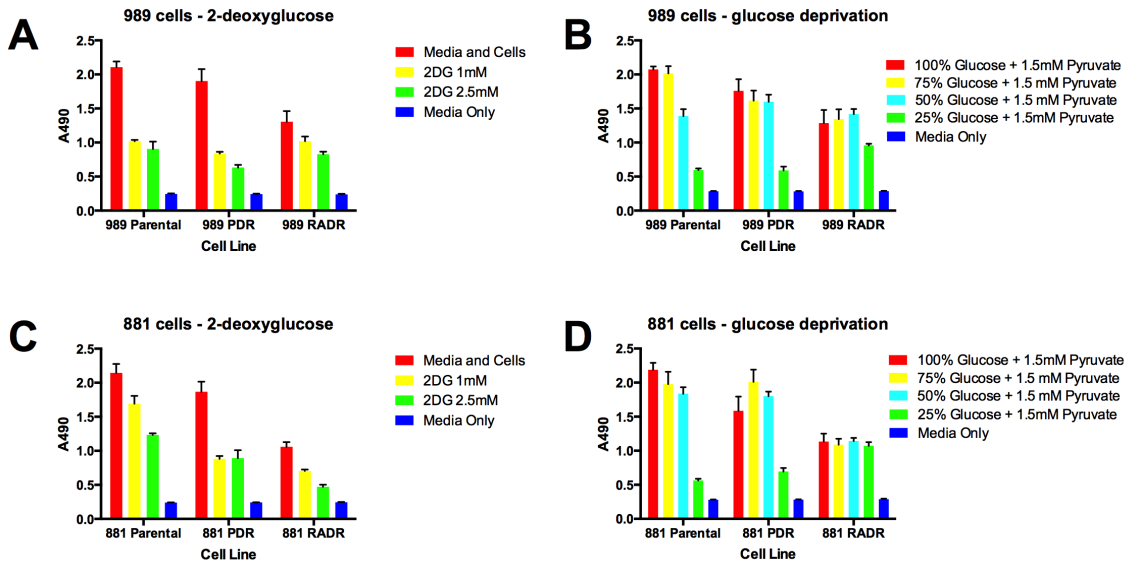


Figure 4. Inhibition of glycolysis in parental and drug-resistant mutant *Nf1* mouse sarcoma lines. (A) Glycolytic inhibition of 989 cells using 2-deoxyglucose. (B) Glycolytic inhibition of 989 cells by glucose deprivation. (C) Glycolytic inhibition of 881 cells using 2-deoxyglucose. (D) Glycolytic inhibition of 881 cells by glucose deprivation.

Inhibiting cellular respiration using oligomycin and phenformin confirmed that 989 parental lines and their drug resistant derivatives showed similar reliance to TCA cycle (Figure 5). In contrast, the proliferation of 881 PD resistant cells seemed to be unaffected by the presence of TCA cycle inhibitors (data not shown). These findings suggest variability in how dependent *Nf1* mutant tumor cells are upon either respiration or glycolysis, however evidence from both lines support that metabolic reprogramming occurs, which can involve either glycolysis (Figure 4) or respiration.

Altogether, measurements of cellular growth in the presence of glycolytic and TCA cycle inhibitors indicated that 881 PD resistant cells relied upon glycolysis (Warburg effect) to fulfil their energy metabolic requirements compared to its respective parental line. This

alteration in energy metabolism from parental line may be linked to mechanisms of drug resistance in 881 PD resistant cells.

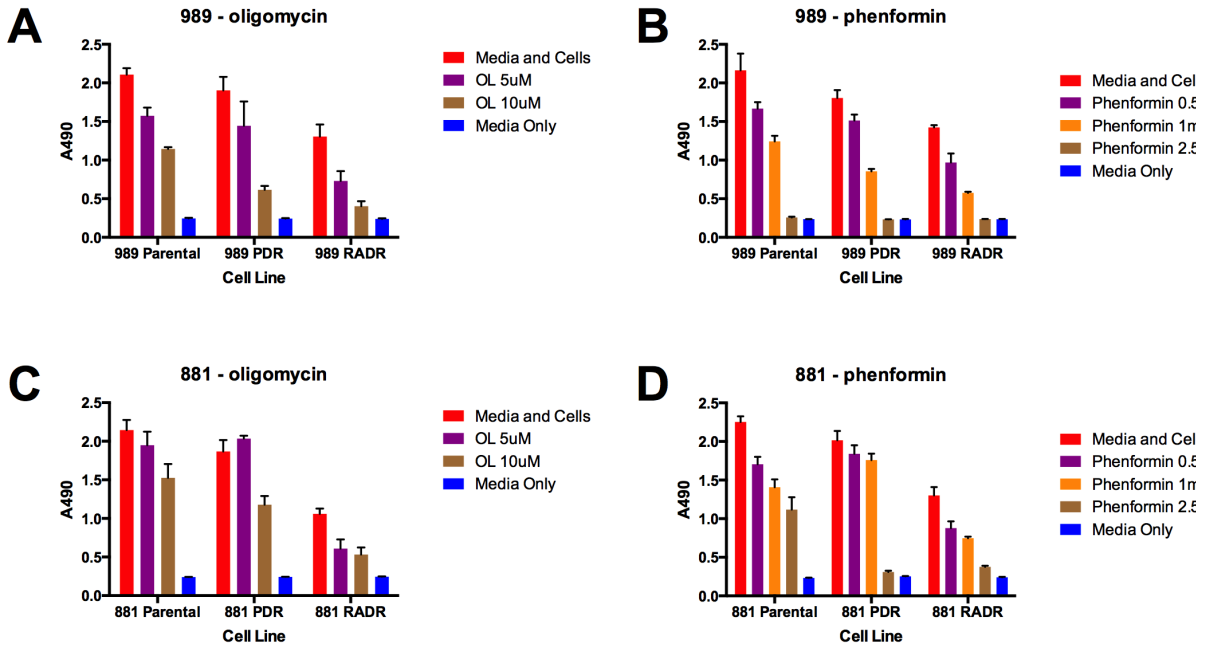


Figure 5. Inhibition of TCA cycle in parental and drug-resistant mutant *Nf1* mouse sarcoma lines. (A) TCA cycle inhibition of 989 cells using oligomycin. (B) TCA cycle inhibition of 989 cells using phenformin. (C) TCA cycle inhibition of 881 cells with oligomycin. (D) TCA cycle inhibition of 881 cells using phenformin.

Conclusion and anticipated follow up studies

Our studies in *Nf1* mutant tumor cells identified candidate genes of potential therapeutic potential that could be studied in follow up studies, and also provide evidence that metabolic dysregulation is associated with drug resistance. These individual molecules, functioning in glycolytic or respiratory metabolism, represent potentially important pathways for tumor growth that will be investigated. Follow up studies that are on-going are measuring discrete metabolite levels in *Nf1* mutant tumor cells to determine how the metabolic network shifts in response to disruption of either glycolysis or respiration. We anticipate that these studies will enable us to improve the specificity of metabolic therapies for *Nf1* mutant tumors.

What opportunities for training and professional development has the project provided?

Professional development and training opportunities were provided to the postdoctoral fellows who worked on this project, Dr. Hiroki Nakaoka and Dr. Danny Laurent. I mentored both of these fellows during the course of this work, mostly in one-on-one teaching.

How were the results disseminated to communities of interest?

Nothing to report.

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

IMPACT:

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

This project's impact is in the area of drug resistance and our results broadly characterized, for the first time, the molecular alterations that distinguish drug-resistant tumors from their original parental cells. Our findings are relevant specifically to those tumors that develop resistance to MEK inhibition or mTOR inhibition, both of which are utilized clinically in individuals with NF1.

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.

This project involved experimental techniques (CRISPRi, FRET-ATP assay, integrated kinome and transcriptome analysis) and demonstrates the utility of these approaches, which can impact other disciplines. By showing feasibility and the utility of these approaches, investigators in other disciplines may increasingly adopt these approaches.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report.

Changes that had a significant impact on expenditures

Nothing to report.

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

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:

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

Journal publications.

PUBLISHED: D. Pucciarelli, S. Angus, C. Zhang, H. Nakaoka, G. Krishnamurthi, B. Huang, S. Bandyopadhyay, K. Shannon, G. Johnson, **J.L. Nakamura**. Inhibition of mTOR or MEK induces transcriptome and kinome re-modeling in *Nf1* mutant tumors Published November 2020 in *Molecular Cancer Therapeutics*. Pages. 2382 – 2395. Acknowledgement of federal support: Yes

N.K. Bennett, M. Darch, M.K. Nguyen, H. Nakaoka, D.Cousineau, M. Hirano, M. Schuelke, M. Emin, M. Kampmann, **J.L. Nakamura**, K. Nakamura. **The ATPome Reveals Cross-Optimization of Metabolic Pathways and Therapeutic Strategies**. *Nature Communications*. 2020 Aug 8; 11(1):4319 PMID: 32859923 Acknowledgement of federal support: yes

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

Nothing to report.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: 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

Name: Danny Laurent
Project Role: Post-doctoral Fellow
Nearest person month worked: 5
Contribution to Project: Dr. Laurent performed experiments involving pharmacologic inhibition of glycolytic and respiratory metabolism.
Funding Support: DHART SPORE

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

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.

I received a one year award (2020-2021) from the Cancer Research Coordinating Committee of the University of California (CRCC). There is no scientific overlap with the present award nor does this compromise my effort.

What other organizations were involved as partners?

NIH/NCI – Financial support through grant funding:
Mitigating long-term treatment-related morbidity in Childhood Cancer Survivors

University of California, San Francisco – Facilities:
The work was performed in the laboratory facilities of UCSF.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES:

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