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13. SUPPLEMENTARY NOTES
Nothing to report

14. ABSTRACT

Malignant Peripheral Nerve Sheath Tumors (MPNSTs) are aggressive, highly chemoresistant sarcomas that are a leading cause of death in patients with Neurofibromatosis Type 1 (NF). NF is caused by germline mutations in the *NF1* gene, which is the key negative regulatory gene of the RAS pathway and is mutated or deleted in a wide range of cancers. Loss of *NF1* leads to deregulated RAS signaling, including the RAF–MEK–ERK pathway. Currently, there is no effective chemotherapy or targeted therapy that is effective in MPNST patients. Even though there have been recent successes with RAF and MEK inhibitors in BRAF-mutated melanoma, innate and acquired resistance to kinase inhibition is a significant clinical issue. Resistance to kinase inhibitors is often promoted by adaptive kinome reprogramming of vital oncogenic signaling networks. The mechanisms and genomic alterations that regulate kinome reprogramming in NF1-deficient cancers are poorly understood. We have demonstrated that *P53* deficiency significantly exacerbates resistance to MEK inhibition in our preclinical MPNST models. These results demonstrate that NF1-related MPNSTs maintain multiple signaling dependencies beyond RAS, and that genomic determinants, such as P53 genomic alterations, profoundly influence therapy response.. In this proposal, we will combine integrated phosphoproteomic/genomic analyses, NF1-MPNST PDX models and a novel targeted NF1 sequencing methodology to 1) untangle the kinome signaling architecture of MPNSTs; 2) determine kinome and genomic events that drive MPNST progression and therapeutic resistance; and 3) identify effective combination therapies for MPNST patients. The results of these studies will significantly advance our understanding of NF1-mediated RAS deregulation and the proteogenomic adaptations that promote therapeutic resistance.

15. SUBJECT TERMS
NF1, MPNST, P53, kinome reprogramming, therapeutic resistance

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1. INTRODUCTION:

Malignant Peripheral Nerve Sheath Tumors (MPNSTs) are aggressive, highly chemoresistant sarcomas that are a leading cause of death in patients with Neurofibromatosis Type 1 (NF). NF is caused by germline mutations in the *NF1* gene, which is the key negative regulatory gene of the RAS pathway and is mutated or deleted in a wide range of cancers. Loss of *NF1* leads to deregulated RAS signaling, including the RAF–MEK–ERK pathway. Currently, there is no effective chemotherapy or targeted therapy that is effective in MPNST patients. Even though there have been recent successes with RAF and MEK inhibitors in BRAF-mutated melanoma, innate and acquired resistance to kinase inhibition is a significant clinical issue. Resistance to kinase inhibitors is often promoted by adaptive kinome reprogramming of vital oncogenic signaling networks. The mechanisms and genomic alterations that regulate kinome reprogramming in NF1-deficient cancers are poorly understood. We have demonstrated that *P53* deficiency significantly exacerbates resistance to MEK inhibition in our preclinical MPNST models. Moreover, we observed that AKT activation may play a critical role in the response to kinase inhibition in p53-deficient MPNSTs. These results demonstrate that NF1-related MPNSTs maintain multiple signaling dependencies beyond RAS, and that genomic determinants, such as P53 genomic alterations, profoundly influence therapy response. Our *hypothesis* is that p53 deficiency promotes kinome reprograming and AKT activation in MPNSTs and that by targeting these adaptive signaling changes a priori, we will greatly improve the sensitivity of MPNSTs to MEK inhibitors. In this proposal, we will combine integrated phosphoproteomic/genomic analyses, NF1-MPNST PDX models and a novel targeted NF1 sequencing methodology to 1) untangle the kinome signaling architecture of MPNSTs; 2) determine kinome and genomic events that drive MPNST progression and therapeutic resistance; and 3) identify effective combination therapies for MPNST patients. The results of these studies will significantly advance our understanding of NF1-mediated RAS deregulation and the proteogenomic adaptations that promote therapeutic resistance.

2. KEYWORDS:

Neurofibromatosis Type 1 (NF), MPNST (Malignant Peripheral Nerve Sheath Tumor), P53, kinome reprogramming, therapeutic resistance, MEK inhibitor, AKT inhibitor

3. ACCOMPLISHMENTS:

See following pages 5-10

Specific Aims	Timeline (Months)	Current status
Specific Aim 1		
SA1a Genetic modification of cell isolates (n=8)	1-6	Completed
SA1b Drug synergy screening	6-18	Completed
SA 1c Integrated proteogenomics	12-36	Completed
Milestone(s) Achieved 1. Establish and characterize cell isolates 2. Complete drug synergy screening 3. Complete proteogenomic analysis of cell isolates and treated cells	1-6 6-18 6-18	Completed
Local IRB/IACUC Approval and ACURO/HRPO Approval	2-3	Completed
Specific Aim 2		
SA2a. Establish METi/AKTi models of drug resistance	12	Completed
SA2b. Drug synergy screening with human MPNST cell lines	12-24	Completed
SA2c. MEKi/AKTi testing in PDX models	6-36	Completed
Milestone(s) Achieved 1. Drug model completion 2. Drug screening and signaling analysis 3. PDX model treatment and characterization	18-24 18-24 18-36	
Specific Aim 3		
SA3a. Long read sequencing characterization *Note - this aim was modified to spatial transcriptomics*	28-48	Completed
SA3b. Integrated transcriptomics	28-48	Completed
Milestone(s) Achieved 1. Finish sequencing and bioinformatics 2. Integrated transcriptomics		

What was accomplished under these goals?

Specific Aim 1: Determine how p53 deficiency modulates kinome signaling in response to MEK inhibition

The completed results of this aim were previously described int the 2022 report. In summary, we developed isogenic MPNST cell lines that we have used to interrogate drug sensitivity and signaling adaptations. The results identified several notable pathways of drug resistance. In this grant, we focused our investigation into the kinome adaptations in P53-deficient MPNST tumors that promote therapeutic resistance. We focused on two striking adaptations: altered signaling of the MET receptor and AKT signaling in response to MEK inhibition. These results point to an important role for p53 in regulating MET signaling, outside of its classic role as a tumor suppressor. We show that p53 regulates expression of several genes involved in MET localization and turnover, resulting in altered signaling kinetics and effector activation. In addition, we observed that p53 influences cell fate determination in MPNST cells, which may partially explain differences in response to drug treatments targeting mTOR, MEK, and MET.

This studies in this aim are complete, have been publsihed in BioRxiv, and the manuscript is currently in a second review at Oncogene.

Grit et al. *p53 modulates kinase inhibitor resistance and lineage plasticity in NF1-related MPNSTs. In review.* https://www.biorxiv.org/content/10.1101/2023.01.18.523629v1

Specific Aim 2: Determine the efficacy of targeting MEK and AKT in p53-deficient MPNSTs.

The completed results of this aim were previously described int the 2022 report. In summary, we demonstrated increased AKT activity was observed in trametinib-resistant tumors in mouse models of NF1-related MPNSTs. To determine whether these cells just lack sensitivity to afuresertib, we also tested the efficacy of the AKT inhibitor, ipatasertib, and mTOR inhibitor, everolimus, in combination with trametinib. The kinome responses to these inhibitors confirmed that inhibition of AKT alone or in addition to MEK inhibition is ineffective in reducing cell viability. This innate resistance to combined AKT and MEK inhibition is supported by the lack clinical translation of this combination. To assess drivers of MPNST resistance, we developed a preclinical model of drug resistance that simulates clinical treatment schedules. We have developed patient-derived xenografts (PDX) from MPNST tissues and also obtained additional models from our collaborator Dr. Angela Hirbe (Washington University). Using a cross-over and a drug holiday design, we evaluated patterns of response and resistance to resumed treatment. This allowed us to not only measure initial treatment response, but also measure the tumor response to drug removal and secondary treatment. In this study, we assessed 1) repeated exposure to the initial treatment (i.e. trametinib → trametinib); 2) treatment targeting a different pathway in the second treatment (i.e. trametinib → everolimus); and 3) efficacy of combination therapy after a drug holiday.

Overall, we observed significant tumor resistance to MEK and/or mTOR inhibition. Commonly we observed an initial response to targeted inhibition; however, tumors quickly rebound when placed onto the drug holiday. The rapid rebound of these tumors mirrors the aggressive nature of MPNST growth in patients. To further understand and validate the heterogeneity in these PDX models, tumors were harvested after the end of the dosing schedule and immunostained to examine any expression changes of proteins that could be used as a route of resistance after treatment. Immunostaining of these tumors revealed an upregulation of YAP after treatment with trametinib, with a subsequent loss in pERK with staining of these two proteins in mutually exclusive regions, revealing distinct populations within the tumors (Figure 1). These results revealed that YAP signaling is a critical signaling node that underlies MPNST treatment resistance.

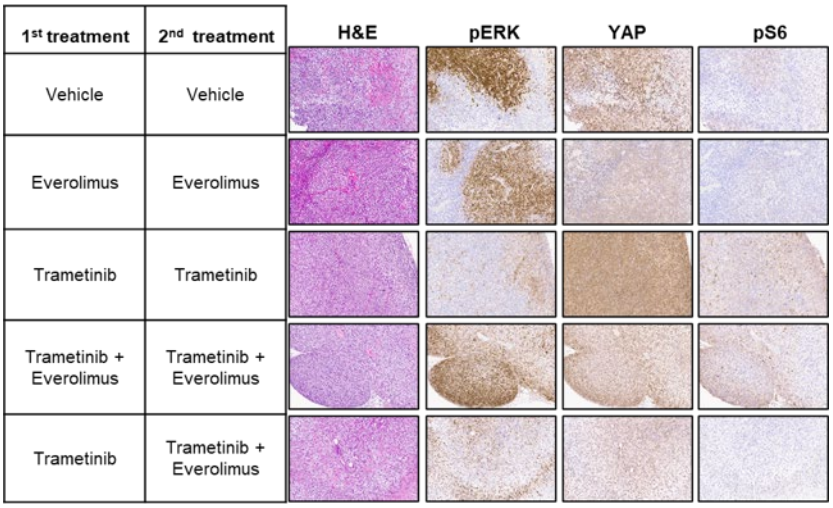

Figure 1: YAP activation present in treatment-resistant MPNST PDX tumors. Immunostaining of tumors from MPNST PDX shown in Figure 4 with pERK, YAP, and pS6.

This studies in this aim are complete and the manuscript is currently in preparation for submission to JCI Insight.

McGee et al. *YAP signaling promotes resistance to MEK and AKT inhibition in NF1-related MPNSTs. In preparation.*

Specific Aim 3: Interrogate genomic determinants of therapy in NF1 in MPNSTs and peripheral nerve sheath tumors.

Because our studies in the above aims revealed that treatment resistance was driven primarily by signaling plasticity than genomic events, we decided to refocus this aim on further interrogating MPNST heterogeneity and signaling plasticity. As discussed in Aim 2, we demonstrated a stark difference in treatment response to

MEK and TEAD inhibitor treatment in MPNST patient-derived xenografts (PDX). Within these responder and non-responder tumors, we identified key signaling adaptations. However there was a lack of understanding on how the level of tumor heterogeneity and clonal evolution that underlies these signaling adaptations. Even though immunostaining of tumors is informative, it does not provide the full story of molecular patterns occurring within subclonal tumor populations as the proteins of interest are pre-defined. Spatial transcriptomics is a newer technology that utilizes the intact tumor tissue on a slide to define the RNA expression profiles of selected populations within the tumor. Here we utilized the Nanostring GeoMx Digital Spatial Profiler (DSP) platform to untangle the tumor heterogeneity, expression changes driving resistance, and the clonal changes throughout MPNST progression. We created a tissue microarray (TMA) of tumor cores from the MEK-mTOR treatment studies described in Aim 2. The staining pattern of YAP and pERK fluorescent markers readily confirmed the heterogeneity within the tumors, as shown in three representative samples taken from the TMA (Figure 2). This approach allowed us to investigate transcriptional changes in response to MEK, and mTOR inhibition. We detected expression differences in several hallmark cancer signatures such as MYC, mTORC, and epithelial to mesenchymal (EMT). Together, these findings reveal the spatial transcriptomic profiles of MPNSTs in response to MEK and mTOR inhibition and importantly, identify gene signatures involved in MPNST resistance.

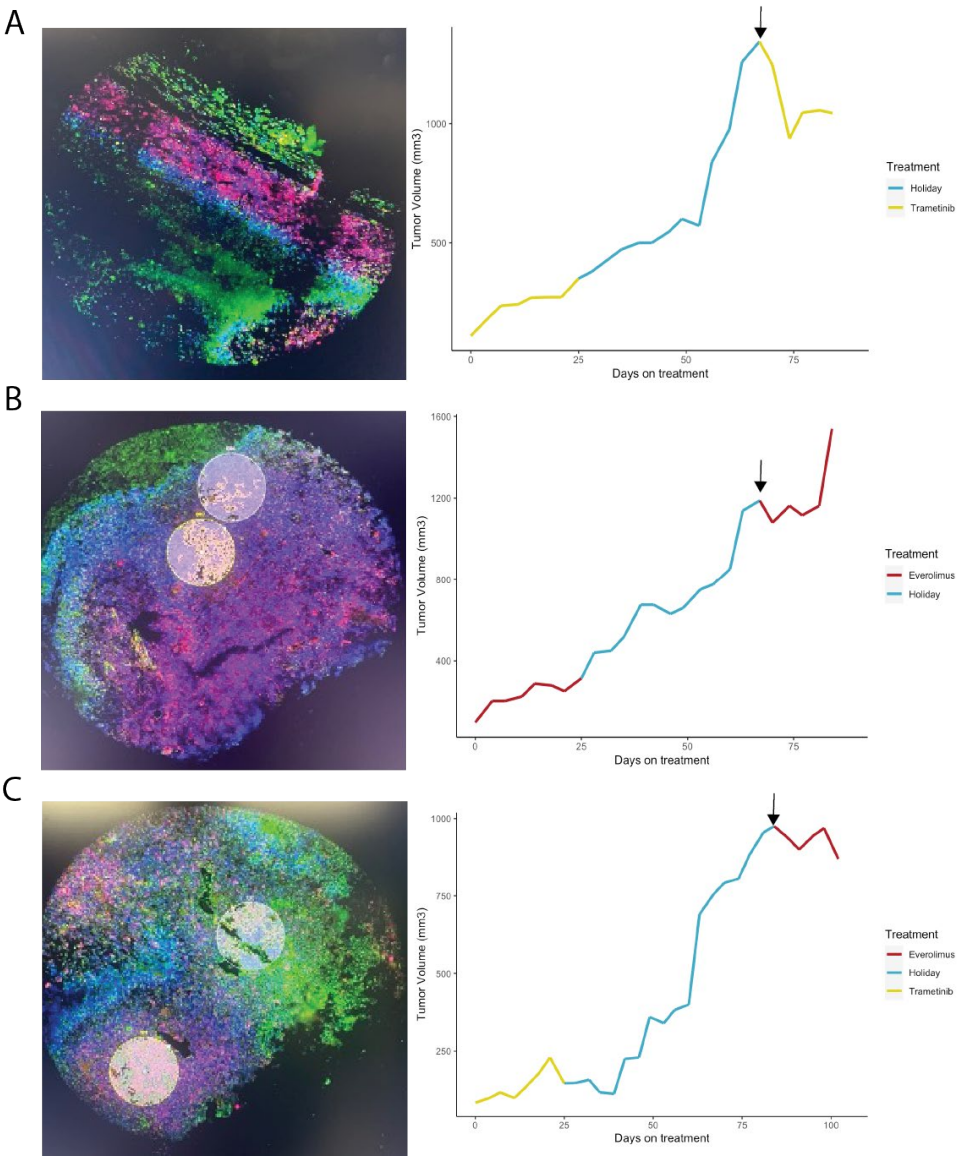

Figure 2. Spatial transcriptomic analysis of MPNST drug response. Selected fluorescent images from GeoMx scan showing YAP (red), pERK (green), Ki67 (light blue), and DAPI (dark blue) staining with associated growth curves. Arrow indicates point when biopsy was taken. Circles within panels B and C indicate selected regions (AOIs) with colors within the circles showing different segments marking cellular populations as defined by fluorescent staining.

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

Because of this funding opportunity, Dr. Jamie Grit was able to acquire knowledge and experience that helped her secure a postdoctoral fellowship in NF1-related research. Our graduate student, Dr. Lauren McGee, presented this work at the Fourth RAS Initiative Symposium 2022 (October 2022) and is now in her postdoctoral fellowship at the National Cancer Institute where her research is focused on sarcoma therapeutics.

How were the results disseminated to communities of interest?

We continue to disseminate our results with our local NF advocacy group (NF Michigan) and at national meetings. Our work has been presented at the AACR Sarcoma Meeting (May 2022) the Children's Tumor Foundation Meeting (July 2022) and we will be presenting at the Fourth RAS Initiative Symposium 2022 (October 2022).

In addition, to our first publication (Grit et al. Kinome Profiling of NF1-Related MPNSTs in Response to Kinase Inhibition and Doxorubicin Reveals Therapeutic Vulnerabilities. Genes. 2020) we are in the process of publishing two additional mansucripts (with one in second review at Oncogene).

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

This grant is completed

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?
The above accomplishments verify the need for broader kinome profiling in preclinical drug studies in MPNSTs. As of today, there are no active agents against NF-related MPNSTs and multiple clinical trials have failed. Better preclinical strategies are needed to justify incorporation into clinical trials. The use of phosphoproteomic and genomic profiling of tumors maybe be a critical method to validate "on target" effects and unavoidable patterns of kinome adaptation. Moreover, AKT inhibition as a single or combined approach does not appear promising. Other pathways such as Hippo or upstream RTK signaling need to be inhibited in addition to MEK for treatment efficacy in MPNSTs.

What was the impact on other disciplines?
We expect these results to advance our understanding of the therapeutic resistance and kinome adaptations in other RAS-deregulated or NF1-mutated cancers, such as non-small cell lung cancer, colorectal, glioblastoma, and pancreatic cancer in other NF-related cancers.

What was the impact on technology transfer?
Nothing to report

What was the impact on society beyond science and technology?
We have an annual meeting with NF-Michigan to update them on our research progress on NF1-related research. This will be held this year in September 2022. These interactions are mutually beneficial. These meetings communicate research progress in NF research and helps us understand the challenges that individuals with NF face.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Our studies were significantly delayed due to Covid-related shutdowns. Covid-related issues particularly delayed completion of the proposed *in vivo* studies and the associated genomic and transcriptomic analysis. We requested and were granted a 12 month no-cost extension in May

2022.

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 *Nothing to report*

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:

Journal publications.

Grit JL, Pridgeon MG, Essenburg CJ, Wolfrum E, Madaj ZB, Turner L, Wulfkuhle J, Petricoin EF 3rd, Graveel CR, Steensma MR. Kinome Profiling of NF1-Related MPNSTs in Response to Kinase Inhibition and Doxorubicin Reveals Therapeutic Vulnerabilities. Genes (Basel). 2020 Mar 20;11(3):331. doi: 10.3390/genes11030331. PMID: 32245042; PMCID: PMC7141129.

Jamie L. Grit, Lauren E. McGee, Elizabeth A. Tovar, Curt J. Essenburg, Emily Wolfrum, Ian Beddows, Kaitlin Williams, Rachael Sheridan, Josh Schipper, Marie Adams, Menusha Arumugam, Thomas Vander Woude, Sharavana Gurunathan, Jeffrey M. Field, Julia Wulfkuhle, Emanuel F. Petricoin III, Carrie R. Graveel, Matthew R. Steensma. p53 modulates kinase inhibitor resistance and lineage plasticity in NF1-related MPNSTs bioRxiv 2023.01.18.523629; doi: https://doi.org/10.1101/2023.01.18.523629

Books or other non-periodical, one-time publications. *Nothing to report*

Other publications, conference papers, and presentations. Poster presentations at Sarcoma Meeting (May 2022) the Children's Tumor Foundation Meeting (July 2022) and we will be presenting at the Fourth RAS Initiative Symposium 2022 (October 2022).

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Matthew Steensma
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	0.96 calendar months or 40% effort of VAI appointment
Contribution to Project:	Guiding the experimental design for the entire project and current overseeing the completion of experiments

Name:	Carrie Graveel
Project Role:	Senior Research Scientist
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3.0 calendar months or 25% effort
Contribution to Project:	Designing, performing, and analyzing the experiments; contributing to the development of research strategies, and preparing the results for presentation and publication.

Name:	Elizabeth Tovar
Project Role:	Research Scientist
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3.6 calendar months or 30% effort
Contribution to Project:	Performing analyzing the experiments; contributing to the development of research strategies and preparing the results for presentation and publication.

Name:	Ian Beddows
Project Role:	Bioinformatics Research Scientist
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2.4 calendar months or 20% effort
Contribution to Project:	Performing bioinformatic analysis of proteomic and genomic data.

Name:	Curt Essenburg
Project Role:	Lab Animal Technologist III
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6.0 calendar months or 50% effort
Contribution to Project:	Performing animal studies

Name:	Jamie Grit
Project Role:	Postdoctoral Fellow
Research Identifier (e.g. ORCID ID):	N/A
Nearest person month worked	1.2 calendar months or 10% effort
Contribution to Project:	Performing analyzing the experiments; contributing to the development of research strategies and preparing the results for presentation and publication.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? Nothing to report

What other organizations were involved as partners? *Nothing to report*

8. Special Reporting Requirements *Nothing to report*

9. Appendices *Nothing to report*